International Journal of Farming and Allied Sciences
Available online at www.ijfas.com
©2014 IJFAS Journal-2014-3-4/402-407/ 30 April, 2014
ISSN 2322-4134 ©2014 IJFAS

Use of RAPD-PCR to reveal genetic proximity of Species of Chrysopidae Family (Insecta, Neuroptera)

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ABSTRACT: RAPD (random amplification of polymorphic DNA) was used for distinguishing the genetic diversity between nine species of Chrysopidae family of Kermanshah province. Many specimens were collected in different locations of Kermanshah province, between them 27 specimens were identified to be of 9 species of Chrysopidae. The wing venation, male genitalia and other morphological characters were used for identification of the species belonging to Chrysopidae family and the determination of species were done by study of male genitalia. The extraction of DNA was done by use of CTAB method. Nineteen primers were used, among them thirteen primers have produced too many polymorph bands. Hundred thirty three bands were obtained by use of primers, among them hundred twenty six bands were polymorph and the rest monomorph. Total polymorphism was 94.7%, and size of the bands had a range of 300-350 bp. The maximum of polymorphism(100%) was obtained using OPA_1, OPA_7, OPA_{10}, PKB b, PKBc, PKBd, OPH_1, OPH_5, OPH_{12},OPH_{20}, POPFe, N_7, N_{13} primers and the minimum of polymorphism (82.86%) was obtained using OPA_2, OPA_3, OPA_{11}, OPA_{18}, POA_{20}, OPBa primers.

***Keywords*:** Genetic similarity, Jacquard index, nine species, Chrysopidae

INTRODUCTION

Chrysopidae (Neuropterida, Neuroptera) generally called as green lacewings are generalist predators, they are active predators of small pests such as aphids, mealybugs, Thrips, whiteflies, spider mites, eggs and nymphs of leafhoppers, leaf miners, larvae of butterflies, Psyllids, etc... The systematics of Chrysopidae of Iran were studied in the past by many authors, among them (Farahbakhsh,1961; Heydari, 1965, 1995; Hölzel, 1966,1967; Shahkarami, 1997;Mirmoayedi,1995,1999a,1999b ,2001, 2002a; Mirmoayedi and Thierry, 2002b;Yassayie and Mirmoayedi 1998; Farahi, 2009; Ghahari, 2010) are to be mentioned here . One of the most important species of Chrysopidae is *Chrysoperla carnea*, which is a generalist predator and is used vastly in biological control and IPM programs in the world. This species was considered at first to be a single species but now there are evidences to prove it's a complex species composed of many cryptic and sibling species collectively called *Carnea* complex. As like of many other insects the identification of Neuroptera species is routinely done by study of morphological characters such as the body color, presence or absence of certain hairs or bristles on particular parts of the body and for the species determination male genitalia are generally used. In addition we can mention other characters based on morphology for identification of species such as the dichotomous keys, but as these keys make use of morphology uniquely so, generally they are the sources of erroneous identifications, because of variations in characters and mistakes which arise in counting or measuring those characters. Therefore, in the past thirty years different entomologists have used molecular analysis to solve the unsolved problems which appeared by the use of

classical methods. Some workers have used RAPD for distinguishing genetic similarities between different populations of insects, for example RAPD was used for separating males of *Telenomus podisi* Ashmead and *Trissolcus* spp. from each other in soybean fields in Brazil(Aljanabi, 1998) and was used for discriminate between natural and inbreed populations of *Orius* in France(Gozlan, 1997). Using RAPD, genetic diversity between three species of ants *Monomorium pharoensis*,*Camponotus maculatus*, *Cataglyphis bicolor* was studied and the acquired results showed the average percentages of monomorphic bands were 33.0% and 39.0% using RAPD and RFLP techniques respectively(Galal, 2009).

RAPD was also used for distinguishing between different populations of a braconid wasp *Diaeretiella rapae*, and helped to reveal genetic diversity between populations located one kilometer distance apart from each other(Vaughn and Antolin, 1998). Genetic variations was studied using RAPD-PCR in forty eight geographic races, infra subspecific forms, cultivated lines, inbred lines of *Kerri laca* a lac producing homoptera in India(Ranjan, 2011). No significant correlation were detected between genetic distance and geographical distance among the populations of *Helicoverpa armigera* in Israel and Turkey, when RAPD was used for analysis of genetic diversity (Zhou, 2000). In an investigtaion made on cotton boll weevil migration in eight US. States by Kim et al.,2004 between 20 RAPD primers which was used, six random primers revealed sufficient polymorphism to characterize genetic variation within and between boll weevil populations indicating that the genetic and geographical distances among all populations were positively correlated. For study of genetic diversity between populations of *Cephus cinctus* , wheat stem borers in three states of Montana, north Dakota and Wyoming, Lou et al.,1998, have collected 182 individual larvae in fifteen natural populations, they have used 20 primers of RAPD and found that band frequencies of 31(52%) varied among collection sites. Pairwise tests for homogeneity of RAPD variance revealed significant divergence among 81 of 105 (77%) of population pairs.

