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MOLECULAR CHARACTERIZATION OF *PHYLLOCINISTIS CITRELLA* (STAINTON) COLLECTED FROM SELECTED DISTRICTS OF KHYBER PAKHTUNKHWA

Muhammad Ismail[1*], Ahmad-Ur-Rahman Saljoqi[2]

[1*,2]Department of Plant Protection, Faculty of Crop Protection Sciences. The University of Agriculture, Peshawar, Pakistan.

***Corresponding Author:** Muhammad Ismail
*E-mail: ismailuop11@gmail.com

Abstract
The study investigated the genetic diversity and population differentiation of *P. citrella* populations from Peshawar, Malakand and Haripur, Pakistan using genomic DNA amplification with three distinct primers (OP-A01, OP-K02, and OP-A02). Consistent amplification patterns across these populations revealed a total of 30 distinct markers, ranging in size from 250 to 2500 base pairs, with each primer generating between 4 to 10 markers. The percentage of polymorphic markers, indicative of genetic diversity, varied among the primers, with OP-A01, OP-A02, and OP-K02 showing 70%, 75%, and 80% polymorphism, respectively, resulting in a mean polymorphism of 75%. The primers yielded five distinct phenotypes, with an average of 7 phenotypes per primer. Genetic distance calculations among the sampled populations revealed the lowest genetic distance between the Malakand and Haripur populations, and the highest between the Haripur and Peshawar populations. A dendrogram constructed using the UPGMA method revealed two distinct clades, grouping populations from Peshawar and Malakand together, and Haripur separately. Principal coordinate analysis reinforced these findings, illustrating significant genetic differentiation. Despite the substantial geographic distance, a relatively small genetic distance was observed between Peshawar and Malakand populations, indicating a weak correlation between geographic and genetic distances. This genetic analysis provides valuable insights into the genetic structure of *P. citrella* populations, informing future research and pest management strategies.

Keywords: *Phyllocnistis citrella*; Genomic DNA amplification; Molecular markers ; Genetic diversity; Polymorphism; Phenotypic analysis; Pest management strategies.

INTRODUCTION

The citrus leaf miner, *Phyllocinistis citrella* (Stainton) is a significant pest of the citrus all over the world (Mustafa *et al*., 2014). The female of this pest lay eggs on lower surfaces of the young leaves of all citrus species, and instantly after hatching, the larvae of the citrus leaf miner penetrates into the foliar tissues and break them. Remain inside the mines on the leaves until their period of development (willink *et al*., 1996). The morphological cycle of the *P. citrella* highly depends upon the temperature and it requires total of 32.7 days from the egg to adult stage. There are many other factors that can affect the cycle (Chagas and Parra 2000).

The direct damage caused by *P. citrella* reduce the leaf area, leaf fall in pre-mature stages as well as results in the reduction of shoots developments, as well as affecting the process of photosynthesis and subsequently the production of the citrus crop (Heppner 1993). Additionally, the *P. citrella* causes the injuries of an extensive nature also provide facilitation to provide a doorway to microorganism, especially the bacterium *Xanthomonas citri* (Subsp), the basic citrus canker causing agent (Hall *et al*., 2010).

In almost all parts of the world *P. citrella* has been reported including Australia, Asia, Africa, United States of America, South America, Central America (Bermudez *et al* 2004). In the Mediterranean Basin, this insect pest has populated most citrus growing areas during the last decade of the 20th century (urbaneja *et al*., 2001). The *P. citrella* in the year 1994 was found and detected in Tunisia, in Tabarka region and was being spread to all the areas and became a significant challenge and threat to all of the citrus species, and its infestation rate reached to 100% (Kheder *et al*., 2002).

