INTRASPECIFIC VARIATION IN THE INTERNAL TRANSCRIBED SPACER (ITS) REGION OF GREEN PEACH APHID *MYZUS PERSICAE* [(SULZER) (HEMIPTERA: APHIDIDAE)] UNDER ELEVATED ATMOSPHERIC CO_2 PRESSURE

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Abstract. The continuously increasing concentrations of atmospheric CO_2 is predicted to affect biological processes at many levels of organisms. Yet, no study exists in the literature attempting to describe that the elevated atmospheric CO_2 (eCO_2) concentration may cause an evolutionary response on nucleotide sequences of ribosomal DNA of *Myzus persicae* [(Sulzer) (Hemiptera: Aphididae)]. Here, we provide a preliminary study to understand how the insect ribosomal DNA sequences are influenced under the elevated CO_2 levels after several generations. Four *M. persicae* populations were established for 35 days under ambient CO_2 (a CO_2) (400 ppm), e CO_2 (600 ppm), e CO_2 (800 ppm) and e CO_2 (1000 ppm) at 29ºC in moisture-controlled greenhouse chambers. Intraspecific variation of *M. periscae* was assessed by the sequencing and analyzing the internal transcribed spacer (ITS) region of *Myzus persicae* (Sulzer) under elevated atmospheric CO_2 pressure. Based on our results, the phylogenetic analysis of ITS sequences differentiated the individuals grown at 800 ppm CO_2 level. The alignment of ITS sequences of all specimens revealed several single-nucleotide substitutions on the nucleotide sequence of *M. persicae* samples grown at 800 ppm CO_2 level. Overall results show that the elevated atmospheric CO_2 levels could be a powerful evolutionary force than expected on *M. persicae* reared on eggplants.
Keywords: *evolution, atmospheric CO_2, alignment, intraspecific variation, nucleotide comparison*

Introduction

Species of the Aphididae (Hemiptera) family species are found almost everywhere in the world, but these pests are more common in temperate regions than in the tropics. It has been reported that there are approximately 5000 species belonging to the Aphididae family in the world and 1600 species in Europe (Nieto Nafria et al., 2013; Blackman and Eastop, 2020). Considering aphids in Turkey, the number of species identified is reported to be 558 (Akyürek et al., 2019; Özdemir, 2020). Aphids are among the most significant pests in agricultural areas around the world. *Myzus persicae* Sulz. (Hemiptera: Aphididae) causes serious damage both in cover crop and the open field (Blackman and Eastop, 2006; Van Emden and Harrington, 2017). Aphids are generally considered polyphagous pests. Among them *M. persicae* feed on phloem tissue of plants (Pollard, 1973). As a result of feeding, the growth of the plant may stunt, produce plant galls, cause deformation of leaves, buds, and flowers and transmit plant virus diseases. *M. persicae* produces large amounts of a sugary liquid waste called "honeydew", and as a result of this secreted substance, saprophyte fungi can grow on honeydew that accumulate on leaves resulting in a decrease in the plant's photosynthesis (Lodos, 1982). This pest completes its life cycle in a short time when there are suitable climate conditions and continues its life throughout the season. Temperature and humidity play an important role in its development.

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M. persicae alone is reported to carry more than 150 plant virus diseases from different crops, including vegetables belonging to the Solanaceae family (Sharma et al., 2008).

