

Fingerprinting of Leafhoppers on Medicinal and Aromatic Plants in Egypt Using ISSRs

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ABSTRACT

Leafhoppers are one of the most important agricultural insect pests. Traditional morphological criterion for leafhoppers identification depending on the presence of males only. So, Inter Simple Sequence Repeats (ISSRs) were used to find diagnostic markers for fingerprinting fifteen leafhoppers species collected from different medicinal and aromatic plants in Egypt. Seven ISSRs primers were successfully produced 72 bands those could be used to differentiate the fifteen different leafhopper species. Also different amplified bands with 65 diagnostic morphological characters were used to determine the phylogenetic relationship among the different species; that divided into two main clusters. ISSR-PCR technique could be successfully used with morphological characters to fingerprint and identify these leafhopper species using any life stage

Keywords: Leafhoppers, fingerprinting, morphology, ISSRs, identification, markers, microsatellites, phylogeny.

INTRODUCTION

Leafhoppers (Hemiptera: Auchenorrhyncha: Cicadellidae). Family Cicadellidae is a globally-distributed group of sap-feeding insects that contains 20000 described species (Dietrich, 2013). They suck plant sap from the xylem, phloem or mesophyll cells (Knight 1983) causing a drying of the leaf tissue. Leafhoppers release their toxic saliva into the plant tissue causing leaves turn yellow, their edges dry and their tissue dies “hopper burn.” and the plant becomes stunted (Ebesu 2004) and cause serious plant injury either directly through feeding or indirectly by transmitting plant pathogens including viruses, bacteria and phytoplasmas (Weintraub and Beanland, 2006). Identification of leafhopper species is mainly based on adult male especially genitalia characters. While another additional characters, such as coloring, details in head and elytral characters are also important in the identification on generic level (Herakly 1970). Identification of Cicadomorphan species is difficult because of their tremendous diversity and the paucity of comprehensive identification keys (Dietrich, 2005). The classical taxonomy proves its reliability but has limitations, such as, requirement of adult specimens especially males for morphological analysis. As well as morphology and high genetic diversity poses problems in phylogenetic studies of insects (Pires and Marinoni, 2010). To solve these problems, DNA based markers have been adopted and are increasingly used as molecular markers for fingerprinting and detecting phylogeny among species (De León and Jones, 2004; De León *et al.*, 2004; De Mandal *et al.*, 2014; Sreejith and Sebastian, 2015). The present work aims at fingerprinting and detecting phylogenetic relationships among different leafhopper species infest medicinal and aromatic plants in Egypt.

MATERIALS AND METHODS

Survey of leafhopper species:

Field survey of leafhopper species was carried out during three successive years from 2013 to 2015 in different localities and different medicinal and aromatic plants of Egypt.

Samples were caught using the sweep net and aspirator from each plant then were transferred to the laboratory where individuals of leafhopper were mounted on slides for identification using available keys. Each species was put in especial tube and preserved at -20°C until molecular analysis.

ISSR-PCR Analysis:

a. DNA Extraction

Fifteen different species of leafhoppers samples were collected and extracted DNA from them. Animal tissues were ground under liquid nitrogen to a fine powder, and then bulked DNA extraction was performed using DNeasy Mini Kit (QIAGEN).

b. Polymerase Chain Reaction (PCR)

PCR amplification was performed using seven Inter Simple Sequence Repeat (ISSR) (Table 1).

Table 1. list of primers, names and their nucleotide sequences used to determine fingerprinting of leafhopper species using ISSR- PCR technique.

