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MOLECULAR SYSTEMATICS OF *Chrysoperla carnea* GROUP (NEUROPTERA: CHRYSOPIDAE) IN PUNJAB, PAKISTAN

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The *Chrysoperla carnea* (Green lacewing) is an important predator of soft bodied insect pests of different crops like wheat, Maize, Cotton and others in Pakistan. Morphology, behavior and ecophysiology studies of *Chrysoperla carnea* were examined by the researcher while molecular study had not been checked. In this experiment the aim was to expand the studies up to molecular level to examine the systematic of *Chrysoperla carnea.* The results indicated that the level of DNA variation was high among the five *Chrysoperla carnea* species. Therefore, RAPD markers can be used successfully for the identification of *Chrysoperla carnea* species. The genetic similarity matrix of RAPD data for the five *Chrysoperla carnea* species was constructed. The genetic similarities of these lacewing species were ranging from 55% to 82%. Genetic similarity co-efficient for the five *C. carnea* species were utilized for the construction of a dandrogram by the UPGMA method. Since DNA polymorphism between the five green lace wing species is high (45%) as revealed by RAPD analysis. Dandrogram analysis showed a wide range of groupings for genetic similarity among five *C. carnea* species.
Keywords: *Chrysoperla carnea*, Molecular systematics, DNA, polymorphic RAPD primers

INTRODUCTION

Green lacewings are insects placed in the family Chrysopidae belonging to the Neuroptera. There are many genera and species in this widely distributed group. Members of the genera *Chrysopa* and *Chrysoperla* are common in Europe and North America; they are very similar and many of their species have been moved from one genus to the other time and again (Agnew *et al*., 1981). Since they are the most familiar neuropterids to many people so they are often simply called "lacewings".

Molecular detection is vital for the analysis handling and manages contamination caused by different pathogens. Today, different types of DNA approaches have been employed. Current findings for DNA analysis have great capability for the detection of species. Amplification in RAPD analysis occurs anywhere in a genome that contains two complimentary sequences to the primer that are within the length-limits of the PCR (Williams *et al.,* 1990). It is fast and easy method for identifying DNA polymorphism generated from several regions of the genome (Huang *et al*., 2003).

Systematics is gaining popularity by the use of molecular protocols to observe insect relationships and analyze species limits. Most of these procedures can be employed on insects preserved as dry form, even those preserved in museum collections. However, mostly need those specimens must be treated so that the molecules are preserved. Generally, specimens observed for molecular work must be collected in 95% or absolute (100%) ethanol (ethyl alcohol). It is paramount if samples are completely dried by altering the alcohol at least a couple of time before storing the specimens for any length of time. It is also wise to keep specimens frozen if possible.

The polymerase chain reaction (PCR) based technique (Welsh and McClelland 1990, Williams *et al*., 1990), provides a fast and successful means of identifying genetic markers to distinguish closely related species. They have been used as a tool for mapping, strain species and systematics on a wide variety of organisms (for example, Multiple fixed RAPD markers easily were obtained to distinguish laboratory populations of the morphologically indistinguishable malaria vector).

MATERIALS AND METHODS

***Collection*:** Adults of *Chrysoperla carnea* were collected using hand net and by vials from following different districts of the Punjab;

- Faisalabad
- Jhang
- Layyah
- Multan
- Sahiwal

***Molecular Techniques*:**

Specimens of *Chrysoperla carnea* from five different districts were selected for molecular characterization. The research study was conducted in Center of Agricultural Biochemistry & Biotechnology (CABB). University of Agriculture, Faisalabad.

DNA extraction:
The collected specimens were immediately transferred to 90% ethanol and stored at -20°C for molecular study (Robert, 2002). DNA extraction was made by using CTAB buffer method (Clark *et al*., 2001).

DNA Extraction Protocol:
CTAB DNA Extraction Method:

- Specimens of *Chrysoperla carnea* were reduced to small particles with the help of crushing in 500 µl CTAB buffer.
- In each sample, Proteinase-K was added at the rate of 5 µl of 20 µg/µl.
- After being vortexed, the samples were placed at 65°C for 1 hr. (vortex at 20 minutes interval).
- Samples were kept cool to room temperature before15µl of 50 µg/µl RNase was added. A vortexing and incubation at 37°C for 2.5 hrs. (Vortexing at 30 minutes interval).
- After incubation samples were placed in the centrifugation machine at 10,000 rpm for 10 minutes at room temperature.
- The upper layer was transferred to a fresh tube. Where 500 µl of Chloroform: Iso-amyl alcohol (24:1) was added.
- The mixture was vortexed and then again performed centrifugation for 10 minutes at 10,000 rpm at room temperature.
- The upper aqueous layer was then transferred to a fresh tube where DNAwas precipitated with 500 µl of 100% ethanol (-20°C).
- The supernatant was removed and the DNA pellet was washed with 70% ethanol.
- DNA pellet was air dried and resuspended in 50 µl of sterile water (d_3H_20).

