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Identification and Reconsidering Phylogeny of Some Aphid Species, (Hemiptera: Aphididae), Based on Molecular DNA Markers Using ISSRs-PCR Technique

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Abstract: Aphids are considered one of the most economically important insect pests worldwide. Successful pest management systems are based on accurate and rapid pest species identification. Traditional morphological identification of closed aphid species may be considered an inaccurate taxonomic process. To overcome the disadvantages of traditional morphological identification, molecular techniques, related to DNA markers and based on polymerase chain reaction (PCR), were approached by using nine ISSRs primers to identify and diagnose fifteen common aphid species that disperse in the Egyptian agro-ecosystem. The examined ISSRs primers could successfully discriminate the tested aphid species that reflected 61.39% polymorphism among them. Moreover, four banding patterns were considered unique bands that could characterize three aphid species (*Aphis gossypii*, *Aphis nerii* and *Myzus persicae*). The highest genetic homology (84.9%) was observed between species *Rhopalosipum padi* and *Schizaphis graminum*. In addition, each of *A. gossypii* and *Aphis citricola* were also genetically homologous species. In contrast, species *Aphis craccivora* and *M. persicae* were analogous genetically with a low similarity percentile (59.8%). High genetic divergence was observed also between *A. nerii* and *M. persicae*. Two alternative molecular branching taxonomic keys were proposed by subjecting the five highest polymorphic ISSRs primers and 29 banding patterns with different molecular sizes.

1 Introduction

Aphids (Hemiptera: Sternorrhyncha: Aphididae) are considered the most economically important and worldwide insect pests (Emden and Harrington 2007), which are more common in temperate zones (Blackman and Eastop 2000). They are invasive pests, that threaten agricultural production and cause severe crop losses to reach 70-80% (Capinera 2002, Blackman and Eastop 2008, Kinyanjui et al 2016). Direct feeding of aphids on plant sap can not only deprive essential nutrients of the host plant but also transmit 30% of plant viral diseases and inject toxic salivary secretions to host plants (Blackman and Eastop 1994, Brault et al 2010). In addition, sooty molds that result in honeydew secretion can also cause effective depletion in the photosynthesis of host plants and reduce the quality and then marketability of crops.

The family Aphididae consists of more than 4000 species. Aphidinae is the largest subfamily of Aphididae that consists of three major Tribes. Macrosiphini is considered the largest tribe of Aphidinae consisting of more than 2000 species, followed by Aphidini with

750 species then Pterocommatini with 50 species (von Dohlen et al 2006, von Dohlen 2009). Aphidina and Rhopalosiphina are only subtribes of Aphidini.

Successful systems of plant quarantine and pest management are based on accurate and rapid pest species identification (Lozier et al 2008, Miller and Foottit 2009). Classification of aphids at various levels has been conducted by using morphological characters, life cycles and host-plant associations (Blackman and Eastop 1994, Kim et al 2011). Although the absence of morphological characteristic keys or the existence of polymorphic, cryptic, small, immature, and damaged specimens complicated the determination process of aphid species (Armstrong and Ball 2005, Lee et al 2011, Kinyanjui et al 2016), morphological traits were used to identify numerous aphid species (Emden and Harrington 2007). Furthermore, morphological determination of aphid species requires taxonomists with specific knowledge, skills and training which may be acquired in many years, thus related aphid species determination, based on traditional morphological characters, may be time-consuming and considered an inaccurate taxonomic process (Coeur d'acier et al 2014).

To overcome the limitation of traditional morphological identification, molecular techniques, related to DNA markers and based on the amplification of specific regions of the genome by using polymerase chain reaction (PCR) techniques, were approached (Mullis and Faloona 1987) and then organisms such as cryptic invertebrates or immature stages as an egg could be identified (Carew et al 2003 and 2005, Hebert et al 2004). Thus, McNeely et al (2001) reported that using a molecular identification system may assist in the identification procedure of difficult or indistinguishable specimens. The most frequently used molecular markers "allozymes, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), DNA microsatellite (SSR, STR), inter-simple sequence repeats (ISSRs), single nucleotide polymorphisms (SNPs), mitochondrial DNA (mtDNA) and barcoding" have been approached and reviewed to be applied in studying populations and genetic diversity of aphid species (Guo et al 2017).

RAPD-PCR, based on the amplification of random regions of the genome by using a single arbitrary ten-mer primer that flanks randomly with DNA, reveals successfully genetic polymorphisms among different insect species (Williams et al 1990). The diagnostic DNA markers, generated by RAPD-PCR, were used to identify different aphid species that belong to the genus *Aphis* and the Tribe Macrosiphini, in Egypt (Amin et al 2013). Furthermore, it was used to diagnose aphid geographical populations such as *Rhopalosiphum padi* in Egypt (Tabikha and Adss 2016). Using appropriate restriction enzymes to digest PCR product by RFLP-PCR assists to determine numerous aphid species (Valenzuela et al 2007 and 2009, Kinyanjui et al 2016).

Finally, ISSRs may be reliable, polymorphic and unexpansive technique (Raina et al 2001), thus ISSRs have been used for studying taxonomic status of various animal populations. Moreover, Gui et al (2008) reported that ISSR is considered as specific technique to detect genetic variability among insects' species. Thus, it could successfully identify two aphid species "*Pemphigus obesinymphae* and *Acyrthosiphon pisum*" (Abbot 2001), characterize cereal aphid species (Helmi and Khafaga 2011), and differentiate geographical populations for each of *Rhopalosiphum padi* (Tabikha and Adss 2016) and *S. graminum* (Tabikha and Adss 2021) in Egypt. In addition, biotypes of insects such as *Schizaphis graminum* could be also differentiated by ISSRs (Weng et al 2007). The ISSRs were also subjected to the study genetic diversity of *Sogatella furcifera* which considered as main species of plant hoppers (Liu et al 2010). Finally, it was employed to find diagnostic markers for fifteen leafhopper species collected from different medicinal and aromatic plants in Egypt (Helmi et al 2016).

Thus, the current research paper aims to pursue finding diagnostic DNA markers and studying the genetic diversity of the most common aphid species in Egypt based on ISSRs-PCR test. Furthermore, the obtained diagnostic DNA markers are also subjected to construct a molecular branching key that distinguishes the fifteen tested aphid species in Egypt.

