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Additional records of the African fig fly *Zaprionus indianus* Gupta, 1970 (Diptera: Drosophilidae) for western Iran supported by DNA barcoding

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Abstract

Zaprionus indianus is a very successful invasive species with a high dispersion capacity. In this paper we present the first host record of the African fig fly pest for western Iran. We use molecular data to support the identification of this species. In total, 250 larval specimens were collected. After immature rearing and adult emergence under laboratory conditions, the specimens were identified as *Z. indianus* based on morphological characters. Then, the morphological identification was confirmed by BLAST analysis of the *COI* nucleotide sequence, which showed 97%–100% identity to *COI* sequences of *Z. indianus*, submitted from different parts of world. This study can provide some insights into the identification, ecology and host preference of *Z. indianus* as a new invasive and potentially major pomegranate pest in western Iran.

Key words: *Punica granatum*, Drosophilidae, DNA barcoding, Phylogenetic tree, Lorestan, Western Iran

Introduction

The genus *Zaprionus* Coquillett, 1901, a vinegar fly in the family Drosophilidae (Khanna and Mohanty, 2017), comprises two subgenera: *Zaprionus* s. str. with a mainly African distribution – 44 species of the genus were described from the Afrotropical region (Brake and Bächli, 2008; Commar et al., 2012) and *Anaprionus* Okada, 1990 (10 species) with an oriental and Australasian distribution (Chassagnard and Kraaijeveld, 1991). *Zaprionus indianus* Gupta, 1970 was first described from specimens collected in India. Now it is a semi-cosmopolitan dipteran species found in all biogeographical realms, including Afrotropical, Australasian, Neotropical, Oriental and Palearctic, except Nearctic (Brake and Bächli, 2008). *Zaprionus indianus* has been known for years as *Z. vittiger* Coquillett, 1902 and since 1980 under the name of *Z. collarti* Tsacas, 1980 (Chassagnard and Kraaijeveld, 1991). As a habitat generalist with broad niche characteristics, this species is able to breed and feed on fallen or decaying fruits and fruits on trees in a wide range of native and cultivated host plants (van der Linde et al., 2006; Lavagnino et al., 2008). *Zaprionus indianus* is known to infest fruits of 74 species in 31 plant families in Africa (Lachaise and Tsacas, 1983), such as mulberry, grapes, peach, nectarine, plum, figs, date palm, sweet orange, sour orange, blackthorn, pomegranate, guava and apple (Alawamleh et al., 2016a). It is a primary fig pest, on *Ficus carica* causing up to 50% crop loss; and may be a secondary pest for other orchard fruits (de Setta and Carareto, 2005; Lasa and Tadeo, 2015; Pfeiffer et al., 2019). Due to its polyphagous behavior, adaptive versatility in new environments, and physiological tolerance, the species is a powerful invader (Parkash and Yadav, 1993; Leão and Tldon, 2004; Molina-Rodríguez and Pérez-Guerrero, 2019). *Zaprionus indianus* is an example of a drosophilid fly with synanthropic status, which is found on a variety of endemic, as well as introduced fruits (Yassin et al., 2008).

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Zaprionus indianus has always been of interest and the subject of many research publications in two last decades (Castro and Valente, 2001; Yassin and Abou-Youssef, 2004; van der Linde et al., 2006; Lavagnino et al., 2008; Carles-Tolrá, 2009; Penariol and Madi-Ravazzi, 2013; Renkema et al., 2013; Joshi et al., 2014; Alawamleh et al., 2016b; Kremmer et al., 2017; Özbek Çatal et al., 2019; Molina-Rodríguez and Pérez-Guerrero, 2019). *Zaprionus indianus* is a very successful invasive species with a high capacity for dispersion, quickly expanding its range from its native distribution in Africa into America and India (de Setta and Carareto, 2005; da Mata et al., 2010; Galego and Carareto, 2010). The number of potential generations was estimated between 12 to 14 per year (Amoudi et al., 1991; Nava et al., 2007). The fruit trade plays a major role in the spread of *Z. indianus* from Africa into Asia (Yassin et al., 2009). Iran, located in western Asia, was invaded by a serious fig pest, *Zaprionus* sp., for the first time in the mid-1980s (Naeem and Akhyani, 1988). Parchami-Araghi and Mohammadi-Khorramabadi (2009) and Parchami-Araghi et al. (2015) briefly recorded the African fig fly feeding on various orchard fruits, such as pomegranate. Recently, Jooybar et al. (2016) reported the presence of *Z. indianus* infesting pomegranate in Fars Province.