Estimation of DNA Concentration: The concentration of total genomic DNA was measured by Spectrophotometer (CECIL, 2021, 2000 SERIES) AT 260 nm wavelength. The genomic DNA was diluted 100 times with a cuevette volume of 500µl.

Agarose gel Electrophoresis: The PCR products were electrophoresed at 80 V in 1.0% agarose gel for approximately 2 hours using 0.5X Tris Boric acid (TBE) buffer containing Ethidium Bromide (0.5 µg/ml) along with a DNA molecular size marker.

Agarose gel Electrophoresis involved the following steps:
An adequate volume of electrophoresis buffer i.e. 0.5X TBE buffer was prepared to fill electrophoresis tank and to prepare the agarose gel.

- Desired amount of agarose (1.0 g) was taken in a flask, containing electrophoresis buffer 0.5X TBE (100 ml), melted in microwave oven for 1.5 minutes, swirled it to ensure even mixing.
- Casting tray was prepared by wrapping mashing tape on its both sides and a suitable comb was adjusted on it.
- The melted agarose was cooled from 55°C to 45°C before pouring on to the gel casting tray and Ethidium Bromide (2.5 µl) was added into it.
- Pour melted agarose on the gel casting tray, gels typically pour between the thickness of 0.5 – 1.0cm.
- Bubbles were removed, underneath the comb or on the surface of the gel and solidified at room temperature.
- After solidification of gel at room temperature, the comb was removed carefully to avoid tearing of well. The mashing tape was also removed from the sides of casting tray.
- The gel casting tray containing the gel was placed in electrophoresis tank, having 0.5X TBE buffer into it.
- Before loading the PCR products in the wells of gel, 4µl of 5X RAPD dye (Bromophenol blue) was added to the PCR products. Only 10 µl of RAPD products were loaded on the gel. Marker of known molecular size was loaded on one side of the gel to compare the size of different amplified fragments of genomic DNA.
- The wires of electrophoresis tank were connected to power supply, adjusted to a certain voltage (80 V). Movement of dye indicated the migration of DNA from anode to cathode through gel.

The fingerprints were examined under UV Transilluminator at 254-300 nm wavelengths and photographed by using the Syngene still video system.

RESULTS

Molecular Study:With regard to genetic variations among green lace wings or the *Chrysoperla carnea* in Pakistan, there have been no references for such kind of scientific study. In the present study five *Chrysoperla carnea* species were molecularly characterized to reveal their genetic variations by using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR).

***Condition Optimization for RAPD Analysis*:** In PCR amplification with RAPD the reaction condition is very important in obtaining reproducible results. The use of standardized RAPD protocol ensures the reproducibility. Before conducting the analysis PCR condition were optimized for $MgCl_2$ *Taq* DNA polymerase and template DNA concentration.

Template DNA Concentration: In condition optimization, template concentration 15ng/µl was found to be optimum. The template concentrations higher than 15ng did not give consistent amplification. Higher template DNA concentration usually increases the yield of non-specific amplification products. Moreover, reagents used in DNA purification procedures (phenol, EDTA, Proteinase-K, etc) are potent inhibitor of *Taq* DNA polymerase activity (Newton and Graham, 1997). Nearly in all routine methods ethanol precipitation of DNA and repetitive treatments of DNA pellets with 70% ethanol is effective in removing

traces of these inhibitors. Even then minute quantities of these inhibitors remained in extracted genomic DNA. These traces are particularly higher when high concentration of DNA is used and can cause hindrance in PCR.

***Annealing Temperature*:** Annealing temperature of 37°C was found optimum in this study. It was also noted that low annealing temperature e.g. 30°C produces less number of bands and high annealing temperature produces occurrence of nonspecific bands.

Primer screening and Molecular characterization of Chrysoperla carnea:
Reactions were duplicated from time to time to check the consistency of amplified products. Only easily resolved bright DNA bands were considered and scored. All the species showed diversity with each other on their amplification profile bases. Of these molecularly characterized 184 DNA fragments amplified by 10 primers, almost all bands showed polymorphism among the five *Chrysoperla carnea* species. These results indicated that the level of DNA variation was high among the five *Chrysoperla carnea* species. Therefore, RAPD markers can be used successfully for the identification of *Chrysoperla carnea* species.

Cluster Analysis: The genetic similarity matrix of RAPD data for the five *Chrysoperla carnea* species was constructed based on Nei and Li's (1979) coefficient of similarities shown in (Table-1). The genetic similarities of these lacewing species were ranging from 55% to 82%. All the five *C. carnea* species were collected from five different districts of the Punjab. The names of the districts are Faisalabad, Jhang, Layyah, Multan, Sahiwal.

The *C. carnea* species *Chrysoperla carnea* and *C. yunusi* Beg. Showed minimum similarity i.e. 55% whereas maximum similarity was observed between the species *Chrysopa leach* and *C. modests*, *C. leach* and *C. corpominus*, *C. modests* and *C. yunusi* Beg, *C. corpominus* and *C. yunusi* Beg i. e. 82%.