Atmospheric CO_2 has increased from about 280 ppm to 400 ppm since the Industrial Revolution in the mid-1700s (Bonan and Doney, 2018). In the inter-country climate panel held in 2014, it was reported that the atmospheric CO_2 rate will increase between approximately 750 and 1300 ppm by the year 2100 (IPCC, 2014). The increase in the CO_2 ratio in the atmosphere in the next century will cause a decrease in the nutrient content in the host of many insect species, thus, it is expected that both larval development time and mortality rate will increase (Carlos and Trumble, 1998). Whether these changes will affect insects' feeding behavior, biology, host preferences, adaptation to climatic conditions and genetics is not clearly known. In the context of climate change, the impact of increased CO_2 on insect species has become a major issue over the last three decades. Elevated CO_2 levels may modify the insect behavior for feeding, but precise effects on insect genetics are poorly known. There have been many studies on the effects of enhanced CO_2 on behavior of insect species (Stiling et al., 2002; Chen et al., 2007; Sudderth and Sudderth, 2014) however, there has been no investigation of the effects of elevated CO_2 levels on intraspecific variation. The purpose of the study was to obtain some initial data regarding the possible effects of ambient and elevated CO_2 levels on intraspecific variation on one example of a species (*Myzus persicae*) using the internal transcribed spacers of the ribosomal DNA (ITS rDNA) region.

Materials and Methods

Source of green peach aphid and eggplants

The colonies of green peach aphids trapped in the eggplant fields. Once aphids were identified, they were maintained in the temperature controlled growing chamber on the same species (*Solanum melongena* L. cv. Pala-49) at 14 h day length alternating at temperature of regimes (29/19±1ºC) under constant relative humidity (60±10% RH with 14:10 h L:D photoperiod at 8-10 klux light intensity photoperiod conditions). The aphids were sourced from the colony cultured on these plants. The eggplants (*Solanum melongena* L. cv. Pala-49) were then transferred to a temperature, humidity and CO_2-controlled greenhouse at the Malatya Turgut Ozal University to provide plants for the experiments. A separate concentration of carbon dioxide level was applied in each compartment of the greenhouse including ambient (400 ppm) and elevated 600 ppm, 800 ppm, and 1000 ppm CO_2 levels, consisting of four compartments.

Extraction of genomic DNA from the green peach aphid

Genomic DNA of single aphid was simply and effectively extracted at room temperature using DNeasy® Blood & Tissue Kit (Qiagen, Germany). Seventeen specimens of *M. persicae* were collected from eggplants grown at ambient (400 ppm) and elevated (600, 800, 1000 ppm) CO_2 levels. The four samples of *M. persicae* from ambient CO_2 level (400 ppm) were served as controls (*Table 1*). In order to eliminate the external microbial contaminants, the aphid samples were rinsed in 70% ethanol for 5 min and then rinsed four times with sterilized water to eliminate the external microbial contaminants. The whole body of an adult aphid was used to purify total genomic DNA (*Table 1*). All DNA preparations were stored at -20°C until use.

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Table 1. *Characteristics of aphid samples used in this study grown under ambient and elevated CO_2 levels*

Number of rooms	CO_2 level	Number of individuals produced	Number of samples sequenced	Number of generations	Temperature of each room	After the third generation duration of rearing (day)
1st room	400 ppm	150	4	4	29/19*	35
2nd room	600 ppm	180	5	4	29/19	35
3rd room	800 ppm	130	4	4	29/19	35
4th room	1000 ppm	140	4	4	29/19	35

*At 14 h day length at temperature of 29 /19ºC (L:D) photoperiod at 8-10 klux light intensity

Elevated CO_2 conditions and experimental design

Five adult aphids maintained in the growing chamber were removed from their host plant and placed on young eggplant leaves grown at ambient (400 ppm) and elevated 600 ppm, 800 ppm and 1000 ppm levels of CO_2 in the green house. The trials were carried out at four-room temperature, humidity and CO_2 controlled greenhouse facility located at Malatya Turgut Ozal University. Each had alternating (29/19±1ºC) temperature regimes under constant relative humidity and photoperiod conditions (60±10% RH with 14:10 h L:D photoperiod) at 8-10 klux light intensity. *M. persicae* stock culture was established on the eggplant plant in each CO_2 level room. Four young eggplant plants were placed in each room and five *M. persicae* individuals were transferred on each plant. The aphid samples were taken from the individuals grown in each room, after 4 generations. Individuals taken from each climate room were stored in sample bags at -80ºC, and total DNA isolation was made from these samples. Genomic DNA isolation was performed from a single individual.