Molecular studies of the pests' population and the valuation of the genetic diversity, identification of the patterns of diversity, determine the history of its migration, trails and infectious biotypes' characterizations, has significant role in developments of Integrated Pest Management techniques and enhancement for improved efficiency and sustainability. The technique used in several studies for genetic diversity is random-amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). It has been used for assessment of the Molecular Characterizations of major threatening pests i.e. potato white fly, *Bemisia tabaci,* date palm bore, *Oryctes Agamemnon* and Mediterranean fruit fly, *Ceratitis capitate* (Abdullah *et al*., 2012). In another study RAPD-PCR was applied to evaluate the genetic diversity and structure of the pest population of *P. citrella* collected from various areas of Tunisia to find out the inner sights of the species ecology and offer some applied information for development of management programs against the pest.

The citrus leaf miner is a foremost persistent pest of citrus in Pakistan. For the development on an effective integrated pest management strategy it is noteworthy to evaluate the population structure and genetic diversity of *P. citrella*. For this purpose, a method called random-amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) is applied worldwide. The random-amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) procedure has been in practice in quite a lot of studies, to assess the genetic multiplicity of some most important persistent pests, such as date palm root borer, *Oryctes agamemnon* Burmeister (Abdallah *et al*., 2012). This study aims to investigate the ecological insights provided by genetic diversity assessments to contribute to the development of effective management programs against *P. citrella* and offer applied information for the enhancement of Integrated Pest Management (IPM) strategies targeting *P. citrella* based on insights gained from the genetic diversity and population structure analysis

2. MATERIALS AND METHODS

2.1 Specimen collection

CLM larvae were collected from citrus trees following the procedures of Fritsch *et al.* (2005) with some modifications. Twenty larvae from each selected district were selected randomly for extraction of DNA isolation to reduce contamination of DNA by endo-parasites (Landry *et al*., 1999) in spring from Peshawar, Haripur and Malakand. The collected specimens were washed and stored in 96% Ethanol before analysis in the laboratory of Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar Pakistan.

2.2 Genomic DNA Extraction

To perform genomic DNA extraction, the Spinklean Genomic DNA Extraction Kit from Thermoscientific® USA was employed, following the procedure outlined by Zimmerman et al. (2000) with necessary adjustments. The specimens were pulverized in liquid nitrogen, and the DNA extraction process adhered to the manufacturer's instructions. In a nutshell, TL Buffer (250 µl) was introduced to the crushed larvae for tissue lysis. After thorough vortexing to ensure proper mixing, 199 units of the enzyme were incorporated and mixed extensively. Next, 220 µl of lysis buffer was

added to the solutions and mixed thoroughly. A total of 560 µl of Buffer TB was introduced to each Eppendorf tube and uniformly mixed by vortexing, followed by an incubation period of 10 minutes at 65°C. Subsequently, 200 µl of absolute ethanol was added. The sample mixtures were passed through a column, which was then placed in a sterile collection tube and subjected to centrifugation at 8000 Xg for 60 seconds. The column underwent two washes with 750 µl of wash buffer 'PS,' each time followed by centrifugation at 8000 Xg for 1 minute. To eliminate any remaining traces of ethanol, the column was centrifuged again at 10,000 Xg for an additional minute.
To extract the DNA, approximately 200 µl of preheated TE buffer was introduced to the column membrane in new tubes, followed by an incubation at room temperature for 2 minutes. The final step involved centrifuging at 10,000 Xg for 1 minute to obtain the DNA, which was then stored at -20°C.

2.3. Polymerase Chain Reaction and Gel Electrophoresis

The Polymerase Chain Reaction (PCR) reactions were conducted in 25 µl reaction volumes, consisting of whole genomic DNA (140 ng), 0.25 mM RAPD primers (Genlink, USA), 50 nM KCL, 1.5 mM MgCl2, 10 mM Tris, 200 µM of each dNTP, and 2.5 units of Taq polymerase (Thermo Scientific). The optimized amplification protocol included an initial denaturation step of 4 minutes at 94°C, followed by 40 cycles, each comprising a 50-second denaturation step at 94°C, a 1-minute annealing step at 280°C, and an additional 1-minute step at 720°C, followed by a final extension of 10 minutes at 72°C. All amplification reactions were performed using the GeneAmp PCR System 2700 programmable thermocycler. The resulting amplification products were visualized on a 2% agarose gel and stained with Ethidium Bromide using a UV trans illuminator.

