GENETIC DIVERSITY OF THREE POPULATIONS OF DUBAS BUGS *OMMATISSUS LYBICUS* DE BERGEVIN (TROPIDUCHIDUE: HOMOPTERA)

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ABSTRACT

Dubas bug (*Ommatissus lybicus* De Bergevin) is a major pest for date palm in several countries and mainly in the Arabian Gulf. The intensive use of insecticides to control the dubas bug might lead to induced resistance. To investigate the diversity of this insect, isolates were collected from three populations from Oman (Batinah and Daklia regions) and Jordan (Safi region). Total genomic DNA was successfully isolated from individual insects using in-house developed freeze-thaw protocol. The isolates were investigated for genetic diversity using an inter-simple sequence repeat (ISSR) marker. The data showed huge diversity within and between the assayed dubas bug populations. Moreover, the Cytochrome c oxidase I (COI) gene was amplified and sequenced for random isolates from all three populations. The sequences were used to generate a phylogenetic tree based on maximum likelihood method. The data confirms the genetic diversity within and between the assayed dubas bug populations. Nonetheless, a group of dubas bug isolates from Oman have clustered in two major clades, indicating their uniqueness among other assayed international isolates.

KEYWORDS:
Dubas bug, Cytochrome c oxidase I, Oman, Jordan

INTRODUCTION

The date palm (*Phoenix dactylifera*) is the main host of dubas bug (*Ommatissus binotatus)* (Homoptera: Tropiduchidae), which is also known as the palm bug. Dubas bug is a destructive pest of date palm and is widely distributed in North Africa (Morocco, Algeria, Tunisia, Libya and Egypt), Sudan, Jordan, Iran and Spain. In addition, it is also an important pest of date palm in Iraq, Kuwait, Bahrain, Qatar, Saudia Arabia, the United Arab Emirates and Sultanate of Oman [1, 2, 3, 4, 5, 6, 7, 8, 9,].

Natural ecosystems provide important habitats for a wide range of organisms. The ability of insects to utilize different host plants has been suggested to be a dynamic and transient phase [10, 9]. Dispersal parameters can be directly estimated using ecological approaches such as mark-release-recapture methods but might not be applicable in studies involving sampling the propagules. In these cases, population genetics approaches provide a better alternative because the information contained in DNA can provide gene flow parameter estimates for different and complementary timescales. Molecular markers are widely used to study the intra-specific genetic diversity. Al-Fawaeer et al. [11] found that populations of *Ceratitus capitata* with common genomes tend to cluster together in the same group, using random amplified polymorphic DNA (RAPD) analysis. Another widely applied sequence is the internal transcribed spacer (ITS) in the ribosomal RNA genes. It was investigated in red palm weevil (*Rhynchophorus ferrugineus* Olivier) populations collected from several Mediterranean countries [12,9]. The discrimination power of ITS1 region was shown to be much higher than ITS2 region for red palm weevil phylogeny. In a phylogenetic study of Fulgoroidea based on 18s rDNA sequence, *Ommatissus binotatus* Fieber was used to represent Tropiduchidae [13]. However, the study covered just one site, utilizing insects reared from eggs collected on palm leaves from southern Spain.

The mitochondrial Cytochrome b (Cyt. b) is another common molecular markers include gene and amplified fragment length polymorphism, which has been applied in both phylogeographic studies and have been widely used for studying genetic diversity in insects [14]. Furthermore, partial DNA sequences of the mitochondrial gene such as Cytochrome c oxidase I (COI) and other molecular markers have been used in phylogenetic studies. Fragment size of COI has been shown to provide high resolution to identify cryptic species, thereby, increasing taxonomy-based biodiversity estimates and its usefulness has been confirmed for Coleoptera, Diptera, Ephemeroptera, Hemiptera, Hymenoptera and Lepidoptera. However, limited studies

has been conducted on the genetic diversity of date palm dubas. A recent study assessed several genomic regions to study the diversity of dubas collected from a number of countries [15]. In addition, a total of 82 partial sequences of the mitochondrial COI gene were deposited in the Genbank [16], which represent dubas collected from several countries in West Asia and North Africa. The aim of this study was to assess genetic diversity between three dubas bug populations from Oman and Jordan using an inter-simple sequence repeat (ISSR) marker and cytochrome oxidase I.