No	Name	Sequence
1	14A	5' CTC TCT CTC TCT CTC TTG 3'
2	44B	5' CTC TCT CTC TCT CTC TGC 3'
3	HB-08	5' GAG AGA GAG AGA GG 3'
4	HB-10	5' GAG AGA GAG AGA CC 3'
5	HB-12	5' CAC CAC CAC GC 3'
6	HB-14	5' CTC CTC CTC GC 3'
7	HB-15	5' GTG GTG GTG GC 3'

Amplification was conducted in 25 µL reaction volume containing the following reagents: 2.5 µL of dNTPs (2.5 mM), 2.5 µL MgCl₂ (2.5 mM), and 2.5 µL of 10 x buffer, 3.0 µL of primer (10 pmol), 3.0 µL of template DNA (25 ng / µL), 1 µL of *Taq* polymerase (1U/ µL) and 10.5 µL of sterile dd H₂O. The DNA amplifications were performed in an automated thermal cycle (model Techno 512). The PCRs were programmed for one cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C. The reaction was finally stored at 72°C for 10 min. Amplified products were size-fractionated using ladder marker 100 bp (1000, 900, 800, 700, 60, 500, 400, 300, 200 and 100 bp) by electrophoresis in 1.5% agarose gels in TBE buffer at 120 V for 30 min. The bands were visualized by ethidium bromide under UV fluorescence and photographed.

Densitometry Scanning and Analysis:

All gels resulted from DNA fingerprints, were scanned using Bio-Rad GelDoc2000 to calculate the pair-wise differences matrix and plot the dendrogram among different leafhopper species.

Phylogenetic relationship among different leafhopper species:

Diagnostic Morphological characters:

For determining the phylogenetic relationships among those leafhopper species, sixty-five diagnostic

morphological characters were compiled according to the previous mentioned identification keys in addition some diagnostic characters were added according to this work (Table 2).

All specimens used for this study were recently collected. The use of this fresh material allowed for thorough manipulation under the binocular for morphological data obtained and for extraction of genetic material.

Table 2. Index of morphological characters used in determining similarity Matrix among fifteen leafhopper species.