2 Materials and Methods

2.1 Specimens' preparation

Molecular genetic analysis for fifteen of the most common and economically important aphid species, classified in Tribes Macrosiphini and Aphidini, were conducted. Viviparous adults of the tested species were monitored on their host plants in different localities of Egypt and were collected and transferred to the laboratory in a glass jar supplemented with parts of the host plant. Presented data in **Table 1** summarize information of taxonomic status, locality, host plant and collection date that related to the tested aphid

Table 1. List of the tested aphid species collection and related data of host plant, geographical information and sampling date of each specimen

Sample Number	Tribe	Species	Host plant	Location	Sampling date
1	Aphidini	*Aphis gossypii* Glover	Eggplant, *Solanum melongena*	31° 25' 20"N, 30° 23' 53"E	May, 2021
2	Aphidini	*Aphis citricola* van der Groot	Orange, *Citrus aurantium*	31° 25' 20"N, 30° 23' 53"E	May, 2021
3	Aphidini	*Aphis nerii* Fonscolombe	Oleander, *Nerium oleander*	31° 12' 17"N, 29° 56' 46"E	June, 2018
4	Aphidini	*Aphis craccivora* Koch	Broad bean, *Vicia faba*	30° 53' 26"N, 29° 58' 56"E	March, 2021
5	Macrosiphini	*Hyperomyzus lactucae* L.	Sowthistle, *Sonchus oleraceus*	31° 19' 42"N, 30° 24' 16"E	April, 2018
6	Macrosiphini	*Brevicoryne brassicae* L.	Cabbage, *Brassica oleracea*	31° 11' 33"N, 30° 30' 52"E	March, 2021
7	Macrosiphini	*Capitophorus elaeagina* Del Guercio	Artichoke, *Cynara scolymus*	31° 05' 22"N, 30° 05' 19"E	March, 2021
8	Aphidini	*Hyalopterus pruni* Geoffry	Peach, *Prunus persica*	31° 25' 20"N, 30° 23' 53"E	May, 2021
9	Macrosiphini	*Pentalonia nigronervosa* Coquerel	Banana, *Musa sapientum*	30° 39' 30"N, 30° 33' 02"E	March, 2021
10	Macrosiphini	*Macrosiphum rosae* L.	Rose, *Rosa hybrid*	31° 01' 46"N, 31° 12' 54"E	June, 2018
11	Macrosiphini	*Sitobion avenae* Fabricius	Wheat, *Triticum aestivum*	31 °19' 42"N, 30° 24' 16"E	March, 2018
12	Aphidini	*Rhopalosipum maidis* Fitch	Wheat, *Triticum aestivum*	30° 01' 03"N, 31° 12' 17"E	March, 2018
13	Aphidini	*Rhopalosipum padi* L.	Wheat, *Triticum aestivum*	28° 06' 44"N, 30° 44' 39"E	March, 2018
14	Aphidini	*Schizaphis graminium* Rondani	Wheat, *Triticum aestivum*	25° 43' 20"N, 32° 37' 43"E	March, 2018
15	Macrosiphini	*Myzus persicae* Sulzer	Potato, *Solanum tuberosum*	30° 01' 03"N, 31° 12' 17"E	March, 2021

specimens. To confirm the taxonomic status of each specimen, mounted alate or apterous forms of the tested aphid adults in swan's gum chloral media on glass slides (Blackman and Eastop 2000), were identified morphologically by using the local taxonomic key of Habib and El-Kady (1961) or universal taxonomic key of Blackman and Eastop (1994) and (2000). Under laboratory conditions (28±2°C, 65±5 RH and 12:12h), a healthy and young adult female from each tested aphid species was caged separately on its host plant to harvest its offspring at age 5 days. The collected offspring were preserved in Eppendorf tubes under -20ºC for use later in molecular studies.

2.2 DNA extraction

Extracted genomic DNA of the fifteen preserved aphid specimens were subjected to a polymerase chain reaction with nine ISSRs primers. Thus, 5-6 individuals of aphid adults were ground to a fine powder, in Eppendorf tubes under liquid nitrogen, the ground material was transferred to tubes (1.5 ml) then 750 µl of extraction buffer "Acetyl Trimethyl Ammonium Bromide (CTAB)" were added as described procedure by Tabikha and Adss (2016). Quantity and quality of extracted DNA was determined by electrophoresis in agarose gel and spectrophotometry, that 5 µl of extracted DNA with 10 µl of loading buffer was mixed and electrophorized in Agarose gel 1.2% with TBE buffer under 80 volts for 30 min.

2.4 ISSRs-PCR preparations and conditions

To differentiate and fingerprint the tested aphid species, PCR was performed by using nine Inter Simple Sequence Repeats (ISSRs) primers with genomic DNA of the tested aphid species. The sequences of ISSRs primers are presented in **Table 2**. The polymerase chain reaction was run in a final volume of 25 µL that contained 2 µL of ISSRs primer, 2 µL DNA, 0.2 µl Taq DNA polymerase (5Uµl^{-1}, Promega Germany), 2.5 µl 4mM dNTPs, 2.5 µl 10x buffer, and 2.5µl 50mM $MgCl_2$.

The thermal conditions of PCR were adjusted to 94°C for 5 min for one cycle (initial denaturation), 40 cycles each cycle includes denaturation at 94°C for 1 min, annealing at 55°C (with ISSRs primers: UBC814, UBC818, UBC840 and UBC880 primers), 57°C (with ISSRs primers UBC817, UBC826, UBC827 and UBC847 primers) or 60°C (with ISSRs primer: UBC812) for 75 sec. then extension at 72°C for 2 min. The Final extension was adjusted to 72°C for 10 min. The yield of PCR was electrophoresed in agarose gel (1%) and TBE buffer under 120 V for 1.5 hours. Ethidium bromide solution was used to visualize DNA banding patterns of different specimens on an agarose gel to be photographed later by using the Bio-Rad Gel documentation system version 2000.

2.5 DNA markers and phylogenetic relationships

Band variations among species with different ISSRs primers were recorded by Gene_Profiler_Eval computer program. To detect DNA markers of different species, generated banding patterns of the fifteen tested aphid species were valued at 0 or 1 according to the absence or presence of a band respectively. To construct molecular taxonomic key for the tested species, unique and polymorphic bands were detected and surveyed. The Phylogenetic relationship and genetic comparison among the fifteen aphid species were concerned with estimating Jaccard's similarity coefficient and using Multi-Variate Statistical Package (MVSP) Version 3.1. According to Sneath and Sokal (1973), the Unweighted Pair Group Method with Arithmetic Means (UPGMA) with estimated Jaccard's similarity coefficient was used to conduct a cluster analysis of the data matrix.