MATERIALS AND METHODS

Total Genomic Isolation. The dubas bug insects used for the genetic diversity study were collected from three populations; Batinah and Daklia regions from Oman and Safi from Jordan (Fig. 1). Three methods were investigated to isolate total genomic DNA from dubas bugs. Promega like (USA) was assayed with some modifications. Individual insects were transferred into 1.5 ml micro-tubes. A small amount of sand was added and a mix of 80 µl nuclei lysis solution, 20 µl 0.5 M EDTA (pH 8) and 4 µl 20 mg/ml proteinase K was added for each isolate. The insects were ground carefully with micro-pestle and incubated at 55ºC on dry bath for 1 h. Thereafter, 3 µl of 10 ml/ µl RNase solution was added and mixed well followed by incubation for 30 min at 37ºC. An amount of 35 µl protein precipitation solution was added. The mixture was vortexed then centrifuged for 4 min. After centrifuge, the supernatant was transferred into a fresh tube and 150 µl of iso-proponal was added. The mixture was mixed gently by inversion mixing then centrifuged for 1 min. Supernatant was removed and 150 µl of ice-cold 70% ethanol was added and mixed then centrifuged as before. The ethanol was aspirated and the pellet was dried for15 min. DNA was rehydrated in 30 µl TE buffer (pH 8).

The SDS was the second method, where individual insects were extracted with 10 µl of 2% SDS by heating at 95ºC in the thermocycler for 20 min then were stored at -20ºC. In the third method, freeze-thaw tandem cycles were applied. Single dubas bug insects were mixed with 40 µl of Tris buffer (pH 8) in 0.2 ml PCR tubes. The tubes were incubated at -20ºC for 30 min followed by heating at 95ºC for 10 min in a thermocycler. The freeze-thaw cycles were repeated four to five times.

Polymerase Chain Reaction. DNA was used to amplify polymorphic ISSR marker UBC807 (Table 1). In addition, primer pair was designed for COI gene (Table 1) based on available dubas bug (*Ommatissus lybicus*) sequences deposited in the Genbank (2018).

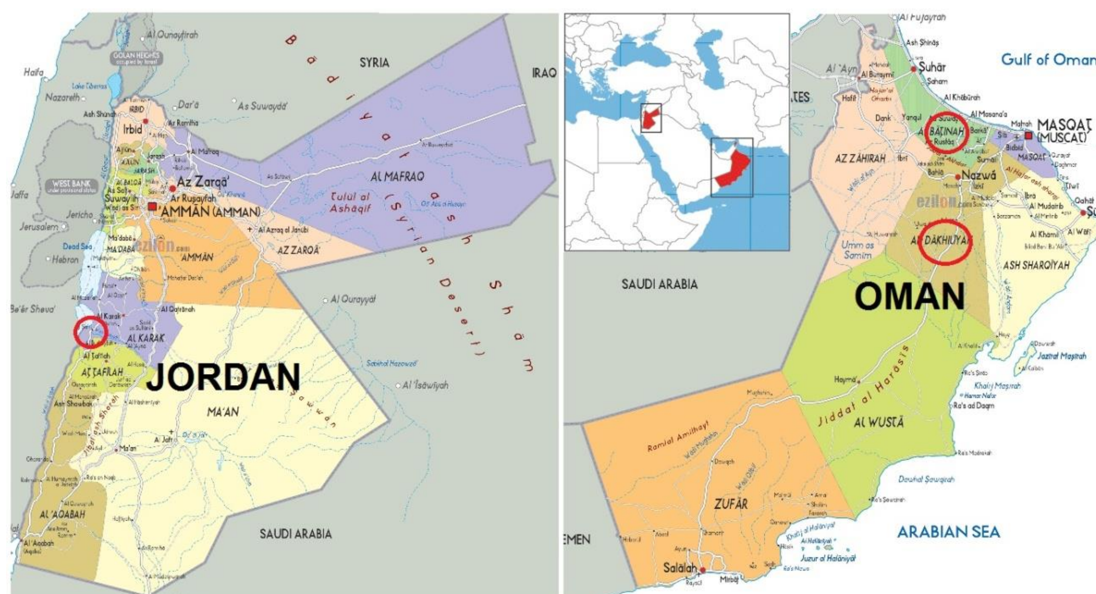

FIGURE 1
Maps of Jordan, Oman and the Middle East. Red circles on the map refer to the sampling points of the *Ommatissus lybicus*. Safi (Jordan), Daklia (Oman) and Batinah (Oman).

