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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 as most economically importance and worldwide insect pests. Successful pest management systems are based on accurate and rapid pests' species identification. Traditional morphological identification of closed aphid species may be considered as inaccurate taxonomic process. For overcoming 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 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 as unique bands which could characterize three aphid species (*Aphis gossypii*, *Aphis nerii* and *Myzus persicae*). Highest genetic homology (84.9%) was observed between species *Rhopalosipum padi* and *Schizaphis graminum*. In additions, each of *A. gossypii* and *Aphis citricola* were also genetically homologous species. In contrast, species *Aphis craccivora* and *M. persicae* were analogous genetically with 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 five highest polymorphic ISSRs primers and 29 banding patterns with different molecular sizes.

1 Introduction

Aphids (Hemiptera: Sternorrhyncha: Aphididae) are considered the most economical importance 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 reach 70-80% (Capinera 2002, Holman 2008, Kinyanjui et al 2016). Direct feeding of aphid on plant sap can cause not only depriving essential nutrients of host plant, but also transmitting 30% of plant viral diseases, and injecting toxic salivary secretions to host plants (Blackman and Eastop 1994 and 2000, Brault et al 2010). In addition, sooty molds that results in honey dew secretion can also cause effective depleting in photosynthesis of host plants and reducing quality and then marketability of crops (Worf et al 1995).

Family Aphididae is consisting of more than 4000 species. Aphidinae is the largest subfamily of Aphididae that consists of three major Tribes. Macrosiphini is considered as the largest tribe of Aphidinae that consists 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 plants quarantine and pests management are based on accurate and rapid pests' species identification (Lozier et al 2008, Miller and Foottit 2009). Classification of aphid at various levels has been conducting by using morphological characters, life cycles and host-plants associations (Blackman and Eastop 1994, Kim et al 2011). Although absence of morphological characteristic keys or 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 required taxonomists with specific knowledge, skills and training which may be acquire in many years, thus related aphid species determination, based on traditional morphological characters, may be time-consuming and considered as an inaccurate taxonomic process (Coeur d'acier et al 2014).

For overcoming the limitation of traditional morphological identification, molecular techniques, related to DNA markers and based on amplification of specific regions of the genome by using polymerase chain reaction (PCR) techniques, were approached (Mullis and Faloona 1987) and then organisms as cryptic invertebrates or immature stages as egg could be identified (Carew et al 2003 and 2005, Hebert et al 2004). Thus, McNeely et al (2001) reported that using molecular identification system may be assist in 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 amplification of random regions of genome by using a single arbitrary tenmer primer that flank 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* (Amin et al 2008) 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 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 leafhoppers species collected from different medicinal and aromatic plants in Egypt (Helmi et al 2016).

Thus, current research paper aims to pursue finding diagnostic DNA markers and studying genetic diversity of 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 economic importance 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 laboratory in glass jar supplemented with parts of host plant. Presented data in **Table 1** summarize information of taxonomic status, locality, host plant and

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*	31º 25' 20"N, 30º 23' 53"E	May, 2021
2	Aphidini	*Aphis citricola* van der Groot	Orange, *Citrus aurantium*	31º 25' 20"N, 30º 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*	31º 25' 20"N, 30º 23' 53"E	May, 2021
9	Macrosiphini	*Pentalonia nigronervosa* Coquerel	Banana, *Musa sapientum*	30º 39' 30"N, 30º 33' 02"E	March, 2021
10	Macrosiphini	*Macrosiphum rosae* L.	Rose, *Rosa hybrid*	31º 01' 46"N, 31º 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

collection date that related to the tested aphid specimens. To confirm taxonomic status of each specimen, mounted alate or apterus forms of the tested aphid adults in swan's gum chloral media on glass slides (Blackman and Eastop 2000), were identified morphologically by using 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), healthy and young adult female from each tested aphid species were caged separately on its host plant to harvest its offsprings in age 5 days. The collected offsprings were preserved in Eppendorf tubes under -20ºC for using later in molecular studies.

2.2 DNA extraction

Extracted genomic DNA of the fifteen preserved aphid specimens were subjected to polymerase chain reaction with nine ISSRs primers. Thus, 5-6 individuals of aphid adults were grinded to a fine powder, in Eppendorftubes under liquid nitrogen, the grinded material were 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 final volume of 25 µL that contained2 µL of ISSRs primer, 2 µL DNA, 0.2 µl Taq DNA polymerase ($5U\mu l^{-1}$, 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 that 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 on 72°C for 10 min. The yield of PCR were 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 agarose gel to be photographed later by using Bio-Rad Gel documentation system version 2000.