2.4. Statistical analysis:

For the statistical analysis of RAPD PCR data, each band was treated as a genetic locus categorized as (1) present or (2) absent, forming a 1-0 matrix. Genetic distances were computed using the UPDMA method, based on Nei and Li's (1979) formula: GD = 1 - (dxy / (dx + dy - dxy)), where GD represents genetic distance, dx is the total bands in genotype 1, dxy is the common loci between genotypes, and dy is the total loci in genotypes. DNA amplification profiles were analyzed using the online software PopGene version 3.1, available at www.ncbi.org.

3. RESULTS

The results of genomic DNA amplification provided consistent amplification patterns for the sampled *P. citrella* populations collected from three distinct districts: Peshawar, Malakand, and Haripur. Employing three different primers, namely OP-A01 and OPK-02 and OP-A02, a total of 30 distinct markers were identified, with marker sizes ranging from 250 to 2000 base pairs for OP-A01, 500 to 1500 base pairs for OP-K02 and 500 to 2500 base pairs. Each primer revealed a unique set of markers, varying from 4 to 10 markers per primer (as illustrated in Table 1).

The percentage of polymorphic markers, a key indicator of genetic diversity, was calculated for each primer. The primers OP-A01, OP-A02 and OP-K02 exhibited polymorphic markers at percentages of 70%, 75% and 80%, respectively. This discrepancy in polymorphism percentages reflects the varying efficacy of these primers in discerning the collected *P. citrella* populations from different geographic locations. When considering both primers collectively, the mean value of % polymorphism (% P) was calculated at 75 % (25 out of 30 markers), indicating a substantial level of genetic diversity within the sampled *P. citrella* populations.

The application of these three primers yielded five distinct phenotypes, with 7 phenotypes generated using OP-A01 and 8 using OP-K02 and 6 using OP-A02, resulting in an average of 7 phenotypes per primer (as indicated in Table 2). This phenotypic diversity highlights the genetic variation present among the studied *P. citrella* populations.

More genetic analysis involved calculating genetic distances between populations. Among the *P. citrella* populations sampled from Peshawar, Haripur, and Malakand, the lowest genetic distance (0.2389) was observed between the Malakand and Haripur populations, while the highest genetic distance (0.3245) was recorded between the Haripur and Peshawar populations (as shown in Table 3).

A dendrogram was constructed using the Unweighted Pair Group of Arithmetic Means (UPGMA) method (Fig 1), revealing the clustering of *P. citrella* populations into two distinct clades. The first group consisted of populations from Peshawar and Malakand, while the second group encompassed populations from Haripur. The principal coordinate analysis (PCO) plot (Fig 3.1) reinforced these findings, with the first axis accounting for 50.55% of the variation and the second axis representing 23.33% and the third representing 26.12%. This plot closely aligned with the UPGMA dendrogram, illustrating the genetic differentiation between *P. citrella* populations collected from different regions. Even though the substantial geographic distance between Peshawar and Malakand, a relatively small genetic distance of 0.0756 was observed between their respective *P. citrella* populations, emphasizing the complex relationship between geographic and genetic distances (as indicated by a weak correlation of 0.31).

The genetic analysis of *P. citrella* populations from different regions revealed significant genetic diversity and population differentiation. The use of molecular markers and genetic distance measurements provided insights into the genetic structure of these populations, which can be valuable for future research and the development of pest management strategies.