TABLE 1
PCR primers utilized in the present study.

Amplicon	Primer code	Sequence (5'–3')
ISSR marker	UBC807	AGAGAGAGAGAGAGAGT
COI gene	O.l. _F01	GGTCAACAAATCATAAAGATATTGG
	O.l._R01	TAAACTTCAGGGTGACCAAAAAATCA

Polymerase chain reaction (PCR) was performed using 2 µl of HOT FIRPOL® Blend Master Mix (Solis BioDyne), 1 µl of each of forward and reverse primers (10 µM), 1 µl of DNA (10 ng/µl) and the volume was completed to 10 µl with water. PCR program for COI was used as follow: initial denaturation at 95Cº for 5 min followed by 35 cycles contain denaturation at 95ºC for 30 sec, annealing at (40, 45, 50, 55 or 60ºC) for 30 sec, extension at 72ºC for 1 min and a separate final extension cycle at 72Cº for 10 min. While the PCR program for ISSR was as follows: 1 cycle of 94°C/5 min, 45 cycles of (94°C/1min, 50°C/45 sec and 72°C/2 min), 1 cycles of 72°C/5 min. PCR products were assayed using 1% agarose gel electrophoresis in 1x TAE buffer. The gels were stained with ethidium bromide and examined by UV light using gel documentation system (Alpha-Innotech, USA). Consequently, gels of generated COI amplicons were stained using SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, USA) and bands were cleaned-up using Wizard® SV Gel and PCR Clean-Up System (Promega, USA), the DNA was eluted with 30 µl TE buffer. PCR products were sequenced with chain-terminating method [17] through Macrogen Company (South Korea).

Data Analysis. ISSR amplicons were scored as present (1) or absent (0) over dubas bug isolated. A data matrix was prepared to determine similarities between each pair of isolates using Jaccard similarity measure. The similarity data was presented as heatmap comparisons within and between populations. A dendrogram was constructed based on un-weighted pair group method with arithmetic mean (UPGMA) using functions supported in the software package SPSS version 11 (IBM, USA).

Bidirectional COI sequences were used to build contigs using *Vector NTI* Advance® 11.5 (Invitrogen, USA). A total of 29 sequences, representing the three populations from Oman and Jordan, were deposited to the Genbank [16]. They were given the accession numbers MK038837-MK038865. COI sequences from several international *Ommatissus lybicus* isolates were retrieved from the Genbank [16] (NCBI, 2018). In addition, one related species *Trypetimorpha occidentalis* (KX702957) was utilized as an outgroup. All these sequences were subjected to multiple alignment along using ClastalW available in the software BioEdit [18]. Aligned sequence were bootstrapped 100 times and utilized for phylogenetic tree construction based on maximum likelihood function available in Phylip [19], and was viewed by TreeView [20] in 1996.

RESULTS AND DISCUSSION

Three populations of dubas infestation were utilized in this study. Omani populations represent decade-long established infestation, while the Jordanian population represent a more recent infestation. Moreover, the selected regions representing the Omani dubas bug populations vary in the degree of infestation. The Batinah region is known for high infestation compared with relatively low infested Daklia region [21, 22].

Three methods were assayed to isolate total genomic DNA form dubas bug. The Promega kit delivered detectable DNA quantities using gel electrophoresis. This was achieved only using a mass sample of 20-30 insects. However, for single insect DNA isolation, it was impractical and DNA was lost, because the kit requires several centrifugation and separation steps followed by DNA precipitation using ethanol. There for it would be practical for larger insects like beetles [12]. Therefore, DNA could not be detected. Likewise, the SDS method was unsuccessful, as it delivers inhibiting concentrations of SDS for the PCR amplifications. On the other hand, the freeze-thaw protocol was the most suitable for single insect extraction. And was adopted for amplification of both ISSR marker and COI gene. Other genomic isolations method such as CTAB were recorded fro dubas bug [15].