2.5 DNA markers and phylogenetic relationships

Bands 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 0 or 1 according to absence or presence of 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 by estimating Jaccard's similarity coefficient and using Multi-Variate Statisical Pakage (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 were used to conduct cluster analysis of the data matrix.

3 Results and Discussion

3.1 Banding patterns analysis

Reaction of the nine ISSR primers with genomic DNA of the tested aphid species generated 101 banding patterns with molecular sizes ranged between 100 - 1100 bp **Fig 1**. Although 39 out of generated banding patterns are monomorphic, the 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 which could characterize three aphid species. Primer UBC814 generated the highest number of DNA fragments (168), on contrary primer UBC817 yielded the lowest number (84 fragments). Primer UBC847 could reflect highest polymorphism (100%) among the examined aphid species. The tested ISSRs primers yielded 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

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	CTCTCTCTCTCTCTCTCTA	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

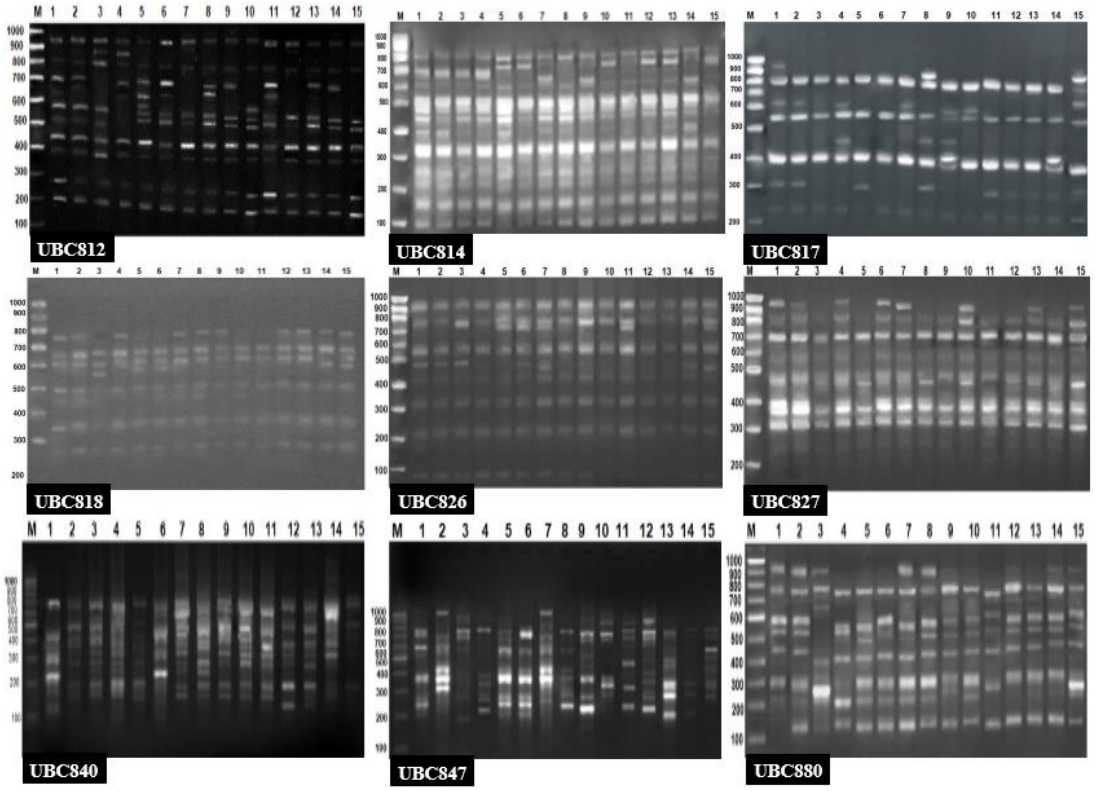

Fig 1. Images of DNA banding patterns for conducting reaction between genomes of the fifteen aphid species [*A. gossypii*(L1), *A. citricola*(L2), *A. nerii*(L3), *A. craccivora*(L4), *H. lactucae*(L5), *B. brassicae*(L6), *C. elaeagina*(L7), *H. pruni*(L8), *P. nigronervosa*(L9), *M. rosae*(L10), *S.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

banding patterns and yielded 145 fragments located between 200 and 950 bp, seven banding patterns were considered as 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*. Absence of the fragment band with molecular size of 550 bp characterized aphid species that belongs to the genus *Aphis*. Moreover, species *A. craccivora* could also be determined by absence of fragment band 530 bp.