Here we record *Z. indianus* on *Punica granatum* (Lythraceae) fruit from western Iran as a new threat to local pomegranate orchards in the study area. The novelty of this study is to report the occurrence of this species from western Iran with molecular evidence for identification.

Material and Methods

Sample collection, DNA extraction and PCR

This investigation was conducted during October to November 2019. Third instar larvae of the fly were collected from the inside of a damaged pomegranate fruits (Fig. 1B) in Zirtang-e Siab, Kuhdasht county of Lorestan Province, western Iran (33°23'51"N 47°11'56"E and 900 m a.s.l.). This site is moderately urbanized, with huge gardens of *P. granatum* and an arboretum in the vicinity (Fig. 1A). It is characterized by a subtropical climate during the collection period as the temperature (°C) was 35.2–18.6 (Max.) and 13.7–4.5 (Min.).

Sampled larvae were reared in plastic containers with adequate ventilation and transparent walls under laboratory conditions (22.5 °C, 60–70% relative humidity). Each container was examined daily and the time of fly emergence from the decayed fruits was recorded. The emerged flies were anaesthetized at -10 °C, then collected with a fine brush and stored in 70% ethanol for further examination. Adult fly specimens were identified to species level under a stereomicroscope (Wild-Heerbrugg M8 Model) according to the morphological characters described by van der Linde (2010). To document the identity of the flies, photos of body parts (head, thorax, abdomen and legs) and overall body shape were captured using a digital camera (Nikon® Coolpix S7000). Voucher specimens are retained in Lorestan Agricultural and Natural Resources Research Center (Khorramabad, Iran). Genomic DNA of a representative specimen was extracted using a CTAB protocol according to Doyle and Doyle (1987). A 524 bp fragment of the cytochrome oxidase subunit I (*COI*) (according to GB accessions EU493688, EU493690-1, JQ679110) was amplified by polymerase chain reaction (PCR) using the primers designed by Simon et al. (1994) including C1-J-1718: 3′- GGA GGA TTT GGA AAT TGA TTA G -5′ and C1-N-2191: 3′- CCC GGT AAA ATT AAA ATA TAA ACT TC -5′ with minor modification in C1-J-1718 as follows: deletion of –TTCC– from the 5′ end. PCR reactions were carried out in a thermocycler (Corbett®, Australia) based on a touchdown temperature profile: 3 minutes at 94°C, 11x [45 s at 94°C, 50 s at 60°C, 60 s at 72°C], followed by 24x [45 s at 94°C, 50 s at 50°C, 60 s at 72°C], 3 minutes at 72°C). PCR for each 25 µl final volume reaction was performed using 12.5 µl RedMaster PCR 2X (Sinaclon®, Iran), 1 µl of each primer (10 pM), 4 µl gDNA template (100 ng/µl), and 6.5 µl ddH_2O. The PCR products were visualized with 1% agarose gel electrophoresis and finally submitted to a third-party service provider (Codon Genetic Group®, Iran) for sequencing using Applied Biosystems-ABI, 3130XL.

Phylogenetic analysis

The DNA sequence was manually checked using FinchTV® software (www.geospiza.com) to correct any sources of error or ambiguities if present. Homologies with the available sequence data in NCBI GenBank were checked using BLAST analysis. Finally, the sequence was submitted to GenBank under accession number MN824026.