The UBC807 ISSR marker was used to investigate genetic diversity within and between investigated populations. Insect isolates from Daklia (Oman) region showed huge similarity with population, while the two other regions revealed huge diversity within populations. Therefore, Batinah (Oman) and Safi (Jordan) populations were advanced for ISSR analysis. The UBC807 ISSR marker was able to generate 17 polymorphic bands. And a total of 561 data entries (155 present and 406 absent bands) were utilized to calculate Jaccard similarity index (Fig. 2). There was huge diversity for insect isolates within Batinah (Oman) population, which was indicated by low similarity values (Fig. 2). Nonetheless, few isolates showed relatively higher similarities, e.g. B10 and B11, B12 and B14. On the other hand the insect isolates from Safi (Jordan) population showed higher within population similarities as compared to (Oman) population, with the highest similarity recorded between J08 and J09 (73%). However, few isolated showed unique ISSR profiles, e.g. J09. This could be due to the intensive insecticide application in date palm orchards in Oman as compared to Jordan [21, 22]. The between population diversity revealed medium similarities (Fig. 2). Some high similarities were achieved between the two populations, J09 and B06 (100%). On the other hand, several isolates did not show any similarity between the two populations, e.g. J04 and B02, J19 and B14.

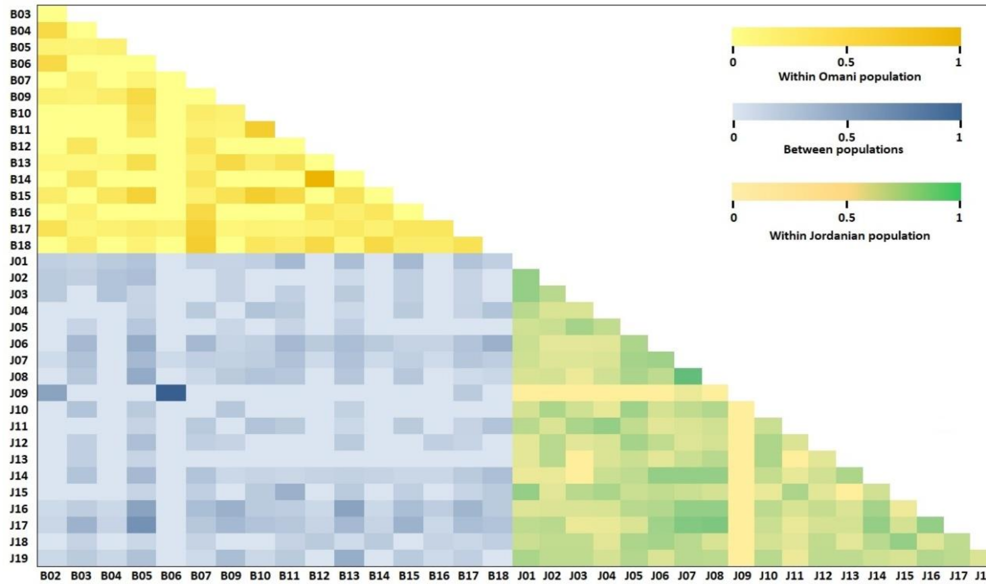

FIGURE 2

Jaccard's similarity index represented as heatmap generated from ISSR marker UBC807 data. Which contains 15 insect isolates for Batinah population from Oman (B) and 19 insect isolates for Safi population from Jordan (J).