PCR amplification of 16S rRNA and sequencing

An approximately 730 bp DNA fragment of ITS region of nuclear ribosomal RNA (nrRNA) gene containing ITS1, 5.8S and ITS2 was amplified by polymerase chain reaction (PCR) with the primers (ITS4:5'-TCCTCCGCTTATTGATATGC-3'and ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990) (*Figure 1*). The PCR mixture consisted of 5 µL of 10×reaction buffer (200 mMTris–HCl pH: 8.4, 500 mMKCl), 2 µL of genomic DNA, 1 µL of dNTPs (10 mM each), 3 µL of $MgCl_2$ (25 mM), 1 µL of each primer (100 pmol), 0.4 µL of DNA polymerase, and 36.6 µL of DNase free sterile water. PCR amplification was performed using the following thermocycling program: a 2 min initial denaturation at 94°C, followed by 36 cycles of 94°C for 1 min, annealing at 55°C for 1 min and an extension of 72°C for 2 min, and a final extension of 72°C for 10 min. The PCR amplified DNA fragments were separated on 2% agarose gel containing fluorescent dye and recovered by agarose gel extraction kit (Bioline, Germany). A total of 17 nuclear ribosomal DNA fragments of *M. persicae* samples, originated from the same population and reared at four levels of CO_2, were sequenced after completing their 4th generations in growing chamber. All the sequences studied in this study are deposited in GenBank database under the accession numbers given in *Table 2*.

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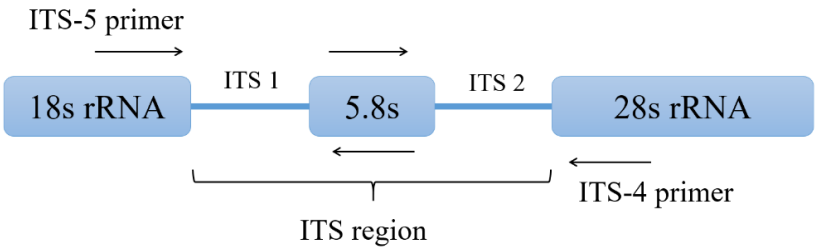

Figure 1. *Diagram of nuclear ribosomal RNA (nrRNA) gene containing ITS1, 5.8S, and ITS2 regions with primer binding sites*

Table 2. *List of ITS sequences of Myzus persicae samples used in this study, length and GenBank accession numbers*

Species	CO_2 level (ppm)	Name of the sample	Length (bp)	Accession no
Myzus persicae	400	Sample 1	698	MW581037
M. persicae	400	Sample 2	699	MW581033
M. persicae	400	Sample 3	699	MW581028
M. persicae	400	Sample 4	699	MW581038
M. persicae	600	Sample 1	699	MW581034
M. persicae	600	Sample 2	699	MW581035
M. persicae	600	Sample 3	698	MW581032
M. persicae	600	Sample 4	698	MW581029
M. persicae	600	Sample 5	699	MW581031
M. persicae	800	Sample 1	699	MW581036
M. persicae	800	Sample 2	699	MW581030
M. persicae	800	Sample 3	698	MW581039
M. persicae	800	Sample 4	699	MW581073
M. persicae	1000	Sample 1	699	MW581070
M. persicae	1000	Sample 2	698	MW581071
M. persicae	1000	Sample 3	699	MW581072
M. persicae	1000	Sample 4	699	MW581069

Bioinformatic analysis

The ITS sequences of aphid samples were initially edited manually than aligned using CLC Main Workbench Version 6.2 (CLC bio, Denmark) software. To determine the intraspecific variation among the seventeen *M. persicae* samples grown in four different levels of CO_2, we constructed a phylogenetic tree using ITS sequences obtained in this study. Intraspecific pairwise alignments of all loci considered (ITS1, 5.8S, ITS2) in this research were generated using CLC Main Workbench Version 6.2 (CLC bio, Denmark) software for all *M. persicae* samples. The phylogenetic tree was built under the neighbor joining algorithm. The relationships were assessed using 1000 bootstrap replicates.