No	Morphological characters	Presence (1) or absence (0).
1	Males only	females and nymphs (0) males (1)
2	Females only	males only and nymphs (0) females (1)
3	Nymphs only	males and females(0) nymphs (1)
4	Vertex shape 1	triangular (1) not triangular (0)
5	Vertex shape 2	rounded (1) not rounded (0)
6	Vertex shape 3	rectangular (1) not rectangular (0)
7	Vertex spots	with spots (1) without spots (0)
8	Vertex band	with band(1) without band (0)
9	Vertex depressions	with depression(1) without depressions (0)
10	Vertex pits	with pits(1) without pits (0)
11	Vertex markings	with markings (1) without markings (0)
12	Vertex with spots 1	more than two spots (1) with two spots (0)
13	Vertex with spots 2	with six spots (1) with three spots (0)
14	Vertex with band	with two bands (1) with one band (0)
15	Vertex with depression	with three depressions (1) with two depressions (0)
16	Head width	as wide as pronotum (1) not as wide as pronotum (0)
17	Head width	wider than pronotum(1) smaller than pronotum (0)
18	Pronotum spots	with spots (1) without spots (0)
19	Pronotum depressions and pits	with depressions and pits (1) without depressions and pits (0)
20	Pronotum band	with band(1) without band (0)
21	Pronotum blotches	with blotches (1) without blotches (0)
22	Pronotum arc	with arc (1) without arc (0)
23	Forewing length in male	very long(1) not very long (0)
24	Fore-wing shape	rounded (0) tapered (1)
25	Appendix	present (1) absent (0)
26	Appendix present	extending around apex (1) restricted to anal margin (0)
27	Number of apical cells	four (1) three (0)
28	Closed subapical cells	with closed subapical cells (1) without closed subapical cells (0)
29	Number of closed subapical cells	two (1) one (0)
30	Open subapical cell	central subapical cell open (1) inner subapical cell open (0)
31	Cross vein	present (1) absent (0)
32	Number in hind wing of apical cells	with more than one apical cell (1) with one apical cell (0)
33	Number in hind wing of apical cells	with four apical cells (1) with three apical cells (0)
34	scutellum spots	with spots (1) without spots (0)
35	Scutellum with shapes	with two a triangular shapes (1) without a triangular shape (0)
36	scutellum depressions and pits	with depressions and pits (1) without depressions and pits (0)
37	Abdominal apodeme	with abdominal apodeme (1) without abdominal apodeme (0)
38	Apodeme length	as long as 4th sternum abdominal segment (1) as long as two abdominal segment (0)
39	Apodeme shape	cresecentically diverging towards apex (1) slightly diverging (0)
40	Pygopher length according to genital plate	long(1) short (0)
41	Pygopher	fused (1)not fused (0)
42	Pygopher macroseatae 1	more than one row (1) reduced one row (0)
43	Pygopher macroseatae 2	uniseriate (1) scattered (0)
44	pygofer macroseatae 3	6long and 4 short (1) 5 long and 3 short (0)
45	Pygopher spines	with spines (1) without spines (0)
46	Pygofer appendages	with tapered appendages (1) without tapered appendages (0)
47	Genital plate spines	with spines (1) without spines (0)
48	Genital plate claw	with sclerified claw (1) without claw (0)
49	Stylus number	with two pair of stylus (1) with one pair of stylus (0)
50	Stylus size	broad (1) narrow (0)
51	Stylus length	long (1) short (0)
52	Stylus preapical lobe	present (1) absent or undeveloped (0)
53	Stylus preapical lobe	long (1) short (0)
54	Stylus apophysis	long (1) short (0)
55	Apophysis shape	claw-like (1) pointed (0)
56	Apophysis	curved (1) not curved (0)
57	Connective	fused to aedeagus (1) articulated with aedeagus (0)
58	Lateral anterior arms of connective	divergent (1) closely appressed anteriorly (0)
59	Lateral anterior arms of connective	divergent y-shaped (1) divergent u-shaped (0)
60	Aedeagus	bifid(1) not bifid (0)
61	Aedeagus bifid arms	long(1) short (0)
62	Aedeagus end	curved(1) not curved (0)
63	Aedeagus end	tapered at the end (1) not tapered at the end (0)
64	Aedeagus spines	with spine (1) without spine (0)
65	Aedeagus spines	with 2 ventral spines (1) with 3 ventral spines (0)

Currently only males can be identified to species. Sometimes, females and nymphs specimens were examined and identified to genus by Dr. Christopher H. Dietrich of the Illinois Natural History Survey, USA.

ISSR-PCR Data analysis:

The similarity matrix was done using Gel Works ID Advanced Software UVP-England Program. The relationships among genotypes as revealed by dendrogram was done using SPSS Windows (Version 10) Program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among species or genus (Yang and Quiros, 1993).

RESULTS AND DISCUSSION

After surveying of leafhoppers individuals from different medicinal and aromatic plants and different localities, they subjected to identification using available keys.

Fifteen leafhopper species were identified and listed alphabetically by scientific name in (Table 3).

Initial screening of many numbers of ISSR markers on fifteen samples of cicadellid resulted in seven ISSR primers those produced informative and polymorphic products resolvable by agarose gel electrophoresis (Fig. 1). These seven markers were amplified 73 bands as follows:

Table 3. Fifteen leafhopper species on medicinal and aromatic plants in Egypt arranged according to their subfamilies alphabetically.

Subfamily	Tribe	
	Chiasmmini	<i>Aconurella prolix</i> (Lethierry)
		<i>Exitianus pondus</i> Ross
		<i>Nephotettix modulates</i> Melichar
	Hecalini	<i>Parabolocralis</i> sp. Evans
		<i>Balclutha frontalis</i> (Ferrari)
		<i>Cicadulina bipunctella zae</i>
Deltocephalinae	Macrostelini	China
		<i>Cicadulina chainai</i> Ghauri, <i>Macrosteles sexnotatus</i> (Fallen)
	Opsiini	<i>Orosius albicinctus</i> Distant
	Paralimnini	<i>Psammotettix alienus</i> (Dahlbom)
	Scaphoideini	<i>Neolimnus aegyptiacus</i>
Typhlocybinae	Typhlocybini	<i>Empoasca decipiens</i> Paoli
		<i>Eupteryx cypria</i> Ribaut
Ulopiinae	Ulopiini	<i>Megulopa sahlbergorum</i> Lindberg
		<i>Austroagallia</i> sp. Evans