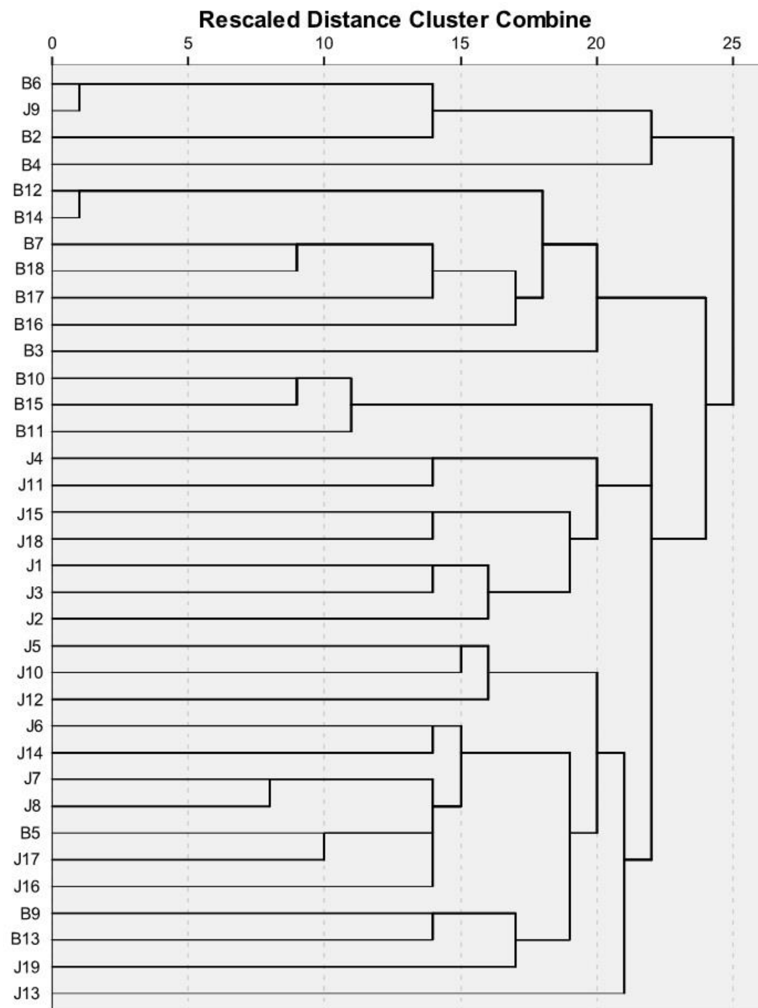

FIGURE 3

A dendrogram constructed with 15 insect isolates for Batinah population from Oman (B) and 19 insect isolates for Safi population from Jordan (J) using UPGMA method.

The UPGMA constructed dendrogram revealed several clustering of dubas bug isolates from Batinah (Oman) and Safi (Jordan) populations (Fig. 3). There was a nice separation between the two populations, where 13 insect isolate out of 15 from Batinah (Oman) were grouped into three major clusters with only one Jordanian isolate (J09). On the other hand, all dubas bug isolates from Safi (Jordan) population (except J09) were clustered together within four major clusters with just three Omani isolates (B5, B9 and B13). This nice separation indicate the effect of intensive pest management including application of insecticides of genetic drift and diversity, which was recorded earlier for dubas bugs in Iran [15] and *Aedes aegypti* in Martinique Island Colombia, respectively [23,24].

The COI primer pair have generated a single clear and reproducible PCR amplicons for all insect isolated from all collection sites (Batinah (Oman), Daklia (Oman) and Safi (Jordan) (Fig. 4).

Therefore, random isolates were picked for each location to sequence the generated COI gens. All delivered bi-directional sequencing data for COI gene were successfully assembled using Vector NTI software (Invitogen, USA). The first phylogenetic tree represents insect isolates from Safi (Jordan) population (Fig. 5).

The tree revealed the separation of the isolate 07 from all other isolates with very high confidence (100% bootstrap value). The remaining isolates were clustered into two major clades, while 01 isolate was not clustered with them. The second phylogenetic tree represents insect isolates from Batinah and Daklia (Oman) regions, in addiiton to some unassigned insect isolates (Fig. 6).

The tree resolved four major clades and twoi un-clustered isolates (Batinah 11 and X 08). The isolates from the two regions did not separate well, but rather there was some integration between the two populations.

When constructing a phylogenetic tree for all populations, we get relatively a better separation between Jordanian Safi population and the two Omani populations (Batinah and Daklia) (Fig. 7).