Secondary structure analyses

In order to predict the most stable secondary structure of the nuclear ribosomal DNA, containing ITS1, 5.8S and ITS2 sequences, the established full sequences were folded

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and visualized using the mfold structure prediction package of CLC RNA Workbench Version 6.2 (CLC bio, Denmark) software by energy minimizing. Each consensus sequence, belonging to a a particular CO_2 level, was folded separately to build the full structure.

In silico virtual RFLP analysis

Delimitation of the start and end points of each sequence was carefully trimmed manually in order to arrange the nucleotide bases at the start and end of the sequences were identical for all individuals. Computer-simulated (*in silico*) RFLP analysis of the ITS sequences of all amplified specimens was performed using pDRAW32 (AcaClone Software). The following 17 restriction enzymes which are commonly used in bench digests and identification *in silico* RFLP analysis for phytoplasmas (Lee et al., 1998; Oksal et al., 2017; Usta et al., 2018) were adapted and screened: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. Among these enzymes, HaeIII, HpaII and TaqI did not cut any of the sequences submitted to digestion, but were retained in the data set for comparison. Following the *in silico* restriction digestion, a virtual 1.0% agarose gel image plotted automatically to the computer screen to capture the RFLP pattern of 16Sr DNA sequences using the program pDRAW32 (AcaClone Software).

Results

An approximately 700 bp single amplified PCR product was obtained in all *M. persicae* samples for the complete rDNA ITS region. The ITS sequence ranged from 698 bp to 699 bp in all accessions. Only three base difference was detected within the 800 ppm CO_2 level of growing condition of *M. persicae*. The divergence in the ITS genes among the individuals grown at 800 ppm tested was low (*c*. 0.4% informative sites) (*Figure 2*). However, the variation among the individuals within the individual grown at ambient OC2 (400 ppm and elevated CO_2 levels (600 and 1000 ppm), appears to be very low, with no informative positions. GenBank accession numbers of the ITS sequences and the origin of samples are given in *Table 2*.

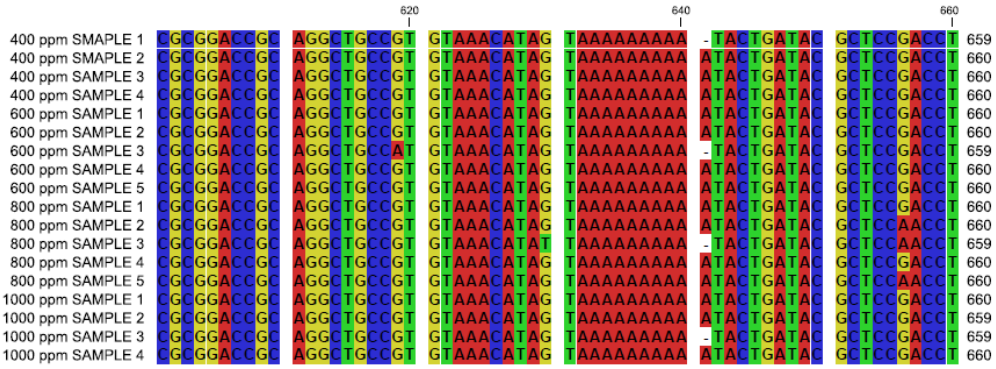

Figure 2. *Sequence alignment of ITS region of M. persicae individuals grown at ambiante and elevated CO_2 levels. Mutational sites of ITS region of M. persicae grown at 800 ppm CO_2 level are boxed*

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The intraspecific variation among the individuals of *M. periscae* resolved by ITS sequence comparisons. Our results show that the intraspecific variability on ITS sequence of *M. persicae* was noticeable at 800 ppm level. In comparison to ambient CO_2, 600 ppm and 1000 ppm CO_2 levels, except one specimen, the ITS sequences of a *M. persica* specimens grown at 800 ppm CO_2 level was phylogenetically diverse. The green peach aphids grown at 800 ppm are clustered in the same branch (Group I) which clearly distinguished from the other individuals grown under ambient CO_2 and 600 and 1000 ppm CO_2 levels (*Figure 3*).