14-A Primer:

The results of ISSR analysis using 14A primer indicated that this primer produced seven bands with molecular sizes ranged between 1337-342bp. All of them were polymorphic (100%) except band with molecular size of 1337 bp. which can be assigned as a positive marker for *C. chinai*. No common band was detected by this primer. The highest number of bands were four which occurred in five species (*E. pounds*, *C. chinai*, *A. prolix*, *N. modulates* and *P. alienus*). No band was recorded for *N. aegyptiacus*.

44-B Primer:

The results of ISSR marker analysis by using 44B primer showed that this primer amplified ten bands with molecular sizes ranged between 919 – 224bp. Nine of them were polymorphic (90%). On the other hand, band with molecular size of 384 bp. was monomorphic (common). The band with molecular size of 307bp. can be assigned as a negative marker for *N. aegyptiacus*. The highest band numbers were nine recorded in *N. modulates* while the lowest band numbers were three detected in *M. sahlbergorum*.

HB-08 Primer:

The results of ISSR analysis using HB-08 primer were indicated that this primer amplified ten bands with molecular sizes ranged between 734 – 174bp. Nine of them were polymorphic (90%). Band with molecular size of 689 bp. was unique so can be assigned as a positive marker for *A. prolix*. While bands with molecular sizes of 332 and 281bp. assigned as negative markers for *Austroagallia* sp. and *C. bipunctella zae*, respectively. No common band was detected by this primer. The highest number of bands was eight detected in *E. pondus*, *N. aegyptiacus*, *E. cypria*, *M. sexnotatus*, *B. frontalis*, *C. chinai* and *O. albicinctus*; while the lowest number of bands was three bands detected in *N. modulates*.

HB-10 Primer:

This primer amplified nine fragments with molecular sizes ranged between 946 – 288bp. All of them were polymorphic (100%). The highest number of bands was 6 bands detected in *A. prolix*, *E. decipiens*, *N. modulates*, *P. alienus* and *M. sahlbergorum*, while the lowest band numbers was two bands in *Austroagallia* sp., *N. aegyptiacus* and *C. bipunctella zae*. No common or unique band was amplified by this primer.

HB-12 Primer:

This primer amplified 12 bands with molecular sizes ranged between 1672 – 167 bp. Six of them were polymorphic. Band with molecular size of 1672 bp. can be assigned as a positive marker and unique band for *Parabolocralis* sp. Also three bands with molecular sizes of 759, 670 and 613bp can be assigned as positive markers and unique bands for *C. chinai*. Bands with molecular sizes of 364 and 276 bp. could be assigned as positive markers and unique bands for *A. prolix*. and *P. alienus*, respectively. The highest number of bands was six detected in *P. alienus*, while the lowest number of band was one band was detected on *Austroagallia* sp., *E. pondus*, *N. aegyptiacus*, *E. cypria*, *M. sexnotatus*, *B. frontalis*, *C. bipunctella zae* and *O. albicinctus*.

HB-14 Primer:

This primer amplified ten bands with molecular sizes ranged between 922 – 252bp. Eight of them were polymorphic (80%) while bands with molecular sizes of 922and 294bp. were unique where these bands can be assigned as positive markers for *Austroagallia* sp. and *C. bipunctella zae* respectively. The highest number of bands was six detected in *Austroagallia* sp. while only one band was detected in *E. pondus*. No common bands were detected.