MATERIALS AND METHODS

123 specimens of Chrysopidae were collected from different locations in Kermanshah province, between them nine species were identified, their species names are as follows; *Chrysoperla kolthoffi*, *Chrysoperla sillemi*, *Chrysoperla lucasina*

Chrysoperla carnea, *Dichochrysa prasina*, *Italochrysa vartianorum*, *Suarius nanus Chrysopa viridana*, *Chrysopa pallens*. Twenty seven specimens totally were used, three specimens from each species were chosen to make molecular analysis. The determination of species was done following morphological characters, then each of them was ground separately using a micropestle in a 1.5 ml microcentifuge tube containig 50 µl of extraction buffer (5 mM Tris-HCl and 0.5 mM EDTA pH 8.0), then washed with a 550 µl of buffer. Each tube was capped and warmed at 65ºC for 50 minutes. The solution was centrifuged at1300 rpm for seven minutes. The supernatant liquid was transferred to a new tube, 550 µl of chloroform was added and the tube was vortexed for a few seconds. Then the new solution was centrifuged at 13000 rpm for 15 minutes, the supernatant was transferred to a new 1.5 ml tube and 750 µl of cold isopropanolol was added. The tube was put in a freezer to stay for an overnight, after this lapse of time the tube was centrifuged again at 13000 rpm for 15 minutes, the supernatant was extracted and 200 µl of 70% cold ethanol was used for washing, then centrifuged at 7000 rpm for 5mn, the obtained DNA was dried in ambient laboratory temperature and was mixed with a 50 µl TE buffer and preserved in -20ºC in a deep freeze freezer for further analysis.

Primers of RAPD

Nineteen, 10 nucleotides RAPD primers were used, 13 of them showed multiple polymorphisms. 133 electrophoresis bands were produced, 126 of them showed polymorphism and totally they have shaped 94.7% of polymorphism. The sizes of bands were between 300-3500 bp. The best primers with maximum (100%) of polymorphism were OPA_1, OPA_7, OPA_{10}, PKB b, PKB c, PKB d, OPH_1, OPH_5, OPH_{12},OPH_{20}, POP Fe, N_7, N_{13} , and those primers which formed the minimum (82.86%) of polymorphism were OPA_2, OPA_3, OPA_{11}, OPA_{18}, POA_{20}, OPB a.

Polymorphism was calculated by dividing the Number of the polymorphic bands to the sum of total bands.

Table 1. The component of a PCR mixture plus DNA from each specimen

Components	Concentration used for each specimen
PCR Buffer (10x)	2.5 mM
dNTP (10 mM	0.55 µM
$MGCL_2$ (50%)	0.3 Unit
Taq polymerase	0.3 unit.
ddH_2O	13-15 µl
Primer(10 µM)	2.5 µl
DNA (10 ng)	5 µl
Total volume	25 µl

RAPD-PCR

We used the method of Williams, (1990) and McClelland and welsh,1994. The commercially prepared PCR mixture kit by Arian Gene Gostar Co. Tehran, was used for PCR (Table 1). All the tubes were sterilized, and 10 ng of DNA of each specimen, was mixed with 20 µl of PCR mixture, and the final solution of PCR was brought to 25 µl. For each specimen, each of 19 primers supplied by Arian Gene Gostar Co. Tehran was used with three replications. The primers have been kept frozen in -20ºC in a deep freeze freezer, each primer was diluted to 10 µL prior to be mixed with PCR mixture. PCR was done in a Master cycler machine, made by Corbett Co.