3 Results and Discussion

3.1 Banding patterns analysis

The reaction of the nine ISSR primers with genomic DNA of the tested aphid species generated 101 banding patterns with molecular sizes ranging between 100 - 1100 bp **Fig 1**. Although 39 out of the generated banding patterns are monomorphic, all used ISSRs primers could successfully discriminate the tested aphid species that reflected 61.39% polymorphism among them. DNA fragment pattern analysis showed that 4 out of the polymorphic banding patterns are unique bands that could characterize three aphid species. Primer UBC814 generated the highest number of DNA fragments (168), on the contrary primer UBC817 yielded the lowest number (84 fragments). Primer UBC847 could reflect the highest polymorphism (100%) among the examined aphid species. The tested ISSRs primers yielded the lowest numbers of DNA band patterns (66 bands) in species *A. nerii* and *Pentalonia nigronervosa* while the highest (80 bands) was in species *Capitophorus elaeagina*. Despite, Primer of UBC812 produced 13 banding patterns

Table 2. Sequences and annealing temperature of the examined ISSRs primers

Codes of ISSRs Primers	Sequencing of Nucleotide 5\ -------------------- 3\	Annealing Temperature ºC
UBC-812	GAGAGAGAGAGAGAGAA	60
UBC-814	CTCTCTCTCTCTCTCTA	55
UBC-817	CACACACACACACACAA	57
UBC-818	CACACACACACACACAG	55
UBC-826	ACACACACACACACACC	57
UBC-827	ACACACACACACACACG	57
UBC-840	GAGAGAGAGAGAGAGATT	55
UBC-847	CACACACACACACACAGC	57
UBC-880	GGAGAGGAGAGGAGA	55

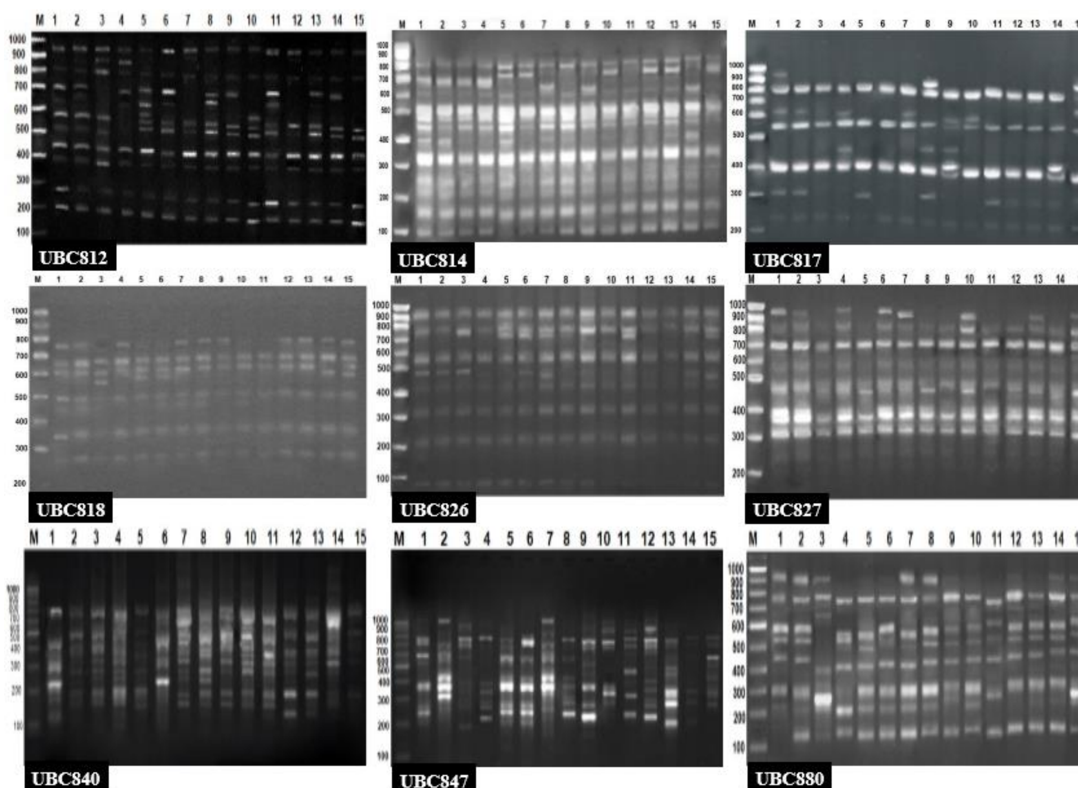

Fig 1. Images of DNA banding patterns for conducting reactions between genomes of the fifteen aphid species [*A. gossypii*(L1), *A. citricola*(L2), *A. nerii*(L3), *A. craccivora*(L4), *H. lactucae*(L5), *B. brassicae*(L6), *C. elaeagina*(L7), *H. pruni*(L8), *P. nigronervosa*(L9), *M. rosae*(L10), *S. avenae*(L11), *R. maidis*(L12), *R. padi*(L13), *S. graminum*(L14), and *M. persicae*(L15)] and nine ISSRs primers

and yielded 145 fragments located between 200 and 950 bp, seven banding patterns were considered polymorphic bands to achieve moderated polymorphism among the examined aphid species (53.85%). The highest numbers of generated band patterns (12 bands) were observed in species *Hyperomyzus lactucae* and *Brevicoryne brassicae*, while the lowest (8 bands) were in species *C. elaeagina* and *M. persicae*. The absence of the fragment band with a molecular size of 550 bp characterized aphid species that belongs to the genus *Aphis*. Moreover, species *A. craccivora* could also be determined by the absence of fragment band 530 bp.

Primer of UBC814 produced also 13 banding patterns situated between 100 and 820 bp; most of the observed bands (9 bands) were monomorphic thus low polymorphism (30.77%) was detected. Generated banding patterns of species *B. brassicae*, *S. avenae* and *M. persicae* were the lowest (10 banding patterns) compared with the other species. Despite the polymorphism of UBC817 primer being moderated (63.64%), it gave two unique bands with molecular sizes of 950 and 660 bp that could characterize species *A. gossypii* and *M. persicae,* respectively. The lowest numbers of generating band patterns (4 bands) were observed in species *A. nerii* and *B. brassicae*.

ISSRs primer of UBC818 gave banding patterns situated in the closest range (270 to 780 bp) that generated 97 fragments in 8 banding patterns with moderated levels of polymorphism (50%) among the tested aphid species. Species *S. graminum* could be determined by the absence of the 610 bp fragment band. The reaction of this primer with the genomic DNA of *P. nigronervosa* yielded the lowest number of band patterns (5 bands). The most observed DNA bands, generated by UBC826, were monomorphic bands thus it produced the lowest level of polymorphism (25%) among the tested aphid species. Wherever banding patterns with molecular sizes of 480 and 760 bp were only considered polymorphic bands.

The ISSRs primer of UBC827 generated 130 fragments of DNA with genomic DNA of the tested aphid species that was graded in eleven banding patterns in rang (250-950 bp). Although the polymorphism of this primer was 36.36%, the band pattern of 680 bp was considered a species-specific band for species *M. persicae*. In addition, three polymorphic bands, with molecular sizes of 380, 460 and 950, were recognized. Each of *Macrosiphum rosae* and *M. persicae* had the highest numbers of banding patterns (10 bands).

UBC840 primer produced 90 polymorphic DNA fragments that were graded in eleven banding patterns in range (130-750 bp), to achieve a high polymorphism level (81.82%). Specimen of *M. persicae* characterized with absence of two fragments bands with molecular sizes of 180 and 430 bp, thus it had the least number of banding patterns (3 bands). Specimen of *A. gossypii* was characterized also with the absence one band of DNA fragment with molecular sizes of 500 bp, which was common in other species.