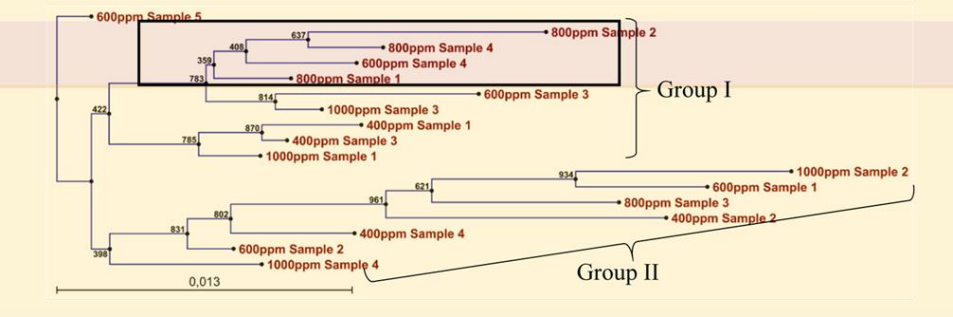

Figure 3. *Phylogram generated from ITS nucleotide sequence data of 17 Myzus persicae specimens grown at elevated CO_2 (600, 800 and 1000ppm) levels and ambient CO_2 level using the Neighbor Joining algorithm. The value of 1000 was used for bootstrap analysis and corresponding values are shown on individual branches. The green peach aphids grown at 800 ppm CO_2 level are boxed*

In *Figure 3*, the neighbor joining phylogram displays the genetic relationships between the aphid samples grown at different CO_2 levels. The Group I composed of 2 subgroups. One of the subgroups consists of 3 green peach aphids all from the same chamber of greenhouse having 800 ppm CO_2 level, indicating their genetic similarity. This cluster received 78% support in the bootstrap analysis. The Group I consist also individuals from ambient and other elevated CO_2 levels, as do Group II, reflecting intraspecific species similarities. However, an aphid sample from the same origin grown at 600 ppm CO_2 level (sample 5) did not grouped together by other aphid samples but instead formed a separate individual branch rooting the tree, indicating its divers feature (*Figure 3*). In Group II, the entire ITS sequence was typically fully conserved within the species, and the variation observed was negligible. In general, no distinct treatment effects were observed on ITS sequences in elevated CO_2 levels among the aphid samples of Group II. Overall results show that the green peach aphid reared on eggplants at different levels of increased atmospheric CO_2 showed a low level of intraspecific variation for Group II members.

Along with nucleotide sequences, the most stabile secondary structure of these sequences was also obtained to compare the topology of sequence paring. What is most significant is the strong distinction in the secondary structure pattern of consensus sequence of individuals grown at 800 ppm CO_2 level (*Figure 4*). The most stable secondary structure alone provided a clear and informative secondary structure topology.

The *in silico* RFLP analyses revealed that the ITS region produced similar banding patterns for all seventeen specimens of *M. persicae*. None of the restriction enzyme used in this study were able to generate different banding patterns (*Figure 5*). The banding patterns obtained from restriction digestion was not sufficient to differentiate the tested specimens with no informative positions.