Electrophoresis

The electrophoresis was done by use of Agarose gel. 0.8 gr of Agarose powder was mixed with 60 ml of TBE, and heated on a heater, after boiling the glass beaker was brought into a ventilated hood and 5 µl of ethidium bromide was added, the hot gel was poured into a buffer filled electrophoresis box and allowed to be cooled and solidified. Electrophoresis was carried out by making standard wells in a gel of agarose, then each single well in the gel was filled with PCR mixture plus one of the 19 primers and for each species this operation was three times repeated. The *h* (Nei's diversity index) was calculated which evaluate more effectively the genetic diversities between specimens (Table-2). Dendrogram of similarity was drawn with use of data obtained by calculating coefficient of Jackard similarity(Fig-2). Phylogenetic analysis using parsimony(PAUP) was used following(Swofford, 1991)

RESULTS AND DISCUSSION

Results

One of the electrophoresis banding pattern obtained when primer OPA2 was used could be seen in Fig-1,
The maximum of genetic similarity based on Jacquard index (Table 2)was observed between *Chrysopa pallens*(H) and *Chrysopa viridana* (I) was 78.5%(=0.785), clustered in one group(Fig-2), the minimum of genetic similarity 49.10% (0.491) was observed between *Chrysoperla viridana*(H) with *Chrysoperla kolthoffi* (A) and also between *Chrysoperla viridana* (H) with *Chrysoperla lucasina*(C). Concerning the species of *Carnea* complex, *Chrysoperla carnea*(D) and *Chrysoperla lucasina* (C) clustered in one group and had a 71% (0.71) of genetic similarity between them while 62.5%(=0.625) of genetic similarity was observed between *Chrysoperla kolthoffi* (A) and *Chrysoperla carnea*(D), more similarity, i.e. 64%(0.642) was observed between *Chrysoperla lucasina*(C) and *Chrysoperla sillemi*(B)

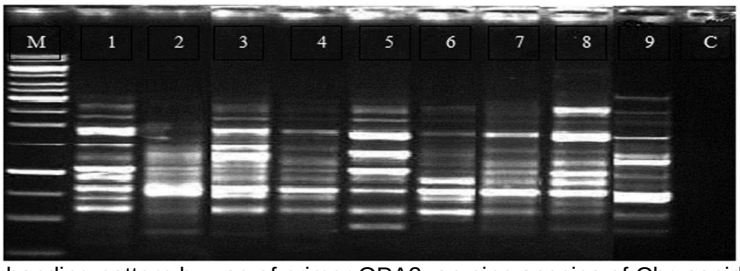

Figure 1. Electrophoresis banding pattern by use of primer OPA2, on nine species of Chrysopidae family in Kermanshah Province. M=Size of DNA(1Kb), C= Ladder, 1-9 are species of Chrysopidae.

Table 2. Matrix of data of species of Chrysopidae of Kermanshah province. The *h* (Nei's diversity index) was used

	Ch.kolthoffi	*Ch.sillemi*	*Ch.lucasina*	*Ch.carnea*	*S. nanus*	*Ch.viridana*	*Ch.pallens*	*D.prasina*	*I.vartianorum*
Ch.kolthoffi	1								
Ch.sillemi	0.732143	1							
Ch.lucasina	0.571429	0.642875	1						
Ch.carnea	0.625	0.660714	0.714286	1					
S. nanus	0.653571	0.589296	0.553671	0.714286	1				
Ch.viridana	0.491071	0.580367	0.491071	0.633929	0.706357	1			
Ch.pallens	0.526786	0.580367	0.526786	0.6875	0.6875	0.785714	1		
D. prasina	0.526786	0.5625	0.526786	0.633929	0.526786	0.607143	0.589286	1	
I.vartianorum	0.526860	0.570367	0.580357	0.6875	0.616071	0.589286	0.680714	0.732143	1

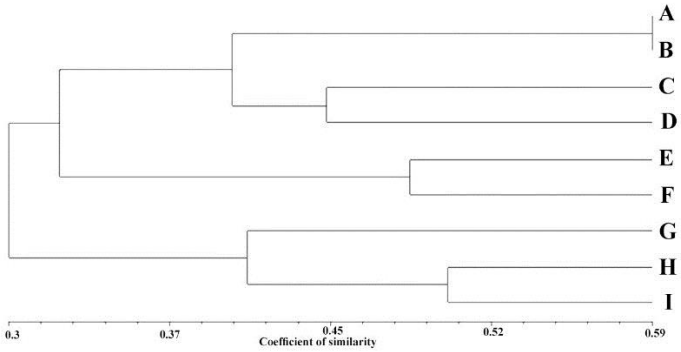

Figure 2. Dendrogram was drawn by the use of coefficient of Jacquard, with Cofting coefficient of 0.819 concerning nine Chrysopidae species.

The abbreviation characters corresponding to the names of nine species which have been found in seven locations of Kermanshah province are as follows; A=*Chrysoperla kolthoffi*, B=*Chrysoperla sillemi*, C=*Chrysoperla lucasina* , D=*Chrysoperla carnea*, E= *Dichochrysa prasina*, F= *Italochrysa vartianorum*, G=*Suarius nanus*, H=*Chrysopa viridana*, I=*Chrysopa pallens*. These species were collected from seven locations in Kermanshah, Sahna, Songhor, Kangavar, Bisetoon, Mahidasht and Islamabad Gharb.