UBC847 primer had the widest banding pattern with molecular sizes ranging from 200 to 1100 bp and the highest polymorphism (100%) compared with the other ISSRs primers, thus nearly 104 polymorphic fragment bands were detected. The DNA fragments of 790 bp were considered as common banding patterns in the tested aphid species except *A. citricola*. Specimen of *S. graminum* had the lowest number of banding pattern (3 bands) followed by *R. padi* (4 bands).

UBC880 primer was also one of the high polymorphic primers (92.31%). It generated 122 fragments of DNA that were phrased in 13 banding patterns with molecular sizes ranging from 140 to 940 bp. Species *A. nerii* could be identified by the presence of one species-specific band in size of 660 bp. One common band with a molecular size of 770 was detected. Absence of DNA fragment with molecular size of 140 bp characterized *A. gossypii*, which was common in the other species. Moreover, each of *A. gossypii*, *Aphis nerii* and *A. citricola was* characterized by the absence of a DNA fragment band with molecular size of 500 bp, while each of *A. gossypii* and *A. citricola was* characterized with presence of DNA fragment with a molecular size of 450 bp. Finally, aphid species of cereal hosts "*S. avenae*, *S. graminum*, *R. padi* and *R. maidis*" were characterized by the absence of DNA fragments with a molecular size of 220 bp.

Insect genetic diversity could be successfully conducted by using techniques of RAPD-PCR or ISSRs (Sartor et al 2008, Perumal et al 2009, Qiu et al 2009). In previous studies, the Technique of RAPD-PCR could differentiate different aphid species "*A. craccivora, A. fabae, A. gossypii, M. persicae, A. pisum*and *R. padi"* (Cenis et al 1993), which belong to the same genus or different genera. In addition, this technique could successfully identify ten aphid species belong to genus *Aphis* (Amin et al 2013) and another 18 aphid species in Tribes Aphidini and Macrosiphini

(Amin et al 2013) in Egypt. The arbitrary ten-mer primers gave DNA markers that could differentiate six aphid species "*A. craccivora, A. faba, A. nerii, A. punicae, A. rumicis* and *A. zizyphi*" in Genus *Aphis*. Furthermore, some aphid species *"A. faba, A. nerii, A. rumicis*and *A. punicae*" could be distinguished by one of the arbitrary primers (Chihadi 2006). Although each of *B. brassicae, C. elaeagina* and *H. pruni* could be distinguished by three DNA bands with primers C15, D2 and I17 in molecular sizes of 1282, 1132 and 1771 bp; B10, D5 and Z1 in molecular sizes of 900, 129 and 2250 bp; and primers D5, I17 and L20 in molecular sizes of 1401, 759 and 2084 bp, respectively, *P. nigronervosa* could be distinguished only by one DNA species–specific marker with primer B10 in molecular size of 830 bp. In contrary, no DNA species–specific bands were detected for *M. rosae* with those primers (Tabikha 2008).

Each of *S. avenae* and *R. padi* could be distinguished by DNA species-specific banding pattern (Lushai et al 1997), which could also be distinguished by other DNA species-specific markers with molecular sizes of 1555 and 225 bp for primers I17 and Z1, respectively (Amin et al 2013). While other cereal aphid species could be distinguished by one DNA species–specific marker in molecular size of 237 bp with primer L12 for *R. maidis* or two DNA species–specific markers in molecular sizes of 626 and 201 bp with primers I17 and UBC75, respectively for *S. graminum* (Tabikha 2008).

In ISSRs, the occurrence of primer-template mismatch is rare, and the annealing temperature is specified which relies on the length of primers. Thus, it is considered a precise technique for detecting polymorphism (Wolff and Morgan-Richards 1998). In evolutionary, genetic, or ecological studies of biotypes, ISSRs technique saves time and costs (Weng et al 2007). Thus, eleven species of cereal aphid were successively identified by five ISSRs primers that yielded 97 and 69 diagnostic markers. *R. maidis* could be distinguished by seven DNA markers that were as follows: 699 and 412bp by HP-09, 854 bp by HP-11, 398 bp by HP-12, 197 and 499 bp by HP-13 and 560 bp by HP-14, while DNA markers of *R. padi* were five (213 bp by HP-09, 716 bp by HP-11, 685 bp by HP-12, 322 bp by HP-13 and 600 bp by HP-14). In addition, each of *S. graminum* and *S. avenae* had higher DNA markers (8 bands) that DNA markers of *S. graminum* were 1109 bp by HP-09, 269 and 1301 bp by HP-11, 127 and 842 bp by HP-12, 507 and 585 bp by HP-13 and 49 bp by HP-14, while in case of *S. avenae* were 210 bp by HP-09, 605 bp by HP-11, 95, 271 and 460bp by HP-12, 993 bp by HP-13 and 223 and 500 bp by HP-14 (Helmi and Khafaga 2011).

Reaction of 6 arbitrary primers and 4 ISSRs primers with genomic DNA of 16 geographical specimens for *R. padi* revealed that those primers produced 416 polymorphic DNA fragments to achieve polymorphism of 47.27% among the examined populations (Tabikha and Adss 2016). Although some banding patterns of UBC826 and UBC827 primers were polymorphic with the tested aphid species, they were monomorphic for most geographic populations of *S. graminum* in Egyptian agro-ecosystems (Tabikha and Adss 2021), which indicated that those primers are more convenient to determine different aphid species than geographical populations of specific species. While in the current study, *S. graminum* could be determined by the absence of the 610 bp band that was generated by ISSRs primer of UBC818. In addition, *S. graminum* had the lowest number of banding patterns (3 bands) followed by *R. padi* (4 bands) which was generated by UBC847 primer. Aphid species of cereal hosts "*S. avenae*, *S. graminum*, *R. padi* and *R. maidis*" were characterized by the absence of DNA fragments with a molecular size of 220 bp that was generated by UBC880 primer.

3.2 Molecular taxonomic keys

The banding patterns of selected ISSRs primers (UBC812, UBC840, UBC818, UBC880 and UBC847), as presented in **Table 3**, that gave adequate levels of polymorphism among the tested species were subjected to construct two alternative molecular branching taxonomic keys. Fourteen banding patterns with different molecular sizes, generated from the reaction of primers UBC880 and UBC847 with the genomic DNA of the tested aphid species, were subjected together to propose a molecular taxonomic key as graphically illustrated in **Fig 2**. Another molecular taxonomic key was also suggested as shown in **Fig 3** that was based on fifteen banding patterns with different molecular sizes generated from another three polymorphic primers such as UBC812, UBC840 and UBC818, consequently. The two suggested molecular taxonomic keys could successfully discriminate the fifteen aphid species. Thus, the discrimination procedure with the suggested molecular taxonomic keys may be precise, easy, simple, rapid, and inexpensive.