Primer of UBC814 produced also 13 banding patterns situated between 100 and 820 bp that most of 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) comparing with the other species. Despite polymorphism of UBC817 primer was 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 closest range (270 to 780 bp) that generated 97 fragments in 8 banding patterns with moderated level of polymorphism (50%) among the tested aphid species. Species *S. graminum* could be determined by absence of the 610 bp fragment band. Reaction of this primer with genomic DNA of *P. nigronervosa* yielded 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 as 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 polymorphism of this primer was 36.36%, the band pattern of 680 bp was considered as 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 graded in eleven banding patterns in rang (130-750 bp), to achieve 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* characterized also with absence one band of DNA fragment with molecular sizes of 500 bp, which was common in other species.

UBC847 primer had widest banding pattern with molecular sizes ranging 200-1100 bp and the highest polymorphism (100%) comparing with the other ISSRs primers, thus nearly 104 polymorphic fragment bands were detected. The DNA fragments of 790 bp were considered as common banding pattern 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 high polymorphic primers (92.31%). It generated 122 fragments of DNA that phrased in 13 banding patterns with molecular sizes ranged from 140 to 940 bp. Species *A. nerii* could be identified by presence one species specific band in size of 660 bp. One common band with 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* characterized with absence of DNA fragment band with molecular size of 500 bp, while each of *A. gossypii* and *A. citricola* characterized with presence of DNA fragment with molecular size of 450 bp. Finally, aphid species of cereal hosts "*S. avenae, S. graminum*, *R. padi* and *R. maidis*" were characterized with absence of DNA fragment with 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, Qiuet al 2009). In previous studies, 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 2008) 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 primer (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 was 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), that could also be distinguished by another 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, occurrence primer-template mismatch is rare, and temperature of annealing is specified that rely on length of primers. Thus, it is considered as precise technique for detecting polymorphism (Wolff and Morgan-Richards 1998. In evolutionary, genetic, or ecological studies of biotypes, ISSRs technique save 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 follow: 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 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 current study, *S. graminum* could be determined by absence of the 610 bp band that was generated by ISSRs primer of UBC818. In addition, *S. graminum* had lowest number of banding pattern (3 bands) followed by *R. padi* (4 bands) that was generated by UBC847 primer. Aphid species of cereal hosts "*S. avenae, S. graminum, R. padi* and *R. maidis*" were characterized with absence of DNA fragment with 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 level 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 reaction of primers UBC880 and UBC847 with the genomic DNA of the tested aphid species, were subjected together to propose 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 to discriminate the fifteen aphid species. Thus, discrimination procedure with the suggested molecular taxonomic keys may be precise, easy, simple, rapid, and inexpensive.

Molecular branching key can be constructed to identify numerous aphid species. Thus, Amin et al (2013) subjected 18 unique DNA marker to construct molecular branching key that differentiate 13 aphid species. Moreover, Tabikha and Adss (2021) proposed