Discussion:

The species of *Chrysoperla carnea*, was considered to be composed of different species known collectively as *Carnea* complex, which was known from previous studies (Tauber and Tauber 1986; Thierry,1992,1998; Wells and Henry, 1992), we have seen that two species belonging to this complex, *Chrysoperla kolthoffi* and *Chrysoperla sillemi* with 0.732 genetic similarity to each other formed a group in one hand and *Chryoperla carnea* and *Chrysoperla lucasina* having the same genetic similarity made another group, all of the four species composed a distinct cluster which was separated from other clusters formed by different species of Chrysopidae family(Fig -2). Mirmoayedi and Thierry 2002(b) have studied morphologically different populations of *Carnea* complex from different locations of Iran and found *lucasina*, *kolthoffi* and *Carnea*, in addition of two new morphs called as iranica and kermanshah morph, these studies was not continued on afterward. In another attempt Mirmoayedi,(2012) used PCR-RAPD and studied 60 specimens composed of males and females of the two genera of *Chrysopa* and *Chrysoperla* of Chrysopidae family from Kermanshah and when Cluster analysis of populations was performed, it showed that the two populations of males and females of *Chrysoperla* cluster into one group near to each other, but the male and female populations of the genus *Chrysopa* have clustered in separate groups, this probably was due to the higher genetic differences between males and females of *Chrysopa* rather than between males and females of *Chrysoperla*. In another research Mirmoayedi et al.,2013, found that all seventeen primers used to study 36 specimens of Myrmeleontidae have produced patterns which was used to distinguish the genetic distance between12 species analyzed. In the present research we have tried to find out the genetic and molecular based family relations between different species belonging to Chrysopidae family found in different locations in Kermanshah province.

Hölzel,(1967) has grouped genera of Chrysopidae based on structure of the sternites 8 and 9 and male genitalia, by using those characters he has classified the following groups; Group *Perla* , which was composed of *Chrysopa perla* ,*Chrysopa dubitans*, *Chrysopa formosa*, *Chrysopa dasyptera* and *Chrysopa astarte*; Group *Carnea* including *Chrysoperla carnea* and *Chrysoperla iranica*; Group *flavifrons* composed of *Chrysopa flavifrons*,*Chrysopa derbendica*, *Chrysopa prasina*; Group *nana* including *Chrysopa nana*,*Chrysopa vartianae* and *Chrysopa*

paghmana. We have found after our molecular researches on some of these species that *Dichochrysa prasina* and *Italochrysa vartianorum* were grouped together in one cluster with a similarity index of 0.732 while (Hölzel,1967) using morphology of male genitalia classified them into two different groups, one consisted of the genus *Italochrysa* and the other was included in the group *flavifrons*. Haruyama, 2008 studying three nuclear genes of the Chrsopidae family have confirmed that the relationship between *Mallada* s.str. *Chrysoperla* and *Dichochrysa* remains uncertain. Besides we have observed in our results that *Chrysopa viridana* and *Chrysopa pallens* were grouped into one cluster with a 0.785 genetic similarity index and *Suarius nanus* located in the nearby branch with genetic similarity indice of 0.6875 to *Chrysopa pallens* and 0.705 to *Chrysopa viridana*, though in Hölzel's classification *Suarius nanus* belonged to another group named as *nana*. Brooks,1997 believed that genus *Suarius* belonged to an unknown affinity. We conclude now that based on our researches, *Carnea* complex has formed a cluster in which each of four species of it was near to each other and separated from other cluster of species of Chrysopidae, so our experiment with RAPD-PCR showed that in identification of specimens of Chrysopidae with the use of RAPD molecular technique we were successful to separate the species belonging to *Carnea* complex in one cluster distinguished from other clusters of other species of this family and to define new inter specific relationship based on molecular methods which potentially could initiate thoughts about the possible modifications in the traditional classification of neuroptera already in routine use by many students and neuropterists around the world. Although we believe that the molecular experiments similar to the work done by us should be repeated by other neuropterists and also equally some other non molecular researches based on morphology should also be done by any other neuropteran taxonomists to arrive to a common consensus to decide whether to change any ranking or classifications in the order of neuropteran and specially of Chrysopidae family. We consider that by use of molecular methods in the future neuropterists can find more clues for the unsolved problems of Neuropteran systematics and specially overcome the classification problems which exist due to the differences and challenges between different neuropterists naming the tribes, genera and species of the Chrysopidae family

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