Table 3. Profile for polymorphic DNA bands of the fifteen aphid species, generated by the selected five ISSRs primers that were used in the construction of the molecular taxonomic keys

Primer	Mol. size	*A. gossypii*	*A. citricola*	*A. nerii*	*A. craccivora*	*H. lactucae*	*B. brassicae*	*C. elaeagina*	*H. pruni*	*P. nigronervosa*	*M. rosae*	*S. avenae*	*R. maidis*	*R. padi*	*S. graminium*	*M. persicae*
UBC812	870	0	1	1	1	0	1	0	0	0	0	0	1	0	0	0
	700	1	1	0	1	1	1	0	1	1	0	1	0	1	1	0
	640	1	0	0	1	1	1	0	1	0	0	1	0	1	0	0
	600	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0
	580	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
	550	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
	530	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
UBC818	780	1	1	0	1	0	1	1	1	0	0	1	1	1	1	1
	610	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
	580	0	0	1	0	1	1	0	1	0	0	0	0	0	1	1
	460	1	1	0	0	0	1	1	1	0	1	0	0	0	0	0
UBC840	720	0	0	0	1	0	1	1	1	1	1	0	0	0	1	0
	500	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	430	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
	360	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0
	320	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0
	280	1	0	1	1	1	0	1	1	0	1	1	0	1	1	0
	230	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0
	180	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
	130	1	0	0	1	0	1	0	0	0	0	1	1	0	0	0
UBC847	1100	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0
	900	0	0	1	0	1	0	1	0	1	1	1	1	1	0	0
	790	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
	650	1	1	1	0	1	1	1	0	0	1	1	0	0	0	1
	600	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1
	520	1	1	0	0	0	0	0	1	0	0	0	1	0	0	1
	420	0	1	0	1	1	1	1	1	0	1	0	0	0	0	0
	380	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
	320	0	1	1	1	1	1	0	1	0	1	1	1	0	0	0
	280	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0
	250	1	0	0	0	1	1	0	1	1	0	1	1	0	0	0
	220	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0
	200	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
UBC880	940	1	1	1	0	0	0	1	1	0	0	0	1	0	1	1
	660	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	600	1	1	0	0	0	1	0	1	1	1	1	1	1	1	1
	580	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1
	550	1	1	0	1	1	1	0	1	0	0	0	1	1	1	1
	500	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
	450	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	390	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
	300	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1
	260	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
	220	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
	140	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1

0 : Absent DNA band **1**: Present DNA band

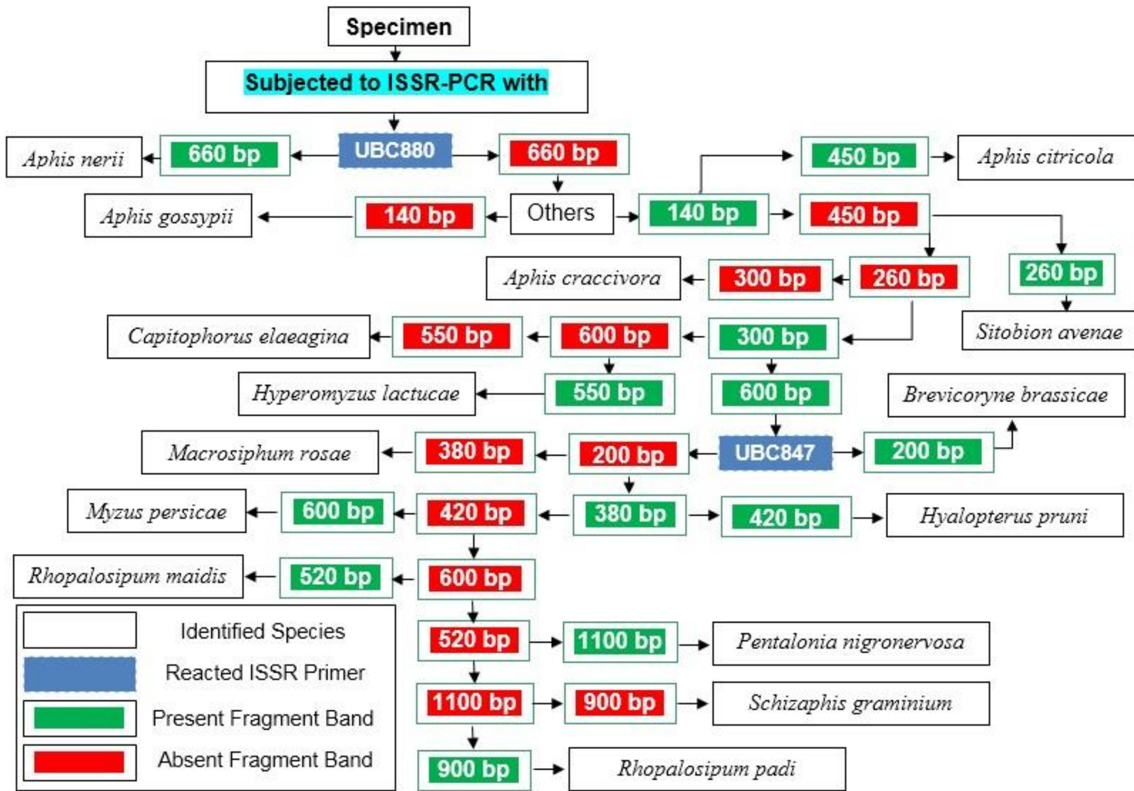

Fig 2. Proposed branching molecular key, based on generated DNA markers by two polymorphic ISSRs primers (USB847 and UBC880) to discriminate the fifteen aphid species

