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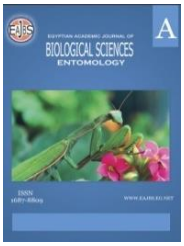

Evolution of Polymorphs in *Rhopalosiphum maidis* (Hemiptera: Aphididae) in Response to Different Seasonal and Host Plant Conditions

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ABSTRACT

The aphid *Rhopalosiphum maidis* is a serious pest against cereal plants. In Egypt, it causes serious damage to many cereal plants. The principal idea of this study was achieved based on the observation of various seasonal invasion rates of this pest on different host plants suggesting climatic adaptation by *R. maidis* polymorphs. The invasion rates on winter plants were higher than those of summer. Morphologically, all collections were belonging to *R. maidis*. The authors assumed that different biotypes of *R. maidis* are present in the studied area. To address this hypothesis, the mitochondrial cytochrome oxidase I (COI) gene was amplified in aphids collected from wheat, barley, sorghum and maize in Assiut, Egypt. Polymerase chain reactions (PCR) identified 687 bps products corresponding to a partial sequence of (COI). Multiple sequence alignment of the sequenced products revealed that at least five haplotypes are present on the tested plants. Phylogenetic analysis using the neighbor-joining method identified five clades that represent different biotypes. The identified clades contained mixtures between summer and winter *R. maidis* collections suggesting that different biotypes are present in mixtures on the same plant. Our findings showed that *R. maidis* develops different polymorphs on various host plants at different climatic conditions.

INTRODUCTION

Molecular taxonomy is a rapidly growing field of science in which molecular techniques are used to define the genetic differences among living organisms even among closely related species. Techniques involved in molecular systematics provide useful information on evolutionary relationships especially when morphological and ecological observations are highly similar (Choe *et al*., 2006). In the last two decades, several molecular markers were identified as promising tools for linking the identity of an organism to its DNA sequences including rRNA from the nuclear genome and the mitochondrial cytochrome b, cytochrome oxidase subunit I (COI) and cytochrome oxidase

subunit II (COII) (Hebert *et al*., 2003a,b; Tautz *et al*., 2003). Aphids (Hemiptera: Aphididae) are characterized by having a unique biological characteristic of biotype evolution in response to the host plant resistance (Smith, 2005). It is believed that the presence of this phenomenon is due to the unique characteristics of the aphid life cycle. For instance, aphids have a symbiotic relationship with several bacterial genera including *Buchnera*, *Rickettsia*, *Regiella* and *Serratia*. These bacteria supply aphids with the essential amino acids leading to biotype adaptation and increasing their resistance to plants (Ruggle and Gutierrez, 1995; Birkle and Douglas, 1999; Moran and Wernegreen, 2000; Wille and Hartman 2009; Oliver *et al*., 2010). Furthermore, the feeding of aphids depends mainly on the juice of plant phloem leading to the induction of plant response against the saliva of the attacking aphid (Mutti *et al*., 2008). It is believed that such a response by the host plant triggers biotype creation in aphid species (Dreyer and Campbell 1984). The aphid *Rhopalosiphum maidis* is a serious pest of many crops worldwide. For instance, it caused serious damage to the corn fields in the northern two-thirds of the USA in 1959, Canada during the period between 1965 and 1970 as well as in Egypt during 1998 (Everley, 1960; Foott and Timmins, 1973; Al-Eryan and El-Tabbakh, 2004). Furthermore, this pest is able to attack many other plants including sorghum and barley (Ortega *et al*., 1980).

This study aims to address the occurrence of different biotypes of *R. maidis* in the Assiut governorate in Egypt. Furthermore, it discusses the correlation between the host plant, the invasion rate of *R. maidis* and the climatic conditions between winter and summer seasons. These approaches are studied based on the molecular sequences of the mitochondrial cytochrome oxidase subunit I gene amplified from randomly selected samples of *R. maidis* collected from wheat, barley, maize and sorghum plants.

MATERIALS AND METHODS

2.1. Plant Cultivation and *Rhopalosiphum maidis* Distribution Survey:

Sorghum, maize, wheat and barley were planted at Assiut University farm (Assiut, Egypt) during the summer and winter seasons of 2017. The apterous adult stage of *R. maidis* was collected from different plants around the peak period of infestation (February and September for winter and summer plants, respectively) and used for survey purposes.

