International Journal of Biochemistry and Biotechnology ISSN 2169-3048 Vol. 9 (6), pp. 001-006, June, 2020.
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Full Length Research Paper

Genetic diversity of *Mayetiola destructor* and *Mayetiola hordei* (Diptera: Cecidomyiidae) by inter-simple sequence repeats (ISSRs)

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Accepted 11 April, 2020

Inter-simple sequence repeats (ISSR) polymorphism was used to reveal genetic variability and phylogenetic relationships within and between three haplotypes of *Mayetiola* species. A set of 14 ISSR primers were screened representing di-, tri, tetra and penta-nucleotide repeats out of which 10 generated scorable bands and three were able to distinguish one of three haplotypes. The consensus tree constructed using binary data from banding patterns generated by ISSR-PCR clustered the two *Mayetiola* species according to their mitochondrial haplotype. Moreover, genetic diversity estimated by the coefficient of variation indicates a high intra and inter-haplotypes polymorphism. Our results indicate that ISSR can be useful as DNA-based molecular markers for studying genetic diversity and phylogenetic relationships of *Mayetiola* haplotypes.

Keywords: Genetic diversity, ISSR markers, *Mayetiola*, haplotypes.

INTRODUCTION

Microsatellites orsimple sequence repeats (SSRs) are short DNA sequences tandemly arranged. They are highly polymorphic and widely distributed in the eukaryotic genome (Tautz, 1989). They have proven to be invaluable sources of markers for population-level studies (Goldstein and Schlotterer, 1999), but can require substantial investment for each taxon studied, since primers often must be uniquely designed for flanking regions. As PCR technology finds increased use in genetic analysis, additional novel variations of this technique are emerging. PCR analysis using anchored simple sequence repeat primers has gained attention recently as an alternative means of characterising complex genome. This approach, named inter-SSR-PCR (ISSR-PCR), employs primers that hybridize with the repeats themselves, rather than in flanking regions (Zietkiewicz et al., 1994). The generated PCR products reveal polymorphic patterns on a single high resolution agarose gel. This strategy is especially attractive because it avoids cloning and sequencing procedure used in the original microsatellite based approach. ISSR has already been applied to study intra and inter-specific variability of many insect such as the silkworm *Bombyx mori* (Reddy et al., 1999), two aphid species *Acyrthosiphon pisum* and *Pemphigus obesinymphae*, the yellow fever mosquito *Aedes aegypti* and a rotifer *Philodina* sp. (Abbot, 2001), and six species of Noctuids (Luque et al., 2002).

In cereals, *Mayetiola hordei* and *Mayetiola destructor* (Diptera: Cecidomyiidae) are the major pests in the Mediterranean region and Europe (Gagné et al., 1991). A gene-for-gene relationship has been demonstrated between resistance in wheat and virulence in *Mayetiola*. Historically, host resistance strategy is the most effective cost efficient way to control this pest (Hatchett and

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Gallun,1970; Ratcliffe and Hatchett, 1997) . Two approaches based on isoenzyme electrophoresis (Makni et al., 2000a) and mitochondrial DNA-RFLP (Mezghani et al., 2002a) used to study genetic diversity in these two *Mayetiola* species revealed relatively little polymorphism. Moreover, cytochrome b gene sequences showed that *M. destructor* is represented by only one mitochondrial haplotype designed A, however *Mayetiola hordei* is characterized by two mitochondrial haplotypes B and C (Mezghani et al., 2002b). Therefore, the search of other markers is required to obtain a deeper comprehension of the genetic organization of *Mayetiola* species. In the present study, ISSR polymorphism was used to determine genetic diversity and phylogenetic relationships of the two species of *Mayetiola.*

MATERIAL AND METHODS

Insect materiel

Adult specimens of *Mayetiola* were collected after emergence from pupae rearing on wheat from several geographical locations where this cereal is the most sown. Infested plants from each field were kept in two separate screened cages and placed into a greenhouse where conditions were favourable for adult emergence (12:12 h photoperiod, 18- 20°C temperature and 60% relative humidity). Emergence was checked every day and *Mayetiola* adults were stored individually in 1.5 microcentrifuge tubes at - 80°C. Insects are then typed individually using the cytochrome b gene following the protocol adopted by Mezghani et al. (2002a). Ten individuals of *M. destructor* of A haplotype coded from A1 to A10, ten from *M. hordei* of B haplotype coded from B1 to B10 and ten of *M. hordei* of C haplotype coded from C1 to C10 were used for ISSR analysis.

ISSR primers

A set of fourteen primers were utilized for this study: nine unanchored repeats $(AG)_{10}$; $(CT)_{10}$; $(TG)_{10}$; $(AGG)_6$; $(CAC)_6$; $(TGGA)_5$; $(ACTG)_4$; $(GACA)_4$ and $(GACAC)_4$, four 3'-anchored di-nucleotide repeat primer $(AG)_{10}T$; $(AG)_{10}C$; $(CT)_{10}A$; $(CT)_{10}G$ and one 5'-anchored tetranucleotide repeat primer $CT(ATCT)_6$. The oligonucleotide primers were synthesized on a DNA synthesizer (Applied Biosystems).