Table 1. Sampling, geographical and meteorological data on the studied *P. citrella* populations, from selected districts of Pakistan

Site	Province	Geographical zone	Sampling date	Annual rainfall (mm)	No of days with rainfall/year
Haripur 33.99° N, 73° E	Khyber Pakhtunkhwa	North-East	11/5/2021	1955	145
Peshawar 34° N, 71.5° E	Khyber Pakhtunkhwa	North-East	12/5/2021	817	71
Malakand 34.5° N, 71.99° E	Khyber Pakhtunkhwa	North-East	13/05/2021	800	58

Table 2. Nucleotide sequences, number of markers generated (N), size range (S), number (P) and percentage (% P) of polymorphic markers, and number of phenotypes generated (PH), of two RAPD-PCR primers used.

Primer code	Nucleotide sequence (5'→3')	N	S (bp)	P	%P	PH
OP-A01	CAGGCCCTTC	10	250-2000	7	70.00	4
OP-K02	GTCTCCGCAA	10	500-1500	8	80.00	6
OP-A02	TGCCGACCTG	10	500-2500	6	75.00	5
Total		30		21		15
Mean	-	-	-	-	75.00	5

Table 3. Pairwise genetic distances matrix between studied populations of *P. citrella*, in Pakistan.

	Peshawar	Malakand	Haripur
Peshawar	**0.3245†**	--	--
Malakand	0.2964	0.0000	0.0000
Haripur	0.3023	**0.02389†**	0.3245

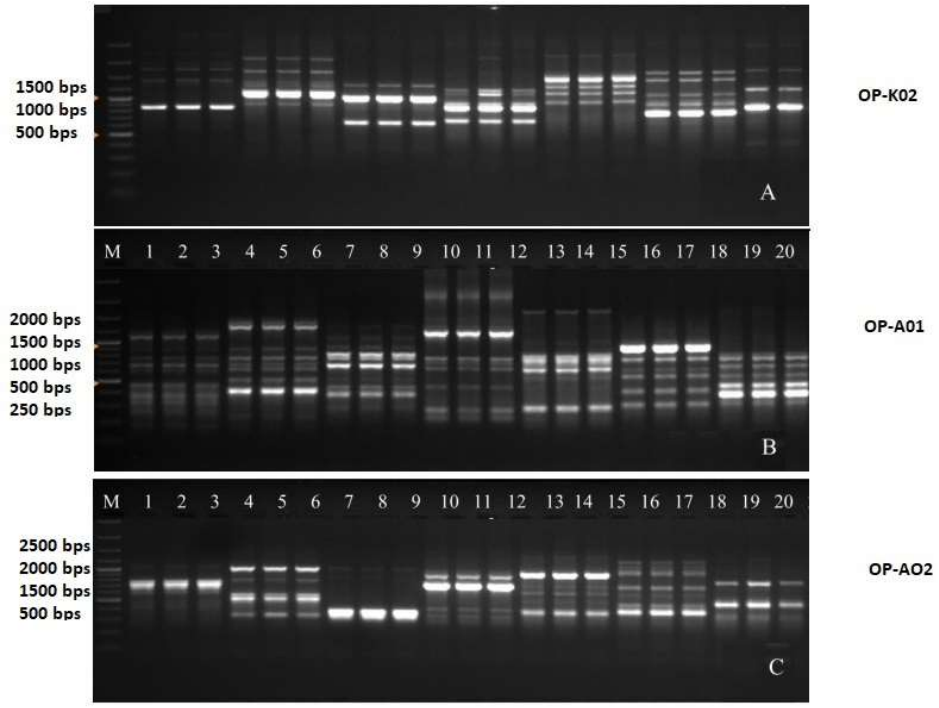

Fig.1. Electrophoretogram showing PCR based amplification products of *P. citrella* populations collected from three districts, (Peshawar, Haripur and Malakand) by using RAPD primers OP-A01, OP-A02 and OP-K01.