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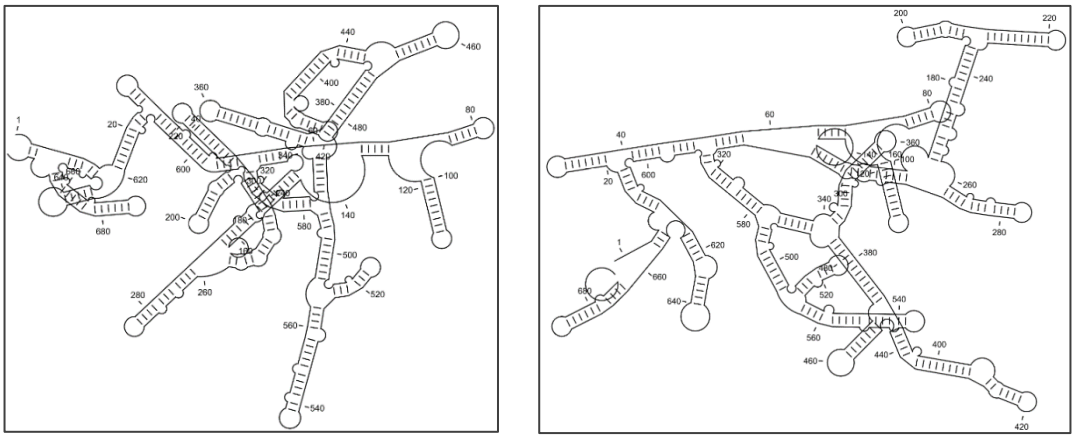

Figure 4. *The most stabile secondary structure based on 730 bp of consensus ITS sequence of green peach aphids grown at ambient CO_2 (A) and elevated (600, 800 (B) and 1000 ppm) CO_2 levels*

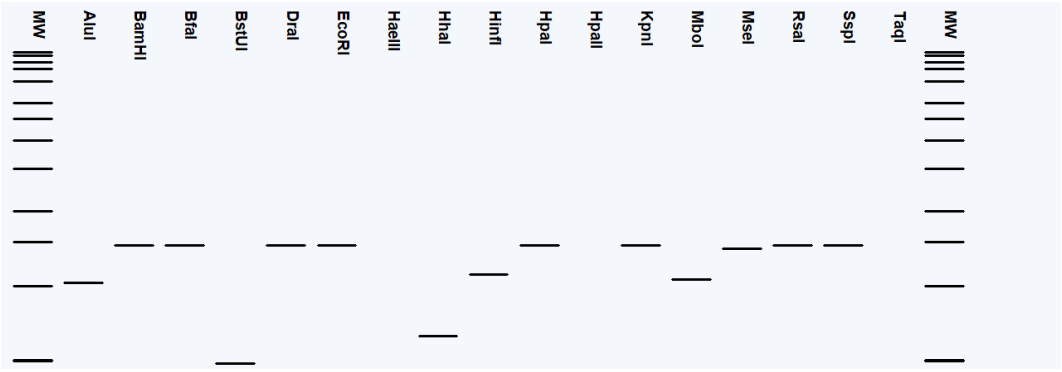

Figure 5. *In silico virtual restriction endonuclease digestion profile of ITS sequence of M. persicae grown at 400 ppm CO_2 level. In the simulated digestions for the recognition sites 17 restriction enzymes were used. The restriction patterns of the all samples were identical. MW: 1 kb DNA ladder*

Discussion

The present study was undertaken to investigate the level of intraspecific variation within the rRNA gene sequences of *M. persicae* based on sequence alignment, secondary structure prediction and *in silico* PCR-RFLP under the CO_2 pressure. It is frequently reported that the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nr DNA) has been widely used for identification and phylogenetic analysis of many microorganism, plant and insect families (Lee et al., 1998; Mir et al., 2009; Keskin et al., 2017; Oksal et al., 2017; Usta et al., 2018).