Genetic similarity co-efficient for the five *C. carnea* species (Table-1) were utilized for the construction of a dandrogram by the UPGMA method. (Figure-1). Since DNA polymorphism between the five green lace wing species is high (45%) as revealed by RAPD analysis. Dandrogram analysis showed a wide range of groupings for genetic similarity among five *C. carnea* species. Dandrogram divided the five green lace wing species into three groups. The first group was obtained among the species *Chrysopa leach* and *C. modests* which showed 82% similarity. It showed that these two species are genetically very close to each other. As these five species were collected from five different distrits of the Punjab and the species *Chrysopa leach* and *C. modests*, were collected from Jhang and Faisalabad, respectively.
The second closest resemblance (82%) was observed among the species *C. corpominus* and *C. yunusi* Beg. As these two species were collected from the districts Multan and Layyah. Among these two groups the species *Chrysopa leach* also showed a close resemblance (82%) with the species *C. corpominus* of other group and the same species *Chrysopa leach* showed 70% similarity with the species *C. yunusi* Beg of other group. On the other hand the species *C. modests* showed 73% similarity with species *C. corpominus* and 82% with species *C. yunusi* Beg, respectively.
The third group comprised of only one species i.e. *Chrysoperla carnea* that is collected from the district Sahiwal. Therefore, this species did not make any group with any other species of different districts.

Table: Similarity matrix of five *Chrysoperla carnea* species obtained from RAPD markers

Species	1	2	3	4	5
Chrysoperla carnea		0.7660	0.6809	0.7234	0.5532
Chrysopa leach			0.8298	0.8298	0.7021
Chrysopa modests				0.7447	0.8298
Chrysopa corpominus					0.8298
Chrysopa yunusi					0.0000

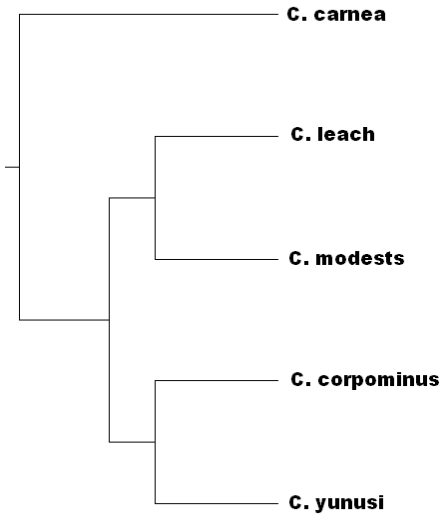

Figure: Dendrogram among five green lace wing species generated through RAPD data using UPGMA method.

DISCUSSION

From the study it was estimated that all the five species showed a significant similarity/diversity. As these five species were collected from five different regions of the Punjab i.e. Multan, Layyah, Jhang, Faisalabad and Sahiwal. The main purpose of collecting different specimens of green lacewing, *Chrysoperla carnea*, is to find whether the specimens of *Chrysoperla carnea* group existing in different districts of Punjab Province are alike or having any kind of diversity. As these species of green lacewing are collected from different regions of the Province so it was expected that there would be dissimilarity at the molecular level which is not clearly evident morphologically. Now the study was extended up to molecular level to observe the systematics of *Chrysoperla carnea.*

The species such as *C. leach* and *C. modests* showed a close resemblance (82%). May be these two species were taken from two close districts i.e. Jhang and Faisalabad. These results were verified by other scientists (Meunier and Grimont. 1993; Reineke *et al*., 1998; Lourence *et al*., 2006) who worked in green lacewing species by using DNA markers.

Similarly the species *C. corpominus* and *C. yunusi* showed a maximum similarity (82%). Also these species were collected from two close districts i.e. Multan and Layyah. But these species have more diversity with species *C. leach* and *C. modests.*

C. yunusi showed 70% similarity with *C. leach*. Similarly the species *C. corpominus* showed 74% similarity with *C. modests*. These results are in agreement with Andras *et al*. 2009, Morales and Freitas, 2010, Haruyama *et al*. 2008 and Winterton and Freitas, 2006).

A third group contained only a single species *Chrysoperla carnea* that did not make any group with rest of the four species and showed a minimum similarity (55%) with species *C. yunusi.*

CONCLUSIONS

The aim is to expand the studies up to molecular level to examine the systematics of *Chrysoperla carnea.* Molecular analysis revealed that there is a marked similarity/diversity within these green lacewing species. All the species showed multiplicity with each other on their amplification profile bases. Of these molecularly characterized 184 DNA fragments amplified by 10 primers, almost all bands showed polymorphism among the five *Chrysoperla carnea* species. These results indicated that the level of DNA variation was high among the five *Chrysoperla carnea* species. Therefore, RAPD markers can be used successfully for the identification of *Chrysoperla carnea* species.

The genetic similarity matrix of RAPD data for the five *Chrysoperla carnea* species was constructed. The genetic similarities of these lacewing species were ranging from 55% to 82.0%. Genetic similarity co-efficient for the five *C. carnea* species were utilized for the construction of a dandrogram by the UPGMA method. Since DNA polymorphism between the five green lace wing species is high (45%) as revealed by RAPD analysis. Dandrogram analysis showed a wide range of groupings for genetic similarity among five *C. carnea* species.

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