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

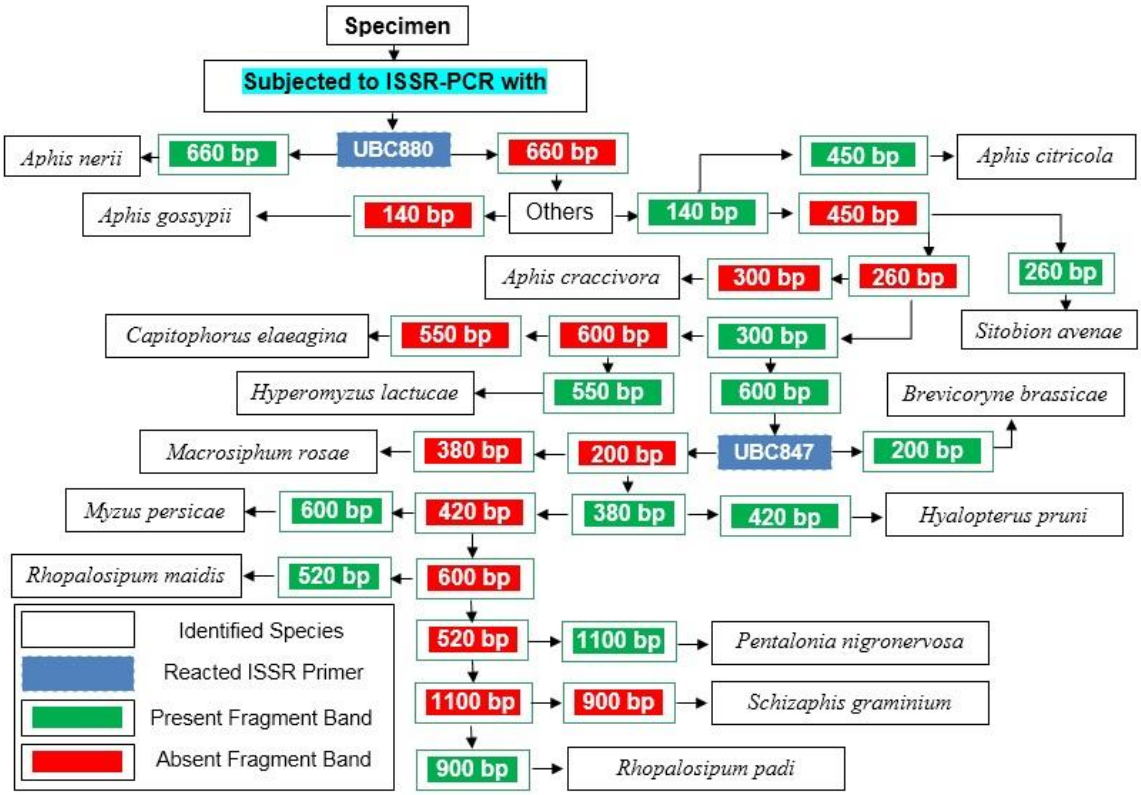

Fig 2. Proposed branching molecular key, based on generated DNA markers by two polymorphic ISSRs primers (USB847 and UBC880) to discriminate the fifteen aphid species

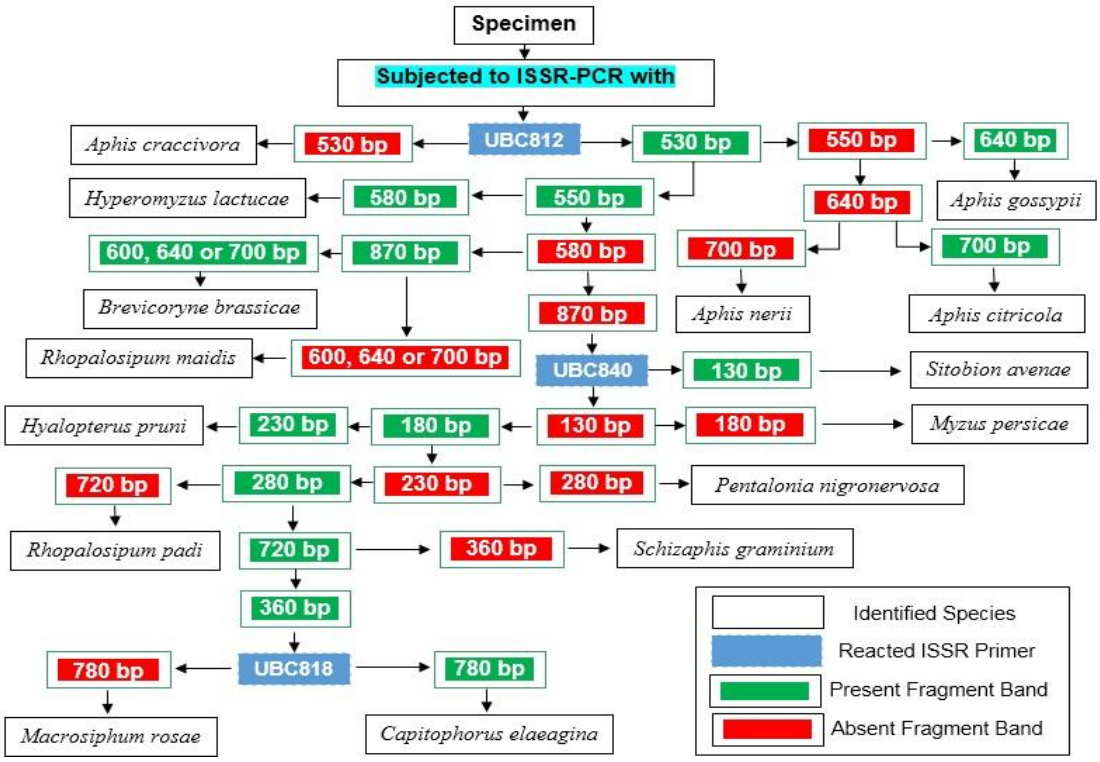

Fig 3. Proposed branching molecular key, based on generated DNA markers by three polymorphic ISSRs primers (USB812, USB818 and UBC840) to discriminate the fifteen aphid species