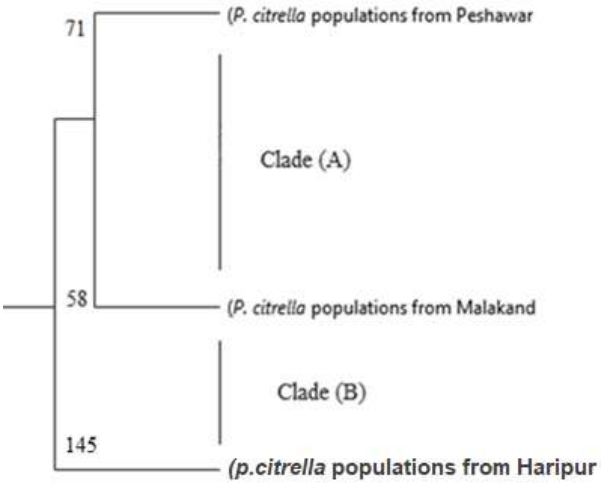

Figure 3.2. UPGMA dendrogram, showing genetic relationships between studied populations of *P. citrella*, from three districts of Pakistan.

4. DISCUSSIONS

The purpose of the study was to record the differentiation levels in the distribution of Molecular characteristics between the populations of *P. citrella* from three districts of Khyber Pakhtunkhwa region that provided a distinct nature of RAPD, polymorphic RAPD fragments' percentage (75.00%), Mean Phenotype numbers generated per primer (7) and Mean genetic distance (0.2964) showed a

slight polymorphism among the collected populations. When DNA bulks were compared, this was the expected fact, revealed by concealed DNA in the population of *P. citrella*.

This resulted polymorphism would imitate a part of the pool of genetic diversity in the *P. citrella*. (Bouktila *et al.*, 2012) quoted previous research work on genetic diversity, as the presence of high genetic diversity among insect's populations is because insect reproducing sexually and have large ecological territories with huge geographical distribution. The characterization of *P. citrella* is based on sexual reproduction, huge number of generations per year, and short life of sexual activity (17-18 days). In addition to it has a long range of host plants including various species of citrus (Jerraya, 2005). These reasons make it a good target to be studied in sexual recombination experiments among the individuals of each collected population of it.

UPGMA and PG analysis indicated that based on the regions of selection of the *P. citrella* position areas were grouped, as the populations of *P. citrella* from Peshawar and Malakand were having similarities and had a clear difference with the population of *P. citrella* from Haripur. Which shows a limited flow of genes taking place between the populations of Peshawar and Malakand with that of Haripur.

The climatic pressure from the weather and climate factors may be the reason of these evolutionary changes occurring due the mutations of genes in these populations. Though the population collected from Peshawar and Malakand had very lower correlation with the genetic distances in the populations from Haripur as per the test conducted. As per UPGMA and PG analysis, within the clades one having populations from Peshawar and Malakand and other populations from Haripur, didn't show any close correspondence. These results recommend that there were clear genetic mutations within the populations of *P. citrella* from these two clades and had a strong selective effect due to geographical differences. (Table 3.1). The selected areas were characterized by the annual rainfall over 1000 mm, while Peshawar and Malakand did not exceed 817 in Peshawar and Malakand districts of Khyber Pakhtunkhwa, Pakistan. Likewise, the number of rainfall days showed contradictions within the selected districts. Haripur was having 145 days, while Peshawar and Malakand was having 817 and 800 days of rainfall.

The results of the experiment shown that there is major potential in the pest to adapt towards a specific environment due to the genetic variations in the populations of *P. citrella.* When it faces with a stressful situation allows its genotypes to adapt resistance towards the environmental situations.

These Genetic variations between the populations should be keep in mind to develop any management strategies against the *P. citrella* in the citrus orchards. More studies should be conducted to eradicate errors in the study by using more genetic markers.