We generated an alignment of 33 *COI* sequences including the sequence of the present study, as well as sequences of other *Zaprionus* species, including *Z. indianus* submitted from Africa (EF632354, EF632355, EF632357, EF632361, EF632362, KC994625, KF736189, MK263238), Europe (EF632359), Asia (EF632365, EF632366, KC994626) and America (KF736182, KJ463786). Sequences were selected according to the similarity revealed by the BLAST algorithm. Outgroups were chosen from the sister groups as well as successively more distant lineages: *Drosophila melanogaster* and *Dichaetophora flatosternata* and *Zaprionus* sp. (*Z. lineosus*). Then, the sequences were aligned using SeaView4 software (Gouy et al., 2010) (Clustal Algorithm calculating pairwise k-tuple distances). Genetic distances among and between various sequences of different *Zaprionus* species were calculated using Maximum Composite Likelihood (MCL) modelled in MEGA7 (Kumar et al., 2016).

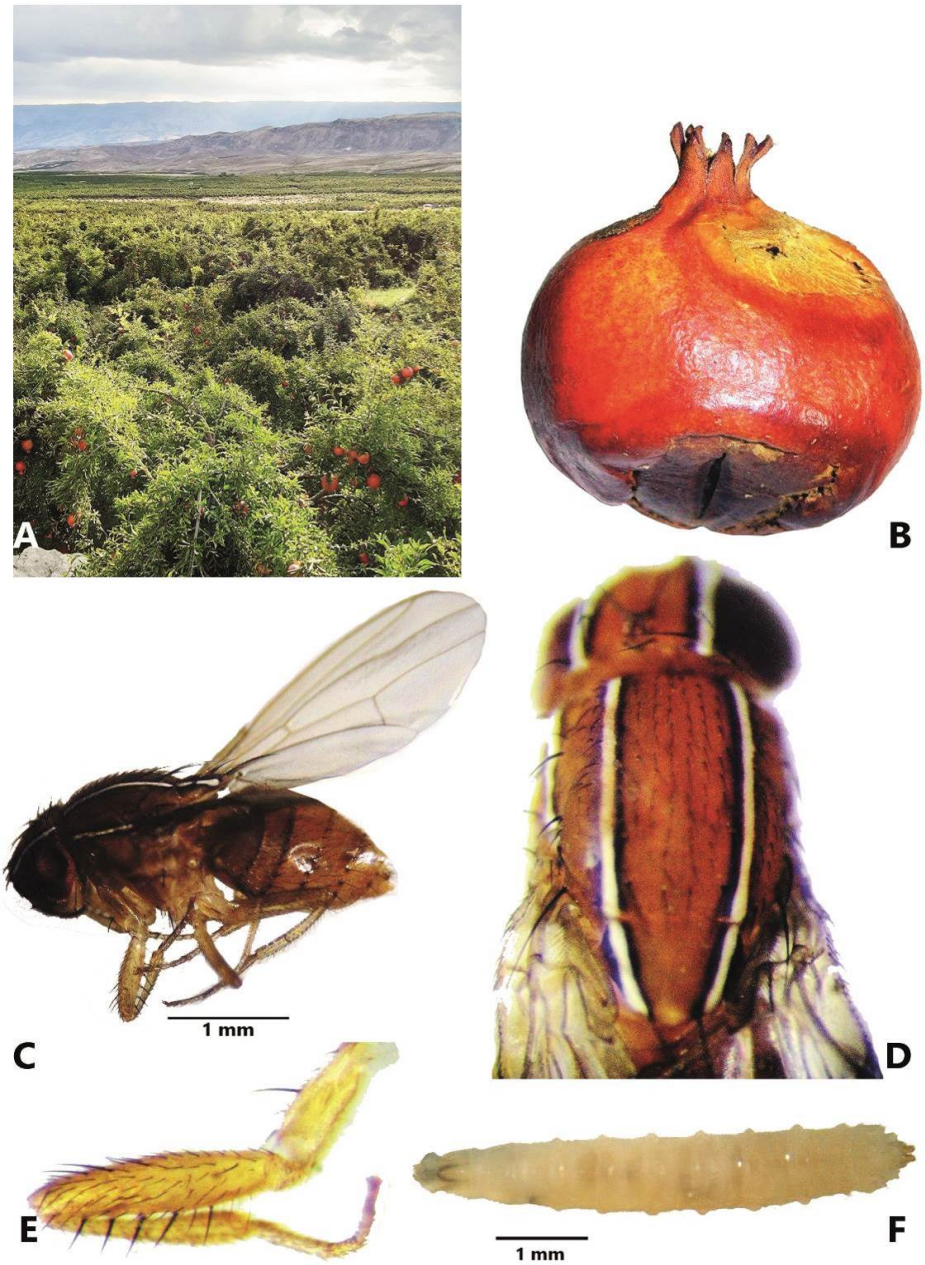

Figure 1: View of the pomegranate orchard in Zirtang-e Siab (A), symptoms of damaged fruit (B), lateral view on *Zaprionus indianus* male (C), dorsal view on thorax (D), leg III (E), 3rd larval instar (F).

The nucleotide sequences were then used to build a phylogenetic tree further confirming the identification. To construct the *COI* phylogenetic tree, a 414 bp alignment sheet was analysed using BEAST v. 2.6.0 based on the Bayesian Inference (BI) method (Model: JC69, 10 million generations). The phylogenetic tree was summarized and visualized using TreeAnnotator and FigTree v. 1.4.4, respectively.

Results

Fly collection, identification and BLAST analysis