a molecular branching key that 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 additions, each of *A. gossypii* and *A. citricola* are genetically homologous species with similarity percentile 84.1%. Despite, aphids' 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 more than 80%. In contrast, species *A. craccivora* and *M. persicae* are analogous genetically with 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 those 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 belongs to Tribe Macrosiphini (*P. nigronervosa, M. rosae* and *C. elaeagina*), while the second division consisted of two clades. Clades of second divisions 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* were 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 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 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

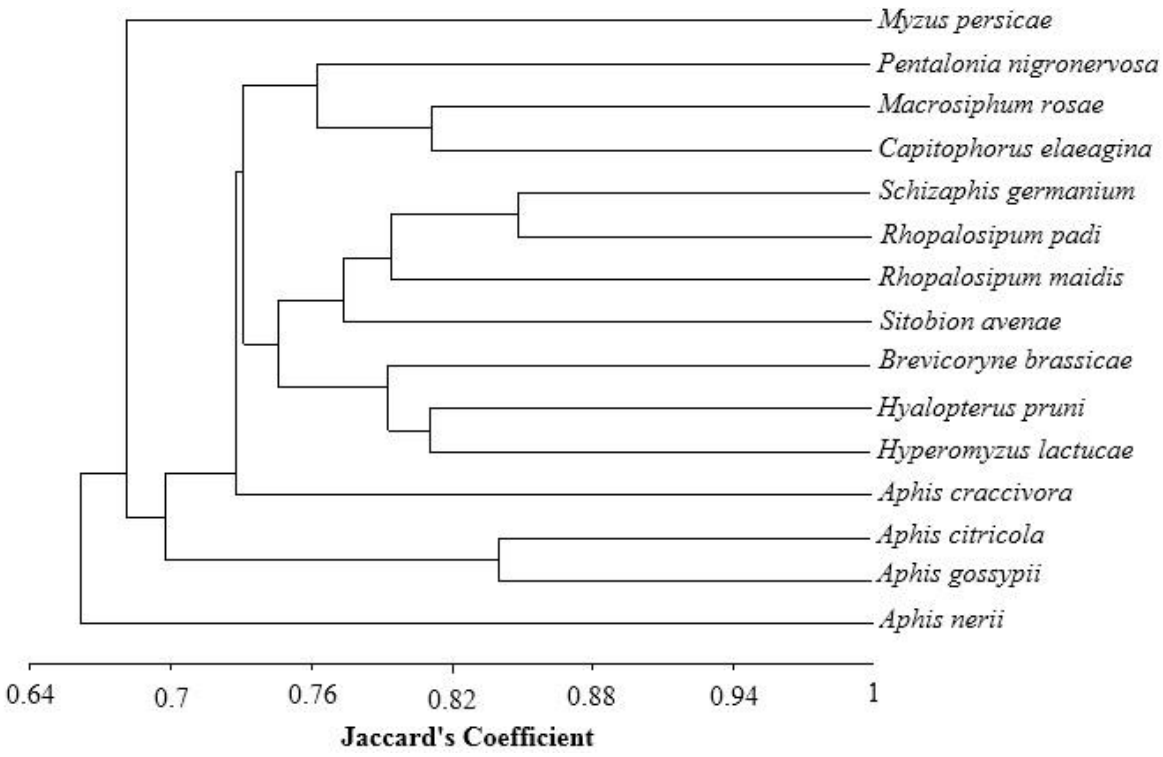

Fig 4. Dendrogram for studying phylogeny of the fifteen aphid species based on analysis of ISSRs similarity matrices

4 Conclusion

The Tested ISSRs succeeded to generate many polymorphic DNA fragments bands with 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 key that discriminate the fifteen aphid species. The phylogenetic relationship among some aphid species confirmed that each of *R. padi* and *S. graminum* in addition each of *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 more than 80%.

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