For comparative purposes, we focused on the prediction of the most stabile secondary structure of ITS sequences and *in silico* virtual RFLP analysis to our consensus sequences of aphids grown at 400, 600, 800 and 1000 ppm CO_2 levels. Point mutations (insertions, deletions or nucleotide substitutions) in ITS sequence of individuals grown at 800 ppm result in a change in secondary structure configuration and fragment size. However, this change did not affect the *in silico* virtual RFLP profiles of aphids tested in this study. The all ITS sequences used in our study showed identical virtual RFLP patterns indicating

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that all these samples cannot be distinguished by this method. The present data suggest that the *in silico* virtual RFLP patterns is not sufficient to understand the intraspecific variation within the species and cannot be perceived by a consideration of virtual RFLP pattern alone. For instance, in this study, the *in silico* approach implementing virtual digestion by 17 key restriction enzymes, did not enabled easy visualization of polymorphisms in ITS sequences of *M. persicae*. However, it may have done for phytoplasma detection and identification and would ultimately facilitate the discovery of new phytoplasma lineages (Lee et al., 1998; Ramdeen and Rampersad, 2012). In species identification and differentiation, recent entomological applications of RFLP studies have focused on mtCOI gene to solve known mitochondrial DNA polymorphism in Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera (Germain et al., 2013; Arimoto and Iwaizumi, 2014; Ovalle et al., 2014; Vesterlund et al., 2014). Comparison of virtual RFLP analysis versus the prediction of secondary structure analysis revealed that the interpretive feature of the later one seems to be higher in understanding of the intraspecific sequence divergence of a given species.

Mutational variation within ITS sequences of *M. persica* specimens grown at 800 ppm CO_2 level probably due to the rate of sexual and asexual reproduction, the number of generations annually, the rate of mutation, and the environmental conditions. Coviella and Trumble (1998) emphasize that the extended period of elevated CO_2 level seems to be a strong evolutionary force in many insect species, who has relatively short generation times and potential for rapid genetic turnover.

The ITS region of nuclear ribosomal is separated into ITS 1 and ITS 2. The ITS 1 is present between 18S and 5.8S rRNA whereas ITS 2 is present between 5.8 and 26s rRNA. 5.8S rRNA is a highly-conserved region (Baldwin et al., 1995). Generally, the *M. persicae* individual grown in ambient and elevated CO_2 levels separated into two groups by phylogenetic analysis of ITS sequences. Except one specimen of 800 ppm CO_2, the aphid samples grown at 800 ppm CO_2 level were clustered in the same group. This is more likely to be related to an elevated CO_2 factor, since some characters are strongly affected by different selection pressures (Mir et al., 2010) and, at some level, expected due to the variable nature of the ITS1 and ITS2 sequences (Nilsson et al., 2009). Here, we used elevated CO_2 concentration as selection pressure, however, the data presented above leave little room for interpretation on the actual influence of elevated CO_2 on ITS sequences, thus the reason that mutational changes increased elevated CO_2 level in this study remain unknown. The present results should be tested with more specimens experimentally. Pillmann et al. (1997) reports that for the measurement of the amount of intraspecific variation within a particular population may not require sampling of many individuals. In the point of view of the conserved nucleotide sequences, long term studies will make critical contribution to our understanding of elevated CO_2 effects on sequence divergence within a species.

Conclusion

Although there are many studies investigating the possible implications of the increasing CO_2 level in the Earth's atmosphere on the genetic characteristics of living organisms, the evolutionary affects remain uncertain. We conducted a preliminary study to analyze the possible changes on the green peach aphid ribosomal DNA sequences grown under ambient CO_2 (a CO_2) (400 ppm), e CO_2 (600 ppm), e CO_2 (800 ppm) and CO_2 (1000 ppm) CO_2 levels. Among the green peach aphid individuals who gave several

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generations after adapting to different CO_2 levels (400 ppm, 600 ppm, 800 ppm and 1000 ppm), striking and exciting differences were found in the ITS region of those who developed at 800 ppm carbon dioxide level. Under the light of these results, it has been seen that elevated atmospheric CO_2 levels may have an evolutionary force on *M. persicae*. However, additional studies are needed to test this hypothesis with more aphid samples and high number of offspring. Continued scientific studies are required to determine the relationship between elevated CO_2 levels and the evolutionary link.

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