In total, 250 larvae were collected (Fig. 1F). Adults were first identified as belonging to the genus *Zaprionus*, based on the presence of characteristic longitudinal white stripes on the frons and the mesonotum. Then, the specimens were recognized as *Z. indianus* based on described morphological characters (van der Linde, 2010), including the overall dark yellowish to light brown thorax and abdomen (Fig. 1C), the narrow (relative to those of the other species) silver bands bordered by black bands across the head, thorax, and scutellum (Fig. 1D), as well as by a row of composite spines fused with long bristles at the base of the anterior femur (Fig. 1E). Then, the morphological identification was confirmed by BLAST analysis of the *COI* nucleotide sequence, which showed 97%–100% sequence identity to *COI*

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sequences of *Z. indianus* submitted from different parts of world (Africa, Europe, Asia, America) (Table 1). The geographic distribution of this species in Iran along with its host range is shown in Figure 2.

Phylogenetic analysis

A phylogenetic tree was constructed based on partial *COI* sequence data (Fig. 3). The *COI* phylogeny of the genus *Zaprionus* is rooted with two drosophilid genera (*Drosophila*, *Dichaetophora*) and *Z. lineosus* as a distantly related *Zaprionus* taxon outside the main *Zaprionus* clade (posterior probability support: 0.99). The *COI* phylogenetic tree shows the species *Z. indianus* as monophyletic in the *Zaprionus* clade (posterior probability support: 1). The genetic distance within *Z. indianus* is < 3% and between *Z. indianus* and other *Zaprionus* species it ranges from 8% to 16%.