2.2. DNA Extraction, COI Amplification and Sequencing:

DNA was extracted from aphids collected from sorghum (S), maize (M), wheat (W) and barley (B) plants using two independent collections for each plant. The genomic DNA of *R. maidis* was extracted using phenol/chloroform extraction and precipitation was achieved by absolute ethanol. The extracted DNA was resuspended in sterilized distilled water and kept at -80ºC for subsequent applications. Polymerase chain reaction amplification of *R. maidis* COI gene was achieved using LCO1490 (5´-GGTCAACAAATCATAAAGATATTGG-3´) and HCO2198 (5´-TAAACTTCAGGCTG ACCAAAAAATCA- 3´) primers (Folmer *et al.*, 1994). The PCR reaction was prepared with a final concentration of 3.5 mmol $MgCl_2$, 1 µmol of dNTPs, 10 µmol of each primer, 0.2 units Taq DNA polymerase and 2µl of template DNA. The cycling conditions started with initial denaturation of the template at 95°C for 5 min followed by 35 cycles of 94°C 30 s, 50°C 30 s, 72°C 40s. The cycles were followed by a final extension step for 3 min at 72°C. Five microliters of the PCR products were separated in a 1.2% agarose gel and stained using ethidium bromide. Polymerase chain reaction products of COI genes were cleaned using PCR cleaning kits and sequenced using the LCO and HCO primers. In order to confirm that our sequencing results lack any reading errors the PCR products were

sequenced using both primers.

2.3 Data Analysis:

Data of *R. maidis* invasion on different plants were analyzed based on 5 independent replicates using SPSS statistical software. All sequence analyses were performed using MEGA X software (https://www.megasoftware.net). Multiple sequence alignments were performed based on clustal W (Thompson *et al*., 1994). The pair-wise genetic distances were estimated using the model of Kimura-2-parameter (K2P) because it supplies better metrics when genetic distances are low (Nei and Kumar, 2000). Conduction of phylogenetic analyses was achieved using the neighbor-joining method (NJ) with 1000 pseudo-replicate bootstrap resampling. Reference (COI) gene sequences from closely related species were downloaded from the gene bank with accession numbers (DQ499048.1 of *R. maidis* isolate 1 partial COI gene and DQ499049.1 of *R. maidis* isolate 2 partial COI gene). *Apis craccivora* KR040128.1 COI gene was used as an outgroup sequence.

RESULTS

3.1. Effect of Seasonal and Host Plant Variations on *Rhopalosiphum maidis* Invasion Rates:

Invasion rates of *R. maidis* on wheat, barley, maize and sorghum were counted during the peak period of invasion (February for wheat and barley as winter plants) and (September for maize and sorghum as summer plants). Infestation rates fluctuated between different plants and seasons (Fig. 1). The mean numbers of *R. maidis* recorded for wheat, barley, maize and sorghum were 784.8, 1683, 933.5 and 2887.3, respectively (Fig. 1).

Fig.1. Mean distribution of *R. maidis* on different plants on a monthly basis.

Aphids were collected weekly during February from wheat and barley and during September from sorghum and maize. Data are plotted as mean values based on the four weekly records (February 2, 9, 18 and 25during 2017; September, 5,12,19 and 26during 2017). All dad based on 5 independent replicates. Data were analyzed using one way ANOVA at $P<0.05$.

3.2. Identification of Various Polymorphs of *Rhopalosiphum maidis:*

Amplification of *R. maidis* partial sequence of COI gene was achieved using universal primers against COI gene and DNA templates obtained from *R. maidis* collections of wheat, barley, maize and sorghum. The produced PCR products were around the expected size showing about 687 bps (Fig. 2). Multiple sequence alignment of the sequenced PCR products using the clustal W algorithm revealed that there are at least 5 polymorphs present in the analyzed samples (Table 1). Wheat plants had two different polymorphs W1 and W2 while barley showed no difference between B1 and B2 samples. The two remaining polymorphs were present as one isolated from sorghum and the other one was collected from sorghum and maize (Table 1).