5. CONCLUSIONS

RAPD markers effectively reveal genetic variations in pest populations, aiding both diversity understanding and targeted pest management strategies. Insights into potential resistance mechanisms against insecticides are invaluable. Such studies facilitate tailored pest control by identifying genetic differences among populations, offering efficient management strategies. Observed genetic distances among *P. citrella* populations highlight environmental influences. Low genetic distance between Peshawar and Malakand may result from shared factors like climate and geography. Higher genetic distance in *P. citrella* populations from Haripur could be due to unique climatic conditions. Elevations and local climate intricacies likely contribute to these genetic disparities.

RAPD markers enhance genetic understanding and pest management strategies. Genetic distances in *P. citrella* populations reveal environmental impacts, particularly the role of climate and geography. These insights are crucial for effective pest management and mitigation strategies.

6. RECOMMENDATIONS

1. While RAPD markers provide valuable insights, consider employing complementary techniques such as DNA sequencing or microsatellite analysis. These methods could enhance the accuracy and depth of genetic characterization.
2. Gather and incorporate detailed environmental data, such as climate, elevation, and vegetation type, to correlate with genetic distances. This will facilitate a more robust analysis of the impact of these factors on population genetics.
3. Collaborate with pest management agencies to integrate genetic data into existing control strategies. Tailor interventions based on the identified genetic differences, potentially leading to more effective and sustainable pest control.
4. Establish long-term monitoring programs to continuously track genetic variations and population dynamics. This will contribute to an ongoing assessment of the effectiveness of management strategies.
5. Collaborate with researchers from other regions to compare genetic variations across a broader scale. Sharing data will contribute to a global understanding of pest dynamics and facilitate more comprehensive management strategies.
6. Investigate the role of other factors, such as host plant preferences, in shaping genetic diversity. Exploring the interaction between genetics and behavior will provide a more holistic perspective on pest dynamics.

LITERATURE CITED

1. Abdallah, N. A., Abdul Kader, M. M., & Abou-Sada, M. A. (2012). Molecular characterization of the Egyptian whitefly Bemisia tabaci (Genn.) (Hemiptera: Aleyrodidae) using RAPD-PCR. Journal of Applied Sciences Research, 8(6), 3116-3121.
2. Bouktila, D., Elleuch, A., Ghrissi, R., Chakroun, M., Ben Jamâa, M. L., & Ferchichi, A. (2012). Genetic diversity of the citrus leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) in Tunisia based on RAPD-PCR. Bulletin of Entomological Research, 102(6), 677-685.
3. Chagas, J. M., & Parra, J. R. P. (2000). Life cycle and population dynamics of *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) in laboratory. Anais da Sociedade Entomológica do Brasil, 29(4), 713-722.
4. Heppner, J. B. (1993). Citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae). In Handbook of Insect Rearing (Vol. 2, pp. 833-837). Elsevier.
5. Hoy, M. A., Nguyen, R., & Boucias, D. (2007). Classical biological control of citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) in Florida. Florida Entomologist, 90(2), 268-271.
6. Kheder, S. M., Saleh, A. A., & Al-Gebaly, A. F. (2002). Correlation of *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) population with climatic parameters. Egyptian Journal of Agricultural Research, 80(3), 1185-1197.
7. Mustafa, R. A., Ashfaq, M., & Tariq, M. (2014). Seasonal incidence and population dynamics of citrus leaf miner, *Phyllocnistis citrella* (Stainton) on Citrus reticulata Blanco in relation to abiotic factors in Faisalabad, Pakistan. Pakistan Journal of Agricultural Sciences, 51(1), 89-94.
8. Nylin, S., & Gotthard, K. (2010). Plasticity in life-history traits. Annual Review of Entomology, 55, 41-61.
9. Willink, E., Lugo, E. A., & Argüello Caro, E. B. (1996). Biology of *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae). Boletin de Sanidad Vegetal, Plagas, 22, 281-292.
10. Jerraya, A. (2005). Host plant diversity and sexual recombination in *Phyllocnistis citrella*: Implications for population genetics. Proceedings of the International Symposium on Citrus Diseases, 123-126.