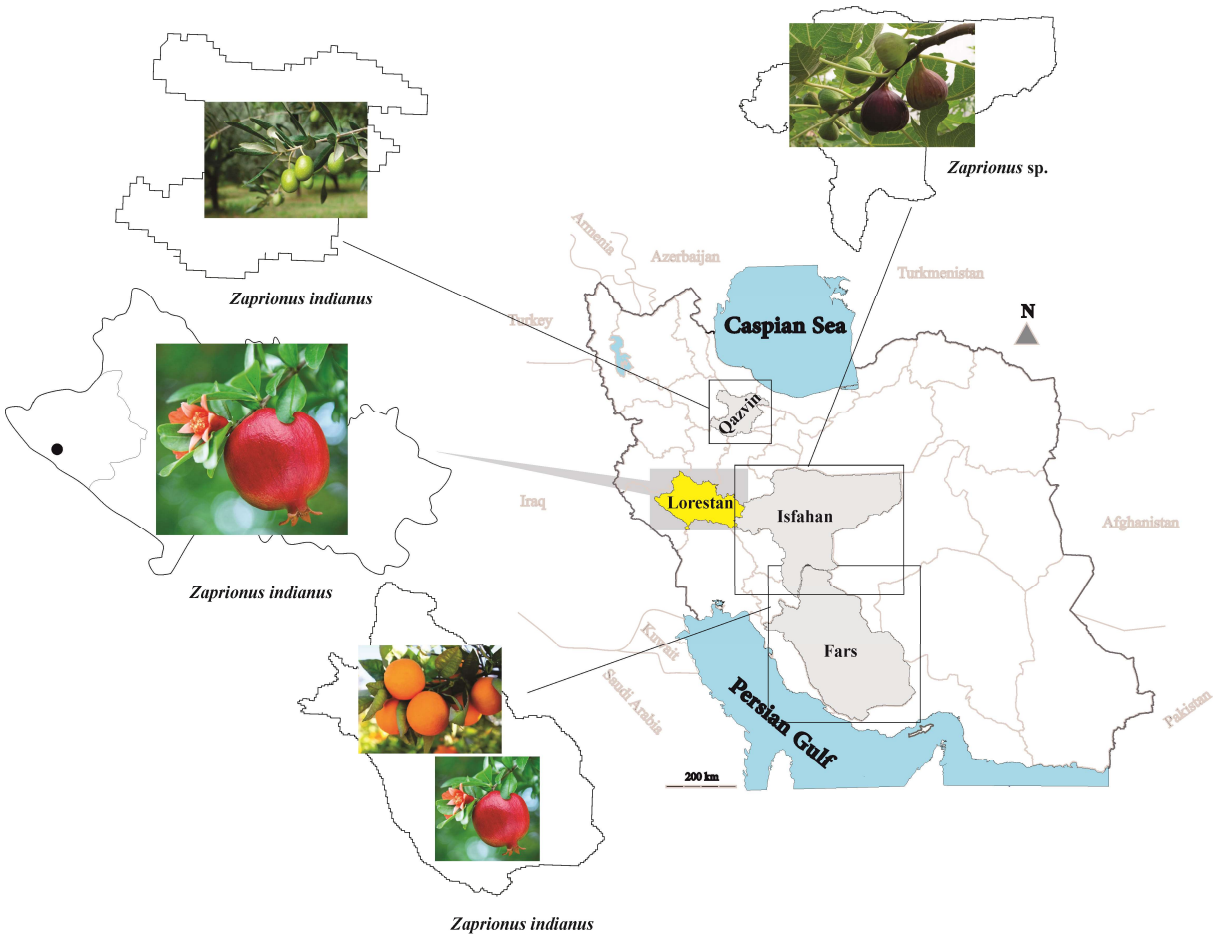

Figure 2: Geographical distribution of *Zaprionus indianus* with the infested hosts; Lorestan Province + pomegranate (this study), Fars Province + pomegranate and oranges (Parchami-Araghi and Mohammadi-Khorramabadi, 2009; Jooybar et al., 2016), Qazvin Province + olives (Parchami-Araghi et al., 2015), Isfahan Province + figs with unknown species probably Z. *indianus* (Naeem and Akhyani, 1988).

Table 1: Percent identity of *COI* sequences of this study (as bold) with *Zaprionus indianus* submitted from different parts of the world.

Species (GenBank Accession Number)- Region	*Z. indianus* (MN824026)	
	Percent identity	Query cover
Zaprionus indianus (KP731065)- Asia, India	97.00%	86%
Zaprionus indianus (EF632353)- Africa, Cote d'Ivoire: Abidjan	97.86%	91%
Zaprionus indianus (KP731059)- Asia, India	98.00%	86%
Zaprionus indianus (MK263238)- South Africa	98.83%	92%
Zaprionus indianus (EF632359)- Europe, Portugal	98.82%	91%
Zaprionus indianus (KF736183)- America, Mexico: Sonora, Alamos	98.55%	90%
Zaprionus indianus (EF632365)- Asia, Saudi Arabia: Raydah	99.76%	91%
Zaprionus indianus (JQ668138)- Asia, Iran	99.75%	85%
Zaprionus indianus (KC994628)- America, Brazil: Sao Luis	99.29%	91%
Zaprionus indianus (EF632366)- Asia, India: Bangalore	100.00%	91%
Zaprionus indianus (KF736189)- Africa, Cameroon: Yokadouma	100.00%	90%