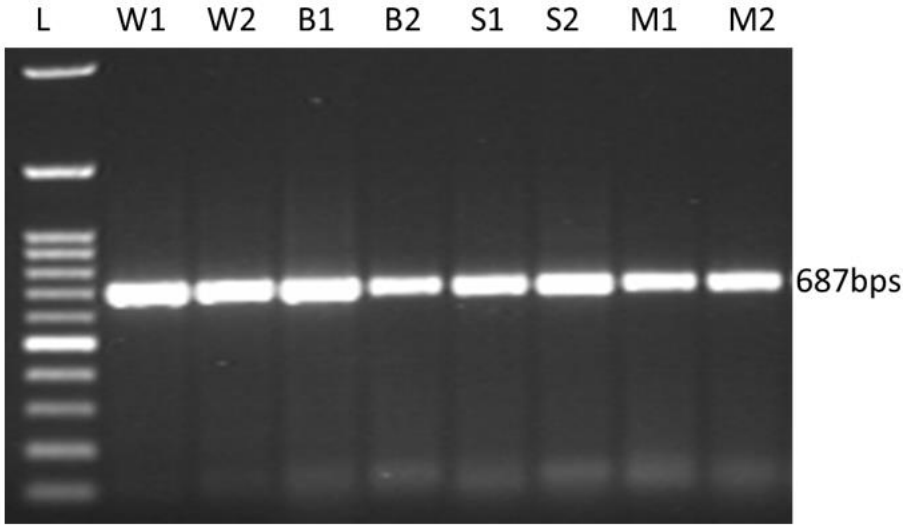

Fig. 2. Amplification of partial cytochrome oxidase subunit I of *R. maidis* using polymerase chain reaction using COI universal primers. DNA templates of *R. maidis* were collected from wheat (W1,W2); barley (B1,B2); sorghum (S1,S2) and maize (M1,M2). (L) represents 100bps DNA ladder.

Table 1. Identification of *R. maidis* haplotypes isolated from wheat (W), barley (B), sorghum (S) and maize (M).

Haplotype	**Polymorphism (bps)***				
	11	**36**	**48**	**53**	**58-59**
Rhopalosiphum maidis S1	T	C	T	T	CA
Rhopalosiphum maidis S2	A	C	-	T	CA
Rhopalosiphum maidis M1	A	C	-	T	CA
Rhopalosiphum maidis M2	A	C	-	T	CA
Rhopalosiphum maidis B1	A	C	C	T	CA
Rhopalosiphum maidis B2	A	C	C	T	CA
Rhopalosiphum maidis W1	A	A	-	G	TG
Rhopalosiphum maidis W2	-	C	T	T	CA

*Polymorphism (bps) was predicted based on multiple sequence alignment using clustal W

3.3. Phylogenetic Relationships among *Rhopalosiphum maidis* Polymorphs:

To address the phylogenetic relationships among different isolates of *R. maidis* from different crops, two isolates of *R. maidis* COI (accession: DQ499049.1 and DQ499048.1) and *Aphis craccivora* (accession: KR040128.1) COI were compared to the isolated sequences of *R. maidis*. Phylogenetic analysis of the sequenced COI gene isolated from different crops using the neighbor-joining method showed that the sequences were

clustered into five different clades that possibly represent different biotypes (Fig. 3). The first clade (I) included a mixture of *R. maidis* collected from both S1 and W2 plants. Clade (II) included a polymorph of the isolated *R. maidis* collected from the W1 plant. Barley polymorph was categorized in (III) while clade (IV) contained a mixture of the isolated *R. maidis* fed on M1 and S2. The fifth clade (V) included a polymorph of the isolated *R. maidis* collected from the M2 plant (Fig. 3). Interestingly, a phylogenetic comparison of COI gene sequences suggested that relationships in the isolated polymorphs; most especially that the isolated *R. maidis* collected from M1 and S2 were more closely related to DQ499049.1 *R. maidis* isolate 2 COI than the other isolated polymorphisms. The range of genetic distances between sequences is shown in (Table 2). The obtained data showed that the distance varied from 0.104 to 0.116 between the studied polymorphs. The highest similarity of distance values was recorded between S2, M1, M2, B2 and B1 and between S1, W1 and W2. The divergences were pairwise indicating that S2, M1, M2, B2 and B1 were closer to the known isolated than S1, W1 and W2.