HB-15 Primer:

This primer amplified fifteen bands with molecular sizes ranged between 1542 – 315bp. Nine of them were polymorphic while bands with molecular sizes of 980 and 538bp. were common bands. On the other hand, band with molecular size of 315bp. can be assigned as a negative marker for *Austroagallia* sp. The highest number of bands was nine detected in *A. prolixa* and *N. modulates*, while the lowest number of bands was three detected in *Austroagallia* sp.

From the above mentioned results it could be stated that six primers out of the seven tested primers had unique bands with certain molecular sizes and can be assigned to identify 7 species out of 15 cicadellid species as follows:

14-A primer at molecular size 1337bp. and HB-12 primer at molecular sizes 759, 670 and 613bp. can be assigned *Cicadulina chinai*. While 44-B primer at 384 bp. could be used to identify *Neolimnus aegyptiacus*. Also, HB-08 primer at 689 bp. and HB-12 primer at 364bp. could be assigned *Aconurella prolixa*. HB-12 primer at 1672 bp. and 276bp. could be assigned *Parabolocralalis* sp. and *Psammotettix alienus*, respectively. While HB-14 primer at 922bp. and HB-15 primer at 315bp. could be identified *Austroagallia* sp. Also, HB-14 primer at 294bp. identified *Cicadulina bipunctella zae*.

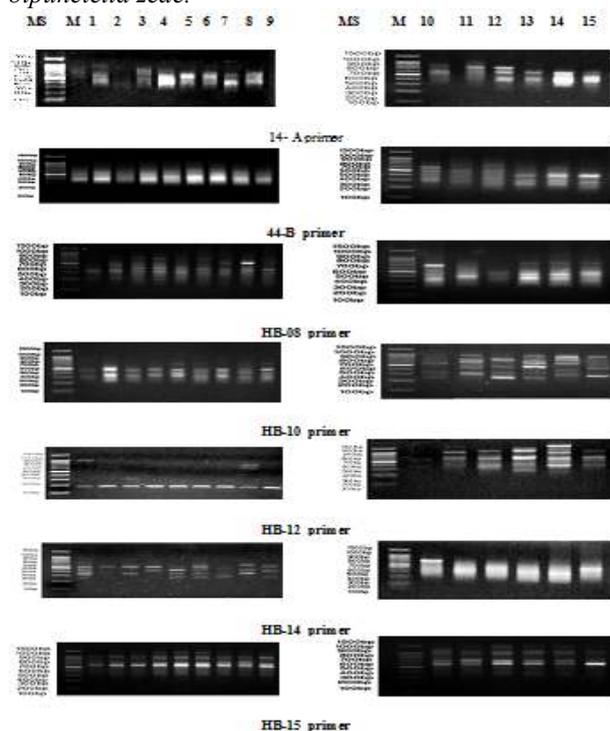


Fig.1. The ISSR-PCR banding patterns of fifteen leafhopper species amplified by seven primers. MS, molecular size; M, marker; 1, *Austroagallia* sp.; 2, *E. pondus*; 3, *N. aegyptiacus*; 4, *E. cypria*; 5, *M. sexnotatus*; 6, *B. frontalis*; 7, *C. bipunctella zae*; 8, *C. chainai*; 9, *O. albicinctus*; 10, *A. prolixa*; 11, *E. decipiens*; 12, *N. modulates*; 13, *P. alienus*; 14, *Parabolocralalis* sp.; 15, *M. sahlbergorum*.

Phylogenetic relationships and similarity matrix based on Morphological Characters and Molecular (ISSR-PCR) analysis:

Genetic similarities and Phylogenetic relationships among the fifteen leafhopper species were based on data gathered from analysis of 65 diagnostic morphological characters and seven molecular markers ISSR-PCR (Table 4). The most close relationship was scored among *C. bipunctella zae* and *C. chinai*. The highest similarity value was 80.3% among the previous two species and the lowest similarity value was 26.3% among *C. bipunctella zae* and *M. sahlbergorum* individuals.