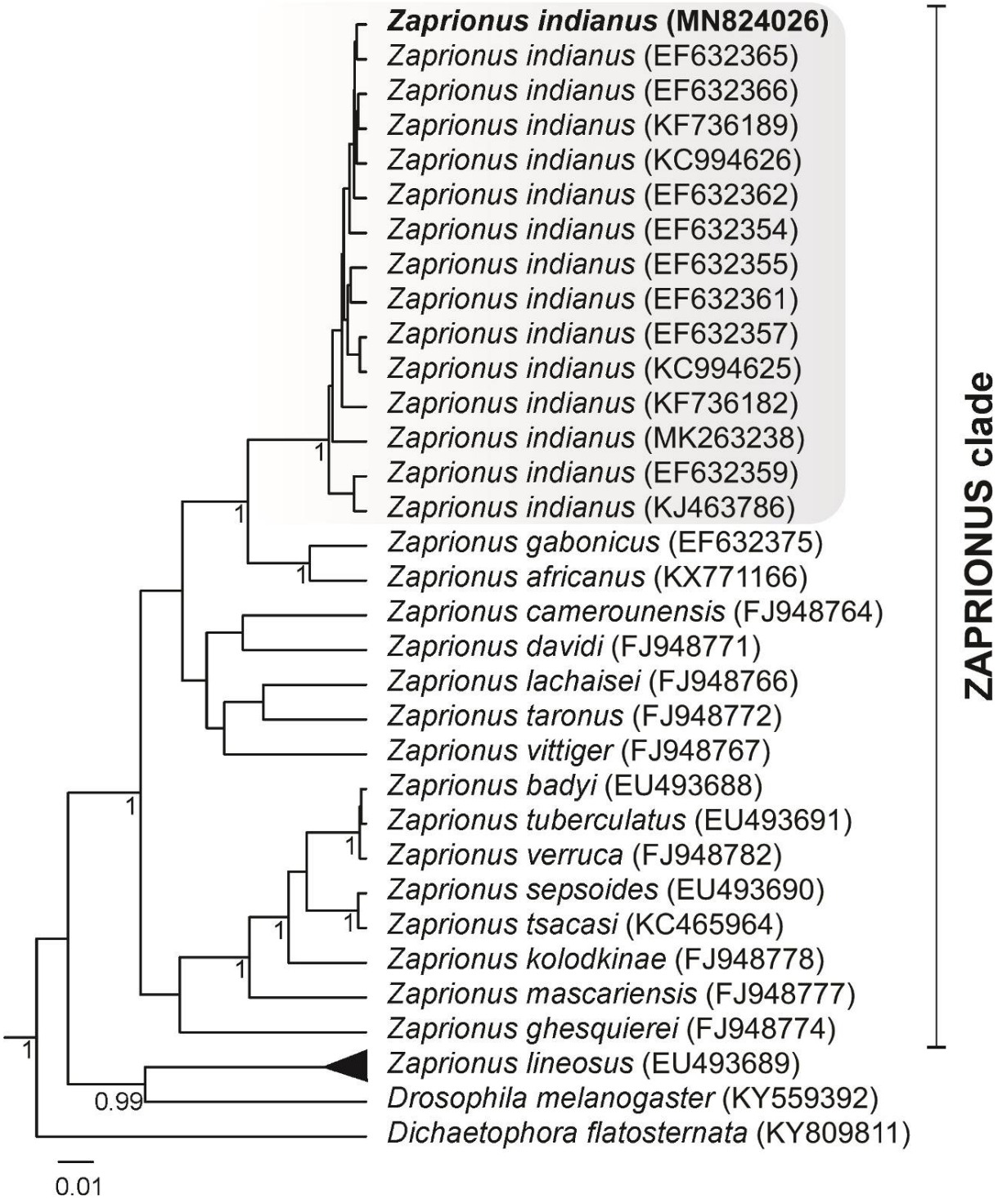

Figure 3: Phylogenetic relationships among *Zaprionus indianus* populations (grey highlighted box) and other *Zaprionus* species derived from the Bayesian inference (BI) based on analysis of partial *COI* sequences; numbers below each node show posterior probability value (10 million reiterations). Taxon labels give the species name followed by GenBank accession numbers in parentheses. The taxon sequenced in the present study is highlighted in bold. Branch lengths are proportional to the evolutionary distances. *Drosophila melanogaster* and *Dichaetophora flatosternata* were used as outgroups and Z. *lineosus* (marked with a solid triangle) was placed outside the *Zaprionus* clade as an out-group