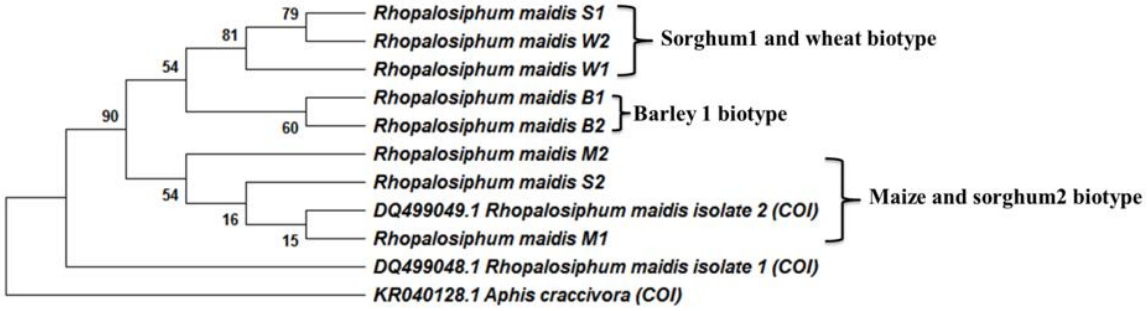

Fig. 3. Neighbour-joining (NJ) phylogenetic relationships between COI sequences using Kimura-2-parameter (K2P) distances. This tree was generated based on the comparison between the isolated sequences of *R. maidis* COI with two known isolates of *R. maidis* COI (accession: DQ499049.1 and DQ499048.1). *Aphis craccivora* (accession: KR040128.1) COI was used as outgroup sequence. Values above the branches are NJ bootstrap values. The bootstrap consensus tree inferred from 1000 replicates. There were a total of 658 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Table 2. Mean and range of genetic distances between sequences of intraspecific nucleotide divergences of COI for *Aphis craccivora* (accession: KR040128.1) and *Rhopalosiphum maidis* isolates 1 and 2 *(*accession*:* DQ499048.1 and DQ499049.1, respectively). Data were estimated by using Kimura's two parameter model (Kimura, M. 1980).

KR040128.1 *Aphis craccivora* (COI)										
DQ499048.1 *Rhopalosiphum maidis* isolate 1 (COI)	0.102									
DQ499049.1 *Rhopalosiphum maidis* isolate 2 (COI)	0.108	0.012								
Rhopalosiphum maidis S1	0.113	0.019	0.012							
Rhopalosiphum maidis S2	0.104	0.009	0.003	0.008						
Rhopalosiphum maidis M1	0.104	0.009	0.003	0.008	0.00					
Rhopalosiphum maidis M2	0.104	0.009	0.003	0.008	0.00	0.00				
Rhopalosiphum maidis B1	0.106	0.012	0.006	0.009	0.003	0.003	0.003			
Rhopalosiphum maidis B2	0.104	0.011	0.004	0.008	0.001	0.001	0.001	0.001		
Rhopalosiphum maidis W1	0.114	0.024	0.017	0.017	0.014	0.014	0.014	0.014	0.014	
Rhopalosiphum maidis W2	0.116	0.021	0.014	0.006	0.009	0.009	0.009	0.012	0.011	0.016

DISCUSSION

This study was conducted mainly to answer a research question related to the occurrence of different biotypes of *R. maidis*, the most widely spread cereal aphid in Egypt, by changing either the collection season or the host plant. The invasion rate of *R. maidis* on different plants varied with different seasons showing higher invasion rates in winter plants rather than summer. Similarly, *R maidis* preferred barley to wheat (winter plants) and sorghum to maize (summer plants). The maximum infestation rate of aphid species in Pakistan was observed in the winter season showing about 65.15% (Mushtaq *et al*., 2013). Furthermore, this species was recorded to prefer barley to both wheat and oat plants (Apablaza and Robinson, 1967). Amplification of partial COI gene using universal primers supplied the expected PCR product around 700bps. Generally, the same primer pair identified a similar size for the partial COI sequence in many genera of aphids including *R. maidis* (Valenzuela *et al*., 2007).