DNA extraction and ISSR amplification

Whole genomic DNA was extracted from single individuals according to the method described by Doyle and Doyle (1987) and its quality and quantity were estimated both spectrophotometrically, as well as visually by ethidium bromide staining on agarose gels. PCR reactions were carried out in a 25 µl reaction mixture containing 20-40 ng total DNA, 2.5 µl of 10X Taq DNA polymerase reaction buffer (Appligene), 1.5 units of *Taq* DNA polymerase, 0.25 mM of each dNTP, 3.75 pM of each primer. Samples were overlaid with 10 µl of mineral oil and reactions were carried out on a "Crocodile III" thermocycler for 35 cycles. After initial denaturation for 5 min at 94ëC, each cycle comprised 1 min denaturation at 94ëC, 90 s at the annealing temperature of each primer, 2 min extension at 72ëC with a final extension for 5 min at 72ëC. Amplified products were mixed with bromophenol blue gel-loading dye and analysed by electrophoresis on 1.4% agarose gel stained with ethidium bromide using 1X Tris Borate EDTA buffer pH 8.0.

Gel scoring and data analysis

Bands profiles generated by PCR were compiled onto a data matrix on the basis of the presence (1) or absence (0) of selected bands. Only those bands that were reproducible and about 0.5 mm apart were considered for scoring. The Nei and Li (1979) coefficient for measuring pairwise band similarities between individuals was calculated as follows: $CS= 2 N_{XY}/ (N_X + N_Y)$ where N_X is the number of total bands in individual X, N_Y is the number of total bands in individual Y and N_{XY} is the number of common bands in X and Y. Individuals X and Y can belong to the same haplotype or to two different haplotypes. The similarity intra-haplotypes (S) is calculated by the similarity mean coefficients (CS) between individual pairwise of the same haplotype. The similarity inter-haplotypes, S_{ij}, is given by the similarity mean coefficient between individual pairwise belonging to i and j haplotypes. The corrected similarity inter-haplotypes, S'_{ij}, is calculated by taking into account the intra-haplotype similarity according to the method of Lynch (1990) $S'_{ij}=1+S_{ij}-0.5(S_i+S_j)$ where S_i and S_j are the value of S for i and j haplotypes, respectively, and S_{ij} is the uncorrected similarity between the pairwise haplotype i and j. The distance values were analysed using the unweighted pair-group method with the arithmetic averaging algorithm (UGMA) by PHYLIP version 3.57 (J. Felsenstein. University of Washington, Seattle. USA). All individuals studied (30) were portrayed graphically in the form of a dendrogram. The coefficient of variation (CV) is used to quantify the genetic distance variation within haplotypes (Kalinouvski, 2002). This parameter is defined as the ratio of the population standard deviation "s" to the population mean "m": CV=s/m. A Mantel test (1967), Z, was performed to evaluate the significance of inter-haplotypes mean distances. Student test "t" was used to estimate the significance of the intra-haplotypes mean distances.

RESULTS

ISSR analysis

To investigate the utility of ISSR-PCR in the genetic analysis of the *Mayetiola* species, we tested 14 primers including five 3' anchored primers, one 5' anchored primer and eight di, tri, tetra and pentanucleotide motifs. Results are summarized in Table 1. Of the fourteen primers tested, $(TG)_{10}$ and $CT(ATCT)_6$ did not show any amplification, indicating that these repeats may be absent in the *Mayetiola* genome or spaced well beyond the capacity of amplification by Taq DNA polymerase. Ten primers gave scorable PCR products and yielded a total number of 141 polymorphic bands ranging from 300bp to 3100 bp. The number of bands observed ranged from 11 with primer $(CAC)_6$ to 16 with primers $(AG)_{10}C$; $(CT)_{10}A$ and $(GACA)_4$. The dinucleotide repeats $(AG)_{10}C$, $(CT)_{10}A$ and $(AG)_{10}T$ gave the best polymorphic and informative patterns. Since $(AG)_{10}$ and $(CT)_{10}T$ primers produced smears, one can conclude that the CT repeats are much abundant in the *Mayetiola* genome.

The amplification products originating from individuals of B haplotype revealed specific bands of 620, 600 and 350 bp obtained by $(AG)_{10}C$, $(CT)_{10}A$ and $(GACAC)_4$, respectively. No specific bands appeared in individuals from the two others haplotypes. Figures 1, 2 and 3 show the amplification profiles generated by the 3' anchored

primer $(AG)_{10}C$ across 10 individuals of haplotype A, B and C. As could be seen, ISSR banding profiles generated by the $(AG)_{10}C$ primer were polymorphic within and between the three haplotypes of *Mayetiola*. In addition, all the other primers revealed polymorphic patterns suggesting that the ISSR procedure constitutes an alternative approach suitable for examination of the *Mayetiola* genetic diversity.