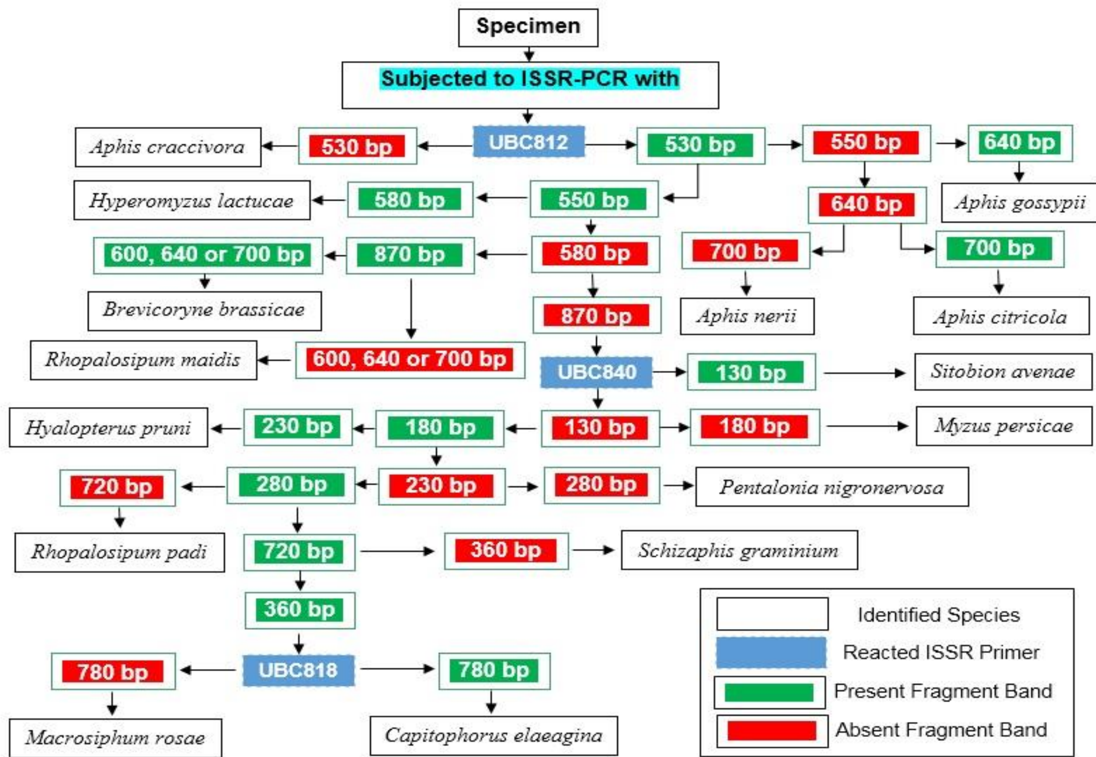

Fig 3. Proposed branching molecular key, based on generated DNA markers by three polymorphic ISSRs primers (USB812, USB818 and UBC840) to discriminate the fifteen aphid species

Molecular branching key can be constructed to identify numerous aphid species. Thus, Amin et al (2013) subjected 18 unique DNA markers to construct a molecular branching key that differentiated 13 aphid species. Moreover, Tabikha and Adss (2021) proposed a molecular branching key that is based on six ISSRs primers (UBC-840, UBC-808, UBC-814, UBC-868, HB-12 and UBC-811) and 16 polymorphic DNA fragments with different molecular sizes, to discriminate 16 geographic populations of *S. graminum* that habitat Egyptian wheat fields.

3.3 Phylogenetic relationships

The similarity matrices of the fifteen examined aphid species, as shown in **Table 4**, reflected that the highest genetic homology (84.9%) was observed between species *R. padi* and *S. graminum*. In addition, each of *A. gossypii* and *A. citricola* are genetically homologous species with a similarity percentile of 84.1%. Despite, aphid species of cereals host plants such as *S. avenae*, *R. maidis*, *R. padi* and *S. graminum* belong to different tribes, they are genetically homologous with similarity percentiles of more than 80%. In contrast, species *A. craccivora* and *M. persicae* are analogous genetically with a low similarity percentile (59.8%). High genetic divergence was observed also between *A. nerii* and *M. persicae*.

The generated phylogenetic tree from the fifteen different aphid species, shown in **Fig 4**, separated species into two main clusters. The first cluster had *A. nerii* while the second one consisted of two sub-clusters. Where the first sub-cluster included *M. persicae* while two main groups appeared from the second sub-cluster. The first main group was for *A. gossypii* and *A. citricola*, while the second included two sub-groups. The first sub-group was for *A. craccivora* while the other sub-group consisted of two division. The first division included one clade for three aphid species belonging to Tribe Macrosiphini (*P. nigronervosa, M. rosae* and *C. elaeagina*), while the second division consisted of two clades. Clades of the second division were for cereal aphids' species "*S. avenae*, *S. graminum*, *R. padi* and *R. maidis*" and three other aphids species"*H. lactucae, Hyalopteruspruni* and *B. brassicae*".

Obtained results agree with Martinez et al (1997) who stated that each of *Myzuspersicae* and *Aphis gossypii* was analogous aphid species while Chihadi (2006) reported that each of *A. citricolla* and *Aphis compositae* were homologous aphid species based on RAPD-PCR with similarity percentile 88.6%.

Genetic similarity indices of eleven cereal aphid species ranged from 73 to 98%, that each of *Anoecia corni* and *H. pruni* were analogous species while each of *H. pruni* and *Saltusaphis scirpus* were homologous species. Thus, aphid species *"S. graminum, R. maidis, R. padi* and *Tetraneura africana"* were placed in separated sub-cluster with a similarity percentile of 84% (Helmi and Khafaga 2011). The results of proximity matrix analysis, based on DNA polymorphism for the eighteen aphid species, showed that *R. maidis* and *R. padi* are homologous species while each of *S. graminum* and *Brachycaudus schwartzi* are analogous species. Furthermore, the aphid species of cereal plants could also be placed in a separated clade from the other species (Amin et al 2013).

Table 4. Similarity matrices of fifteen aphid species based on ISSRs banding patterns analysis

	A. gossypii	*A. citricola*	*A. nerii*	*A. craccivora*	*H. lactucae*	*B. brassicae*	*C. elaeagina*	*H. pruni*	*P. nigronervosa*	*M. rosae*	*S. avenae*	*R. maidis*	*R. padi*	*S. graminium*	*M. persicae*
A. gossypii	1.000														
A. citricola	0.841	1.000													
A. nerii	0.644	0.687	1.000												
A. craccivora	0.701	0.706	0.671	1.000											
H. lactucae	0.681	0.685	0.711	0.709	1.000										
B. brassicae	0.707	0.711	0.659	0.776	0.793	1.000									
C. elaeagina	0.756	0.783	0.728	0.747	0.744	0.750	1.000								
H. pruni	0.742	0.747	0.636	0.733	0.812	0.795	0.747	1.000							
P. nigronervosa	0.663	0.667	0.692	0.713	0.690	0.678	0.772	0.674	1.000						
M. rosae	0.644	0.686	0.691	0.732	0.771	0.736	0.813	0.753	0.756	1.000					
S. avenae	0.678	0.644	0.709	0.750	0.790	0.753	0.723	0.729	0.731	0.728	1.000				
R. maidis	0.686	0.711	0.654	0.695	0.714	0.721	0.711	0.738	0.740	0.695	0.779	1.000			
R. padi	0.714	0.699	0.642	0.747	0.788	0.729	0.763	0.768	0.750	0.769	0.813	0.824	1.000		
S. graminium	0.686	0.690	0.654	0.695	0.735	0.701	0.753	0.802	0.763	0.716	0.734	0.766	**0.849**	1.000	
M. persicae	0.648	0.690	0.614	**0.598**	0.674	0.721	0.711	0.718	0.634	0.635	0.651	0.744	0.731	0.722	1.000