The phylogenetic dendrogram in **Figure 2** branched into two main clusters the first cluster divided into two sub-clusters. The first sub-cluster separated into two sub-sub-clusters. The first sub-sub-cluster, *E. decipiens* was found alone. The second sub-sub-cluster divided into two clades within the first clade *A. prolixa* was standed alone while the second clade included *P. alienus* and *N. modulates*. The second sub-cluster included *M. sahlbergorum* and *Parabolocralalis* sp. which were grouped together. The second cluster separated into two sub-clusters. The first sub-cluster *Austroagallia* sp. was located alone. Meanwhile, the second sub-cluster separated into two sub-sub-clusters. The first sub-sub-cluster *N. aegyptiacus* and *E. pondus* were grouped together. The second sub-sub-cluster divided into two clade the first clades, contained *M. sexnotatus* and *E. cypria* which were grouped together and the second clade divided into two sub-clades. The first sub-clade *O. albicinctus* and *B. frontalis* were grouped together. The second sub-clade contained *C. chinai* and *C. bipunctella zae*.

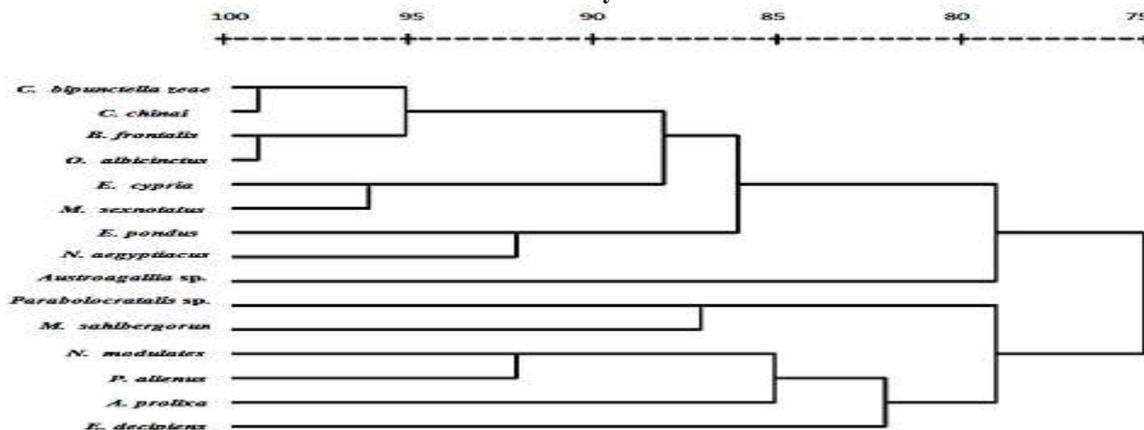
In addition the combined data markers represented in Table 4 could be concluded that the both criteria could be used to discriminate between leafhopper species that belong to the same genus. Also it could be successfully separated between the fourteen genera that included the fifteen leafhopper species.

Inter Simple Sequence Repeats (ISSRs or ‘microsatellites’) has shown much promise for the study of the plant population (Clausing *et al.*, 2000; Hess *et al.*, 2000). Also ISSR-primers have been widely used for DNA fingerprinting and assessing genetic diversity in closely related germplasm (Blair *et al.*, 1999; Charters *et al.*, 1996). While in animals, ISSR technique broadly used as intraspecific markers for animal populations (Abbot, 2001; Ardeh, 2013; De León and Jones, 2004; De León *et al.*, 2004a& b; Kostia *et al.*, 2000 and Reddy *et al.*, 1999). On the other hand few researchers used ISSR markers as interspecific markers (Helmi and Khafaga, 2011 and Luque *et al.*, 2002). However study of ISSRs used only to detect DNA polymorphisms in *Homalodisca coagulata* populations (De León and Jones, 2004; De León *et al.*, 2004). ISSR-primers can be used as universal primers, which do not need to be adapted to individual species like in microsatellite marker. Consequently, the production of large numbers of fragments, reproducibility, and low cost are considered as advantages of the ISSR primers (Moreno *et al.*, 1998 and Weng *et al.*, 2007).