Discussion

Pomegranate is one of the important horticultural products cultivated in some dry and subtropical regions of Iran, e.g. Lorestan Province (Mohseni, 2009). Iran is one of the richest areas in terms of genetic diversity of pomegranate, with 764 varieties and genotypes of *P. granatum* (Basaki et al., 2016).

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Apparently Iran and its surroundings could even be considered the region of origin of this fruit (Holland et al., 2009). Pomegranates are susceptible to various pests, such as insects. The definition of primary and secondary pests varies between the different geographical regions (Holland et al., 2009). While some pests are a big problem in one place (primary pest), they are not harmful or absent from other regions (secondary pest).

Here, we report *Z. indianus* for the first time from western Iran (Zirtang-e Siab in Lorestan Province). Zirtang-e Siab is the largest localized pomegranate growing area in the country with 1,000 ha area under cultivation (Mr. Rashidzadeh, Jihad-e Keshavarzi Kuhnani, 2019, pers. comm., 19 Dec.). The topography and favorable climatic conditions have made the area one of the most desirable pomegranate areas in the country. In the present study, a so-called secondary pest, *Z. indianus*, was collected on *P. granatum* fruit. The species represents a great threat to agriculture: the larvae enter the fruit and feed on the seed flesh, causing crushing, spoilage, crop rot and lack of marketability. On the margin of the pomegranate orchards there are other hosts of *Z. indianus*, such as fig and olive trees. *Zaprionus indianus* was also reported from olive fruit in northern Iran (Parchami-Araghi et al., 2015). It was also reported on pomegranate fruits in nearby countries, such as Pakistan (Shakoori and Butt, 1979), and Saudi Arabia (Amoudi et al., 1991) and on persimmon, blackberry, fig, cherry, mulberry, peach and plum in Turkey (Özbek Çatal et al., 2019).

In this study, *Z. indianus* was identified according to BLAST analysis of *COI* sequences. *Zaprionus indianus* belongs to the *Z. vittiger* subgroup within the *Z. armatus* group. The accurate identification of various species should be taken using *COI* barcoding; up to 97% can be correctly identified (Hebert and Gregory, 2005). Since all *Zaprionus* species possess a number of identical morphological characters, such as bright white or silvery stripes extending longitudinally from the fronto-orbital plates down the mesonotum to the scutellum (Markow and O'Grady, 2005), we decided to confirm the species identity using a genetic marker. The genetic distance among different sequences of the *Z. indianus* clade was calculated < 3% and between *Z. indianus* and other *Zaprionus* species ranging from 8% to 16%. A smaller genetic distance among various geographical populations of *Z. indianus* was reported between 0.22% to 0.68% in the past (Yassin et al., 2008). In this study, 100% genetic identity was found between a *Z. indianus* sequence of this study with sequences submitted from eastern Mediterranean, India and Cameroon, as well as, 98% identity with sequences of *Z. indianus* originated from USA, Portugal and South Africa. Nevertheless, all *COI* sequences of *Z. indianus* clustered in a monophyletic and robust clade according to our Bayesian Inferences analysis. Markow et al. (2014) presented six *COI* haplotypes in *Z. indianus* from Mexico and Panama that clustered into three distinct clades. The genus *Zaprionus* appeared to be phylogenetically close to the genus *Drosophila* (Robe et al., 2005; Sarswat et al., 2016), as well as in terms of overall nucleotide content (da Silva et al., 2009); an issue reflected in our *COI* phylogenetic tree. This study can provide some insights into the identification, molecular phylogeny and biology (host record) of *Z. indianus* as a new invasive and potentially major pomegranate pest in western Iran.

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Conflict of interest

All the authors declare that there are no conflicting issues related to this research article.

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