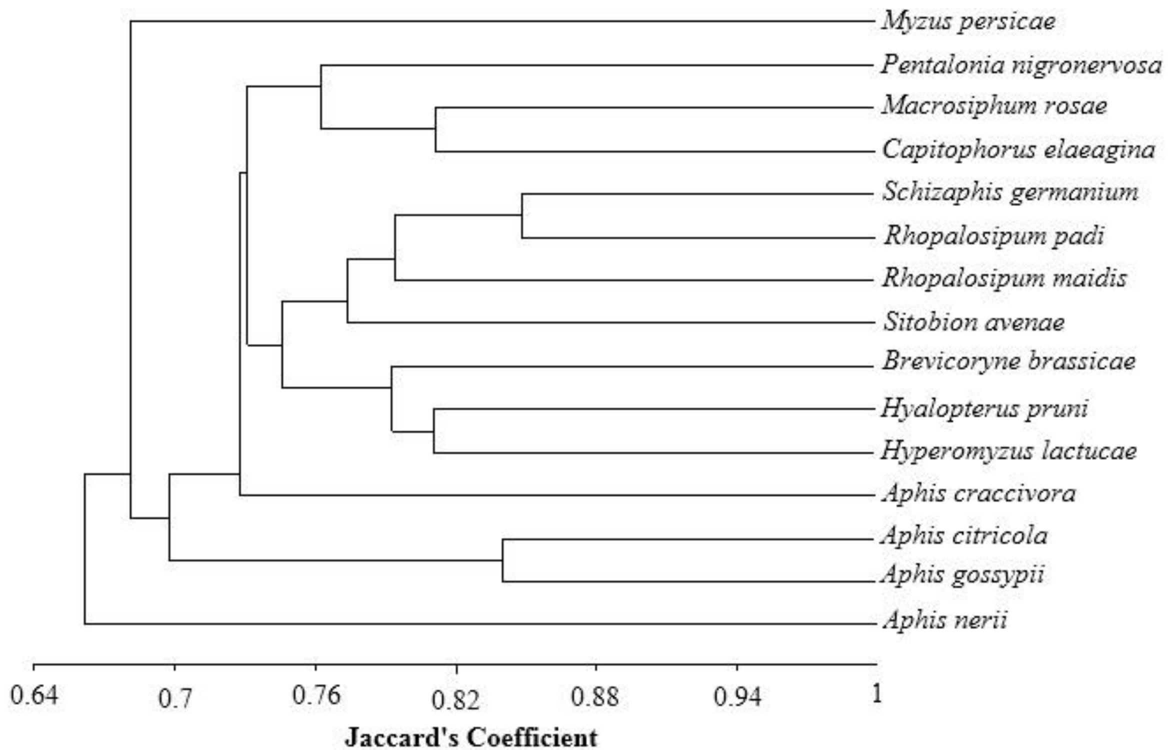

Fig 4. Dendrogram for studying phylogeny of the fifteen aphid species based on analysis of ISSRs similarity matrices

4 Conclusion

The Tested ISSRs succeeded in generating many polymorphic DNA fragment bands with a wide range of molecular sizes, four of them were species-specific markers for three aphid species. The other polymorphic banding patterns were subjected successfully to construct two alternative molecular taxonomic keys that discriminate the fifteen aphid species. The phylogenetic relationship among some aphid species confirmed that each of *R. padi* and *S. graminum* in addition to *A. gossypii* and *A. citricola* are genetically homologous species, while *A. craccivora* and *M. persicae* are analogous genetically species. Although the tested aphid species of cereals host plants belong to different tribes, they are genetically homologous with similarity percentiles of more than 80%.

References

Abbot P (2001) Individual and population variation in invertebrates revealed by Inter-simple Sequence Repeats (ISSRs). *Journal of Insect Science* 1, 8. https://doi.org/10.1093/jis/1.1.8

Amin AH, Draz KA, Soliman, KA, et al (2013) Genetic fingerprints and phylogenetic relationships of eighteen aphid species from Egypt (Hemiptera: Sternorrhyncha: Aphididae). *Munis Entomology and Zoology* 8, 387-406.

Armstrong KF, Ball SL (2005) DNA barcodes for biosecurity: Invasive species identification. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences* 360, 1813–1823. https://doi.org/10.1098%2Frstb.2005.1713

Blackman RL, Eastop VF (1994) Aphids on the World's Trees: An Identification and Information Guide. *CAB International, Wallingford, Oxon* 1024 pp. https://doi.org/10.1017/S0007485300034465

Blackman RL, Eastop VF (2000) Aphids on the World's Crops: An Identification and Information Guide. 2nd (ed). John Wiley & Sons, Chichester 476 P

Blackman RL, Eastop VF (2008) Aphids on the world's herbaceous plants and shrubs, Vol (1): Host Lists and Keys Vol (2): The aphids. John Wiley & Sons. 1456 p. https://rb.gy/ggwiu9

Brault V, Tanguy S, Reinbold C, et al (2010) Transcriptomic analysis of intestinal genes following acquisition of pea enation mosaic virus by the pea aphid *A cyrthosiphonpisum*. *Journal of General Virology* 91, 802-808. https://doi.org/10.1099/vir.0.012856-0

Capinera JL (2002) North American vegetable pests: The pattern of invasion. *American Entomologist* 48, 20-39. https://doi.org/10.1093/ae/48.1.20

Carew ME, Pettigrove V, Hoffmann AA (2003) Identifying chironomids (Diptera: Chironomidae) for biological monitoring with PCR-RFLP. *Bulletin of Entomological Research* 93, 483–490. https://doi.org/10.1079/BER2003268

Carew ME, Pettigrove V, Hoffmann AA (2005) The utility of DNA markers in classical taxonomy: Using cytochrome Oxidase I markers to differentiate Australian *Cladopelma* (Diptera: Chironomidae) midges. *Annals of the Entomological Society of America* 98, 587–594. https://doi.org/10.1603/0013-8746(2005)098[0587:TUODMI]2.0.CO;2

Cenis JL, Perez P, Fereres A (1993) Identification of Aphid (Homoptera: Aphididae) species and clones by Random Amplified Polymorphic DNA. *Annals of The Entomological Society of America* 86, 545-550. https://doi.org/10.1093/aesa/86.5.545

Chihadi FH (2006) Taxonomical studies on aphids by using modern techniques. Ph.D. in Economic Entomology, Plant Protection Department, Faculty of Agriculture, Ain Shams University, Egypt. 83pp.

Coeur d'acier A, Cruaud A, Artige E, et al (2014) DNA barcoding and the associated PhylAphidB @ se website for the identification of European aphids (Hemiptera: Aphididae). *PLoS ONE* 9, e97620.https://doi.org/10.1371/journal.pone.0097620

Emden HFV, Harrington R (2007) Aphids as Crop Pests. CAB International, Oxford, UK. 762p. https://rb.gy/7jayn5

Gui FR, Wan FH, Guo JY (2008) Population genetics of *Ageratinaa denophora* using inter-simple sequence repeat (ISSR) molecular markers in China. *Plant Biosystems- An International Journal Dealing with all Aspects of Plant Biology* 142, 255-263. https://doi.org/10.1080/11263500802150399

Guo J, Wang Z, Francis F (2017) Use of molecular markers for entomological diversity assessment and their application in population study of aphids. *Faunistic Entomology (Faunistic Entomology)* 70, 49-62 https://doi.org/10.25518/2030-6318.3647

Habib A, El-kady E (1961) The Aphididae of Egypt. *Bulletin of The Entomological Society of Egypt* 45, 1-137.