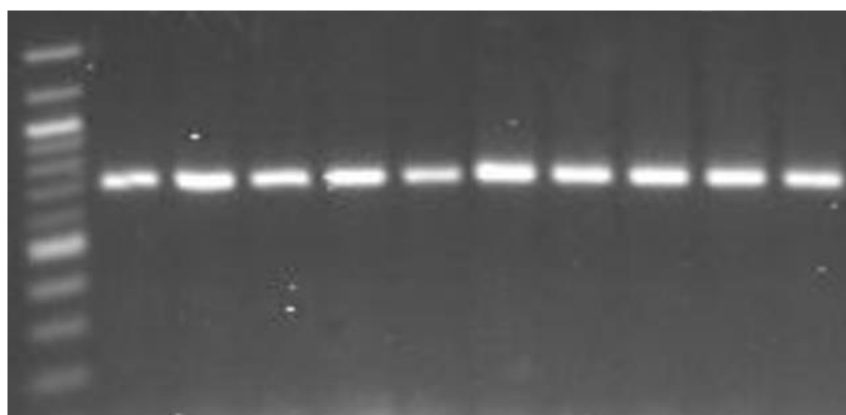

FIGURE 4
Gel electrophoresis for COI gene using dubas bugs collected from Jordan. Left lane represents 1 kb DNA ladder.

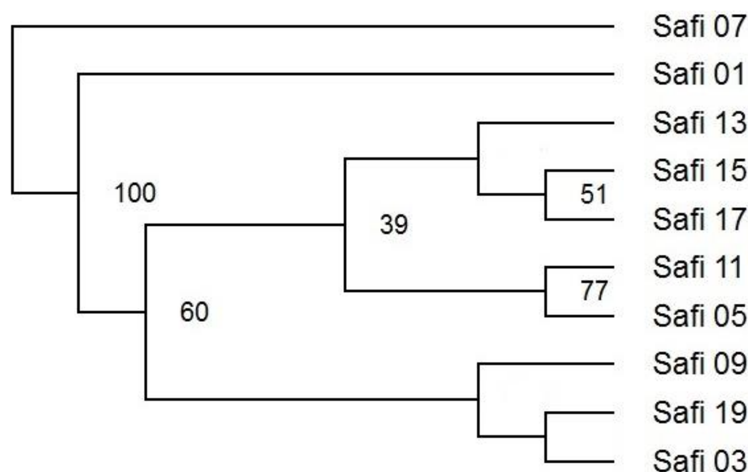

FIGURE 5
COI gene-based maximum likelihood phylogenetic tree for ten insect isolates for Safi population from Jordan. Numbers represent bootstrap values (100 times), values <30% were omitted.

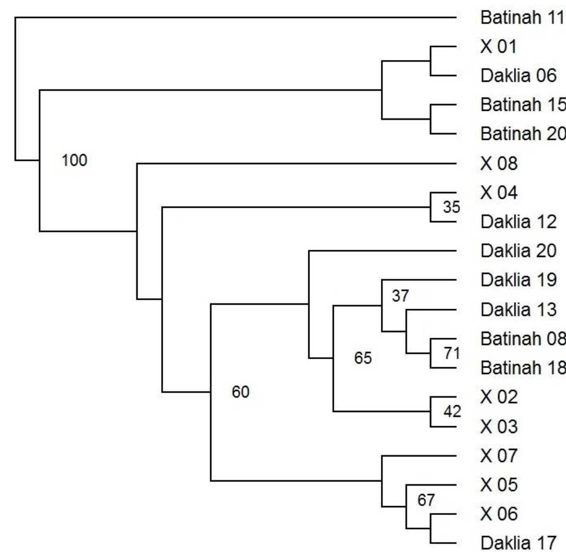

FIGURE 6
COI gene-based maximum likelihood phylogenetic tree for six, five and eight insect isolates for Daklia, Batinah and unassigned populations from Oman. Numbers represent bootstrap values (100 times), values <30% were omitted.