Several methods have been used to assess genetic relationships among aphid biotypes such as identification methods based on morphological characters and isozymes (Abid *et al*., 1989; Starks and Burton, 1977). However, these methods suffered several limitations in identifying all biotypes. Mitochondrial COI sequences could effectively distinguish different aphid species, and further different haplotypes within an aphid species (Krishnamurthy and Francis, 2012; Li *et al*., 2014). Furthermore, the variation in the rDNA internal transcribed spacers (ITSs) in the green peach aphid *Myzus persicae* identified two different haplotypes that were present on the same genome (Fenton *et al*., 1998). Sequence analysis of *R. maidis* COI data identified 5 different polymorphs present in wheat, barley, maize and sorghum plants. Identification of polymorphs usually requires RFLP analysis to confirm the sequencing results. The running work suffered from difficulties in finding appropriate restriction cutting sites at the suitable sites of most COI products. Furthermore, the polymorphism sites on *R. maidis* COI were highly directed toward the 5´-end leading to difficulties in visualizing the cutting products. Nucleotide polymorphisms identified three haplotypes in corn leaf aphids (Li *et al*., 2014). Generally, biotypes refer to an intraspecific group of organisms that are not morphologically different but vary in their biological characteristics (Eastop, 1973). The development of biotypes in aphids is believed to be a response to avoid plant resistance mechanisms. Generation of resistant plant cultivars should be proceeded by biotype identification (Anstead *et al*., 2002). For instance, in green bug aphids over 20 biotypes were identified based on host plant preference and their ability to produce selective damage to specific plant cultivars (Burd and Porter, 2006). Several biotypes of *S. graminum* were identified using the sequencing data of the mitochondrial COI gene (Kharrat *et al*., 2012). Several plant breeders use different plant cultivars especially those which are tolerant to pests. However, any changes or rapid evolutionary rate in the populations of aphids (i. e. biotypes) can tolerate the host-plant resistance leading to resistance loss in the host plant causing limitations in the usefulness of resistant plant cultivars (Weiland *et al*., 2008).

Several reports indicated that genetic differences were detectable between aphid biotypes (Black *et al*., 1992; Shufran *et al*., 1992; Black 1993; Anstead *et al*., 2002). Approximately 50% of the recognized insect biotypes on agricultural crops are aphids (Saxena and Barrion, 1987). In contrast, Aikhobare *et al*. 1998 with USA populations, and Lopes-Da-Silva *et al*. (2004) with Brazilian populations, could not distinguish biotypes C and E of green bugs by RAPD markers. Li *et al*., 2014 revealed that no polymorphism was found in the mitochondrial DNA from different sorghum aphid and green bug populations. Bulman *et al*., 2005 developed PCR tests to distinguish species and populations of New Zealand *Rhopalosiphum* aphids and detected two ITS haplotypes in aphid species. Lee *et*

al., 2011 confirmed that DNA barcodes are highly effective identification tools for species of the family, aphididae. RAPD-PCR has been successfully applied to reveal distinctive patterns among some green bug biotypes (Black *et al*., 1992; Aikhionbare *et al*., 1998; Lopes-Da-Silva *et al*., 2004). Based on RAPD-PCR polymorphism, Amin *et al*., 2013 recorded the highest similarity value between *R. maidis* and *R. Padi* and each of them can also separate the Graminaceae host plant aphid from others.

Although increasing numbers of aphid molecular phylogenetic studies have been made (Moran *et al*., 1999; Normark, 2000), a few gene sequence-based tools have been developed for the differentiation of aphid species. Our results showed that using Phylogenetic analysis, the isolated sequences of *R. maidis* clustered into five clades that possibly represent different biotypes. Similar results were reported by (Kharrat *et al*., 2012) who said that the isolated sequences of *Schizaphis graminum* were clustered into two clades, they distinguished between agricultural and non-agricultural biotypes of *S. graminum.* Xin *et al*., 2014 classified the *Sitobion avenae* aphid populations into three clusters using the UPGMA method. Bulman *et al*., 2005 confirmed that *Rhopalosiphum* species formed a monophyletic group with *Rhopalosiphum sp.* T lineage clustering mostly closely with *R. near insertum*. Results showed that polymorphisms infesting sorghum plants mixtures with the other polymorphs fed on maize and wheat plants. This may be due to the adaptation of sorghum plants to climate change conditions, consequently, their polymorphs inhabited winter and summer plants and adapted to the climatic changes (Li *et al*., 2014). This adaptation clearly affected the infestation rate of *R. maidis* on sorghum plants which fluctuated between the rate found on wheat and maize plants. Unlike, independent polymorphs were recorded on barley plants that have the most infestation rate. These results contribute to Whitlock (1992) who reported that genetic differentiation might be accelerated by the high level of variation in population size. Also, Guo Wei *et al*., 2005 recorded that wheat aphid populations frequently exhibit high levels of genetic variations.

Conclusion

In conclusion, the variable of the season and host plant mean that identification of different biotypes of aphids can be difficult. In difficult identification cases, molecular DNA techniques may usefully be applied to aid aphid diagnostics. DNA barcoding is designed purely to aid the recognition and identification of known species. However, it has also been used as a method for both the discovery of new species and revising taxonomies. Moreover, DNA sequence information will provide an essential phylogenetic backbone for aphid identification and classification. Using DNA sequences, we have demonstrated that different biotypes of *R. maidis* infesting different host plants of cereal crops at different climatic conditions.

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