Hebert PDN, Penton EH, Burns JM, et al (2004) Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America* 101, 14812-14817. https://doi.org/10.1073/pnas.0406166101

Helmi A, Khafaga AF (2011) Molecular fingerprinting of certain cereal aphids in Egypt (Hemiptera: Sternorrhyncha: Aphididae) using RAPD and ISSR markers. *Journal of Entomology* 8, 327-340. https://doi.org/10.3923/je.2011.327.340

Helmi A, Sharaf AA, Ibrahim HE (2016) Fingerprinting of leafhoppers on medicinal and aromatic plants in Egypt using ISSRs. *Journal of Plant Protection and Pathology* 7, 751-756. https://doi.org/10.21608/jppp.2016.52137

Kim H, Lee S, Jang Y (2011) Macroevolutionary patterns in the Aphidini aphids (Hemiptera: Aphididae): Diversification, host association, and biogeographic origins. *PloS ONE* 6, e24749. https://doi.org/10.1371/journal.pone.0024749

Kinyanjui G, Khamis FM, Mohamed S, et al (2016) Identification of aphid (Hemiptera: Aphididae) species of economic importance in Kenya using DNA barcodes and PCR-RFLP-based approach. *Bulletin of Entomological Research* 106, 63-72. https://doi.org/10.1017/s0007485315000796

Lee W, Kim H, Lim J, et al (2011) Barcoding aphids (Hemiptera: Aphididae) of the Korean Peninsula: updating the global data set. *Molecular Ecology Resources* 11, 32–37. https://doi.org/10.1111/j.1755-0998.2010.02877.x

Liu J, Gui F, Li Z (2010) Genetic diversity of the planthopper, *Sogatella furcifera* in the Greater Mekong Subregion detected by inter-simple sequence repeats (ISSR) markers. *Journal of Insect Science* 10, 52. https://doi.org/10.1673/031.010.5201

Lozier JD, Foottit RG, Miller GL, et al (2008) Molecular and morphological evaluation of the aphid genus *Hyalopterus* Koch (Insecta: Hemiptera: Aphididae), with a description of a new species. *Zootaxa* 1688, 1-19. https://doi.org/10.5281/zenodo.180576

Lushai G, Loxdale HD, Brookes CP, et al (1997) Genotypic variation among different phenotypes within aphid clones. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 264, 725-730. https://doi.org/10.1098/rspb.1997.0103

Martinez TD, Moya A, Hebert PDN, et al (1997) Geographic distribution and seasonal variation of mitochondrial DNA haplotypes in the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae). *Bulletin of Entomological Research* 87, 161-167. https://doi.org/10.1017/S0007485300027309

McNeely JA, Mooney HA, Neville LE, et al (2001) A Global Strategy on Invasive Alien Species. International Union for Conservation of Nature. International Council for Science (ICSU), Scientific Committee on Problems of the Environment (SCOPE), IUCN, CAB International. Retrieved from: https://rb.gy/8reu7z

Miller GL, Foottit RG (2009) The taxonomy of crop pests: The Aphids. In: Foottit, RG, Adler PH (Eds) Insect Biodiversity: Science and Society. Oxford, Wiley-Blackwell. pp. 463-473.

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polvmerase catalyzed chain reaction. *Methods in Enzymology* 155, 335-350. https://doi.org/10.1016/0076-6879(87)55023-6

Perumal Y, Marimuthu M, Salim AP, et al (2009) Host plant mediated population variations of cotton whitefly *Bemisiatabaci* (Gennadius) (Aleyrodidae: Homoptera) characterized with random DNA markers. *American Journal of Biochemistry and Biotechnology* 5, 40-46. https://doi.org/10.3844/ajbbsp.2009.40.46

Qiu B, Chen Y, Liu L, et al (2009) Identification of three major *Bemisia tabaci* biotypes in China based on morphological and DNA polymorphisms. *Progress in Natural Science* 19, 713-718. http://dx.doi.org/10.1016%2Fj.pnsc.2008.08.013

Raina SN, Rani V, Kojima T, et al (2001) RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome* 44, 763-772. https://doi.org/10.1139/g01-064

Sartor C, Demichelis S, Cenis JL, et al (2008) Genetic variability of *Bemisia tabaci*in the Mediterranean and Sahel Regions. *Bulletin of Insectology* 61, 161-162.

Sneath PHA, Sokal RR (1973) Numerical Taxonomy - The Principles and Practice of Numerical Classification. WH Freeman, San Francisco 537p. https://philpapers.org/rec/SNENTT

Tabikha RM, Mansour RM, Soliman EA et al (2008) Taxonomical studies on some aphid species by using modern techniques. Ph.D. in Economic Entomology, Plant Protection Department, Faculty of Agriculture, Alexandria University, Damanhour Branch, Egypt.

Tabikha RM, Adss IA (2016) Genetic and morphological variations among geographical populations of *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) in Egypt, using RAPD and ISSR markers. *Munis Entomology and Zoology* 11, 695-708.

Tabikha RM, Adss IA (2021) Morphologic and genetic analysis for geographic populations of greenbug *Schizaphis graminum* (Hemiptera: Aphididae) in Egypt . *Biologia* 76, 77-89. https://doi.org/10.2478/s11756-020-00501-2

Valenzuela I, Eastop VF, Ridland PM, et al (2009) Molecular and morphometric data indicate a new species of the aphid Genus Rhopalosiphum (Hemiptera: Aphididae). *Annals of the Entomological Society of America* 102, 914-924. https://doi.org/10.1603/008.102.0602

Valenzuela I, Hoffmann AA, Malipatil MB, et al (2007) Identification of aphid species (Hemiptera: Aphididae: Aphidinae) using a rapid polymerase chain reaction restriction fragment length polymorphism method based on the cytochrome oxidase subunit I gene. *Australian Journal of Entomology* 46, 305–312. https://doi.org/10.1111/j.1440-6055.2007.00615.x

von Dohlen CD (2009) Aphid molecular systematics: history, progress and prospects. *Redia* 92, 39-45.

von Dohlen CD, Rowe CA, Heie OE (2006) A test of morphological hypotheses for tribal and subtribal relationships of Aphidinae (Insecta: Hemiptera: Aphididae) using DNA sequences. *Molecular Phylogenetics and Evolution* 38, 316-329. https://doi.org/10.1016/j.ympev.2005.04.035

Weng Y, Azhaguvel P, Michels GJ, et al (2007) Cross-species transferability of microsatellite markers from six aphid (Hemiptera : Aphididae) species and their use for evaluating biotypic diversity in two cereal aphids. *Insect Molecular Biology* 16, 613-622. https://doi.org/10.1111/j.1365-2583.2007.00757.x

Williams JGK, Kubelik AR, Livak KJ, et al (1990) DNA polymorphism amplified by arbitrary primers are useful genetic markers. *Nucleic Acid Research* 18, 6531- 6533. https://doi.org/10.1093/nar/18.22.6531

Wolff K, Morgan-Richards M (1998) PCR markers distinguish Plantago major subspecies. *Theoretical and Applied Genetics* 96, 282-286. https://doi.org/10.1007/s001220050737