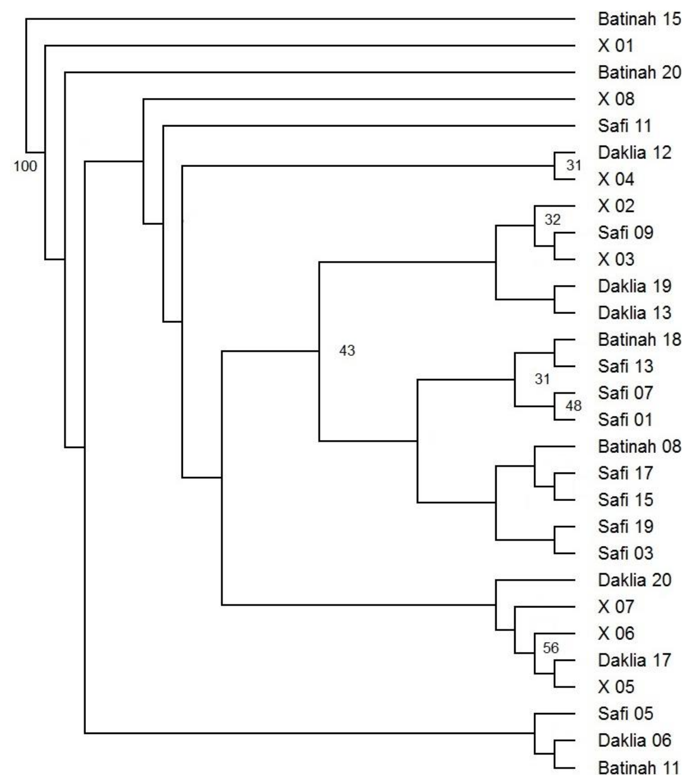

FIGURE 7
COI gene-based maximum likelihood phylogenetic tree for six, five and eight insect isolates for Daklia, Batinah and unassigned populations from Oman, in addition to ten insect isolates for Safi population from Jordan. Numbers represent bootstrap values (100 times), values <30% were omitted.

However, it did not have that strong separation as revealed in the ISSR dendrogram (Fig. 2). This is presumably possible due the higher genetic resolution delivered by ISSR [25, 26] as compared to the more conservative COI with limited haplotypes generated by limited number of mutations as recorded for dubas bug earlier [15].

The final phylogenetic tree represents insect isolated from Oman populations (Batinah and Daklia) and Jordan population (Safi), along with international isolates Egypt, Tunisia, Iran and Pakistan from sequences deposited Genbank (Fig. 8).