Table 4. Similarity matrix percentages among fifteen leafhopper species based on both diagnostic morphological characters and ISSRs markers

Species	<i>Austroagallia</i> sp.	<i>E. pondus</i>	<i>N. aegyptiacus</i>	<i>E. cypria</i>	<i>M. sexnotatus</i>	<i>B. frontalis</i>	<i>C. bipunctella zeae</i>	<i>C. chinai</i>	<i>O. albicinctus</i>	<i>A. prolixa</i>	<i>E. decipiens</i>	<i>N. modulatus</i>	<i>P. alienus</i>	<i>Parabolocrotalis</i> sp.	<i>M. sahbergerorum</i>
<i>Austroagallia</i> sp.															
<i>E. pondus</i>	53.8														
<i>N. aegyptiacus</i>	52.4	69													
<i>E. cypria</i>	48.9	58	58												
<i>M. sexnotatus</i>	58.7	61	62	75											
<i>B. frontalis</i>	49	65	63	62.7	72										
<i>C. bipunctella zeae</i>	45.8	59	55	56.9	64	79									
<i>C. chinai</i>	43.4	56	52	52.4	64	73.1	80.3								
<i>O. albicinctus</i>	47.4	62	67	56.4	74	80	73.2	70.7							
<i>A. prolixa</i>	41.2	56	41	44.3	48	58.5	54.7	55.1	57.4						
<i>E. decipiens</i>	31.3	40	31	56.3	42	37.8	36.7	38.7	32.7	52.2					
<i>N. modulatus</i>	46	47	40	44.9	55	48.7	37.2	43.9	50.9	53.8	56				
<i>P. alienus</i>	40	53	45	56	58	57.1	48.9	53.9	59.1	62.8	54	68.9			
<i>Parabolocrotalis</i> sp.	28.2	36	31	41.8	45	32.3	24.7	33.6	38.8	42.7	52	59.1	56.6		
<i>M. sahbergerorum</i>	32.9	38	30	32.3	38	31.7	26.3	36.7	32	38.1	47	53.3	50	62.2	

Fig . 2. Dendrogram of phylogenetic relationships for the fifteen leafhopper species based on morphological characters and seven ISSR-PCR markers analysis.



CONCLUSION

Molecular fingerprint of fifteen leafhopper species collected from different medicinal and aromatic plants in Egypt were carried out using ISSR-PCR technique. This technique successfully generated many molecular markers for different leafhopper species; therefore they could be assigned leafhopper species and to differentiate among them in any life stage. This technique in addition to diagnostic morphological characters could be used to detect the Phylogenetic relationship among the fifteen leafhopper species.

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البصمة الوراثية لنشاطات الاوراق على النباتات الطبية والعطرية باستخدام تقنية تتابع التكرارات البينية البسيطة

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تلعب النباتات الطبية والعطرية دورا قيما وهاما من الناحية الاقتصادية . يعتمد تعريف وتمييز أنواع نشاطات الأوراق على الشكل الظاهري لأعضاء التناسل في الذكور لذلك تم استخدام تتابع التكرارات البينية البسيطة ISSR لعمل البصمة الوراثية لخمس عشرة نوعاً من نشاطات الأوراق من النباتات الطبية والعطرية في مصر . انتج ٧ برايمر ٧٢ حزمة ويمكن استخدامها للفرقة بين هذه الانواع . استخدمت ايضا حزم مكونة من ٦٥ صفة تشخيصية مورفولوجية لتحديد صلة القرابة بين الانواع وبعضها . يمكن استخدام تقنية تتابع التكرارات البينية البسيطة مع الصفات المورفولوجية لعمل البصمة الوراثية وتعريف نشاطات الاوراق في اى مرحلة عمرية .