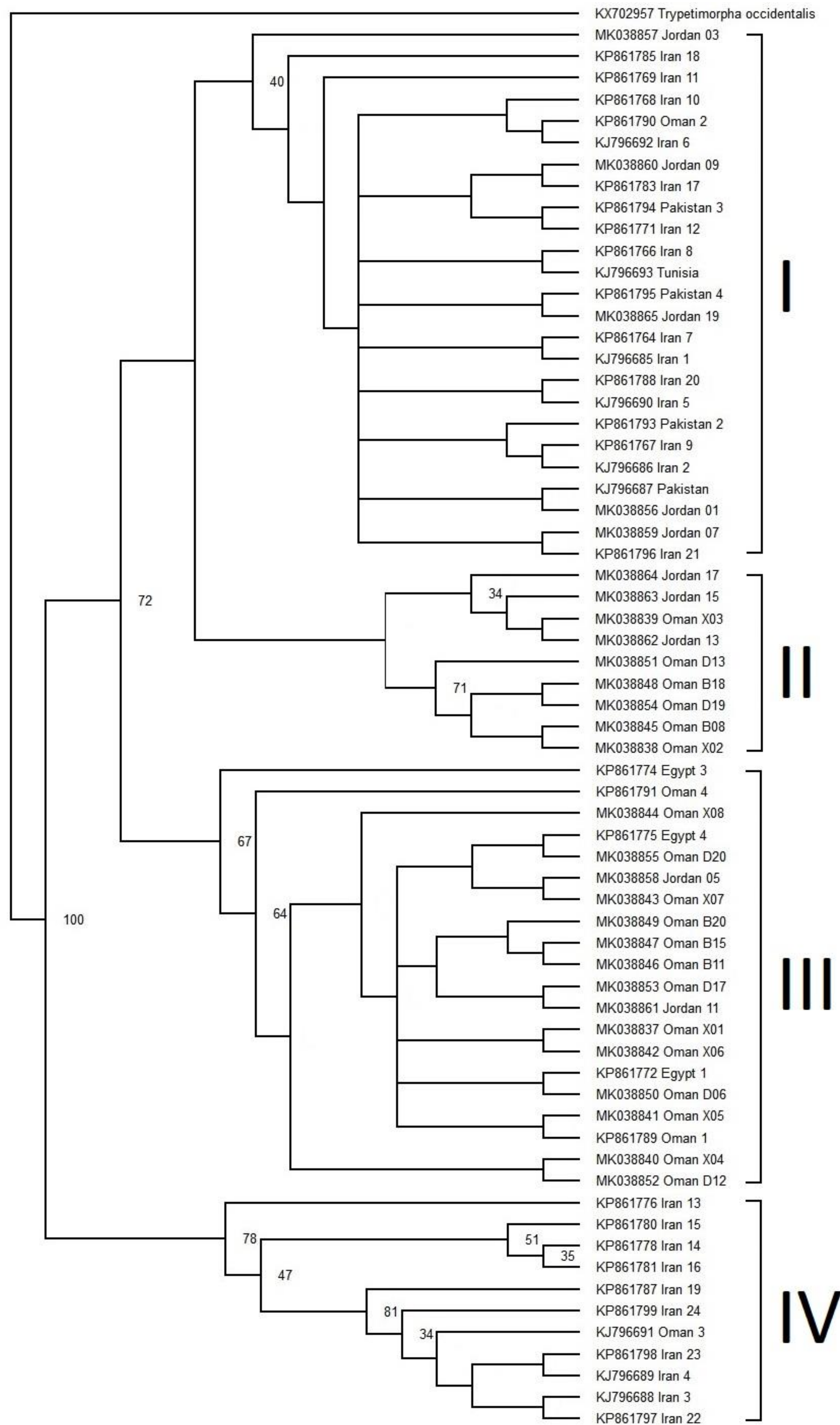

FIGURE 8

COI gene-based maximum likelihood phylogenetic tree for six, five and eight insect isolates for Daklia, Batinah and unassigned populations from Oman and ten insect isolates for Safi population from Jordan, in addition to international isolates from several countries. The *Trypetimorpha occidentalis* (KX702957) served as an outgroup entry. Numbers represent bootstrap values (100 times), values <30% were omitted.

COI of *Trypetimorpha occidentalis* (KX702957) served as an outgroup entry and was successfully separated and did not cluster with any other isolates. The insect isolates were clustered into four major clades. The first clade (I) clustered dubas bug isolates from Oman, Jordan, Tunisia, Iran and Pakistan but not from Egypt. However, both the Jordanina isolate 03 and the Iranian isolate 18 did mot grouped within the same clade with any other isolate, indicating their diverse nature. The second clade (II), was clustering isolated only from Oman and Jordan. This clade was divided into two subclades, one has three Jordanian and one Omani isolate, while the other one has pure Omani isolates (five of them). The third clade (III) revealed more interesting group, where 15 Omani isolates were clustered with three Egyptian isolates and one Jordanian isolate. The final clade (IV) was also almost pure, where ten Iranian dubs bug isolates were clustered with single Omani isolate. Our data agrees with earlier reports [15], nonetheless, were deliver more detailed information about the Omani dubas bug populations. The huge diversification between the most investigated dubs bug population from Iran (Clade IV) and Oman (Clade III), could be related to the geographical separation between the two populations and the lack of long distance flight ability of *O. lybicus* [27], which may intensify the fast evolution process and formation of diverged populations undergoing intensive pesticides application [15].

CONCLUSIONS

In the present study a versatile genomic DNA isolation procedure (Freeze-thaw method) from small-size insect isolates were developed, which worked well with both alcohol preserved and dried insect isolates. The ISSR DNA marker analysis revealed incredible genetic diversity within and between investigated insect populations from Oman and Jordan. The phylogenetic analysis based on COI gene sequence confirms the immense genetic diversity within and between investigated insect populations from Oman and Jordan, while revealing the uniqueness of Omani populations as compared with other international dubas bug isolates. It is worth further investigating the diversity of dubas bud base on polymorphism within the ribosomal DNA sequence as it would deliver additional diversity window and would resolved clustered international isolated based on COI gene.

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The authors have declared no conflicts of interest.

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