Table 1. Summary of DNA patterns amplified par ISSR primers in *Mayetiola*

Primer sequences	Annealing (^{O}C)	Patterns
$(AG)_{10}$	60°C	S
$(CT)_{10}T$	60°C	S
$(TG)_{10}$	60°C	-
$(AG)_{10}T$	57°C	13
$(AG)_{10}C$	60°C	16
$(CT)_{10}A$	57°C	16
$(CT)_{10}G$	60°C	15
$(CAC)_6$	57°C	11
$CT(ATCT)_6$	60°C	-
$(AGG)_6$	50°C	14
$(TGGA)_5$	55°C	13
$(ACTG)_4$	45°C	13
$(GACA)_4$	45°C	16
$(GACAC)_4$	55°C	14

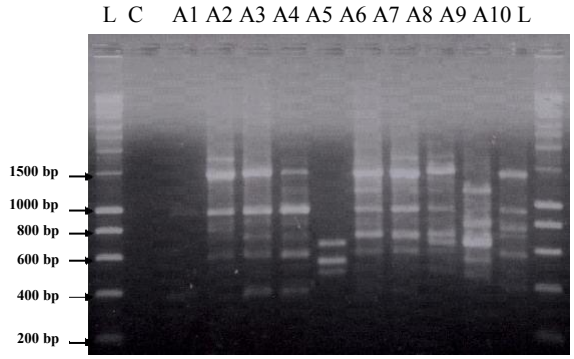

Figure 1. ISSR banding patterns in *M. destructor* of A haplotype generated by the 3´-anchored primer $(AG)_8C$. L: Standard molecular size (1Kb Ladder). C: negative control.

Cluster analysis: inter - and intra-haplotypes relationship

Based on the distance matrix expressed as similarity coefficients, a phenogram was generated by the UPGMA method (Figure 4). The dendrogram clustered all individuals into two major groups representing *M. destructor* and *M. hordei* species. Two different subgroups were formed within *M. hordei* group,

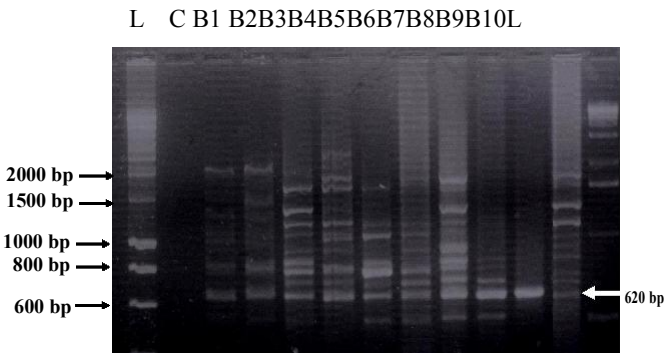

Figure 2. ISSR banding patterns in *M. hordei* of B haplotype generated by the 3´-anchored primer $(AG)_8C$. L: Standard molecular size (1Kb Ladder). C: control. The white arrow marked the specific band of B haplotype.

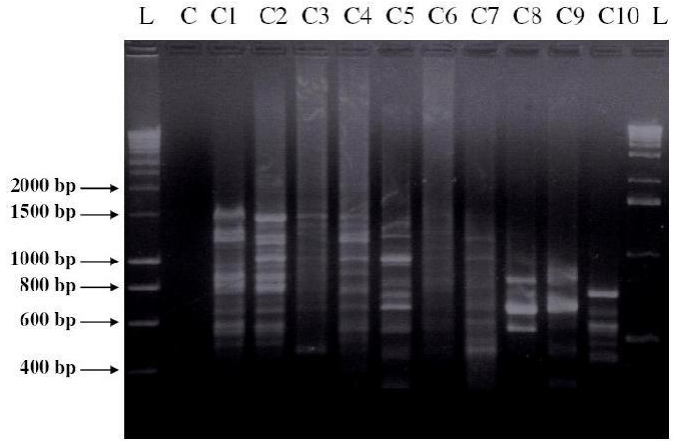

Figure 3. ISSR banding patterns in *M. hordei* of C haplotype generated by the 3´-anchored primer $(AG)_8$ C. L: Standard molecular size (1 kb Ladder). C: Negative control.

representing the two haplotypes B and C. Analysis of the obtained tree revealed a high level of variability intra-haplotypes; this is confirmed by the estimation of standard deviation correlated to the mean distances (Table 2). The A haplotype has the highest standard deviation (0.014), followed by the B haplotype (0.010) and the C haplotype (0.004). The variation of the Nei and Li (1979) genetic distances within the three haplotypes of *Mayetiola* is also quantified by the coefficient of variation (Table 3). The A haplotype is characterized by an important ISSR variability showed that for the three haplotypes the mean genetic distance is significant at *P=0.05* (CV=42.42%), however the C haplotype is the less polymorphic with a coefficient of variation of 23.53%. The B haplotype has an intermediate level of variation (CV=38.46%).

The intra-haplotype extends variation, particularly for A and B haplotypes, necessitated us to test the significance of the mean genetic distances per haplotype with the Student test. Results given in Table 2 showed that for the

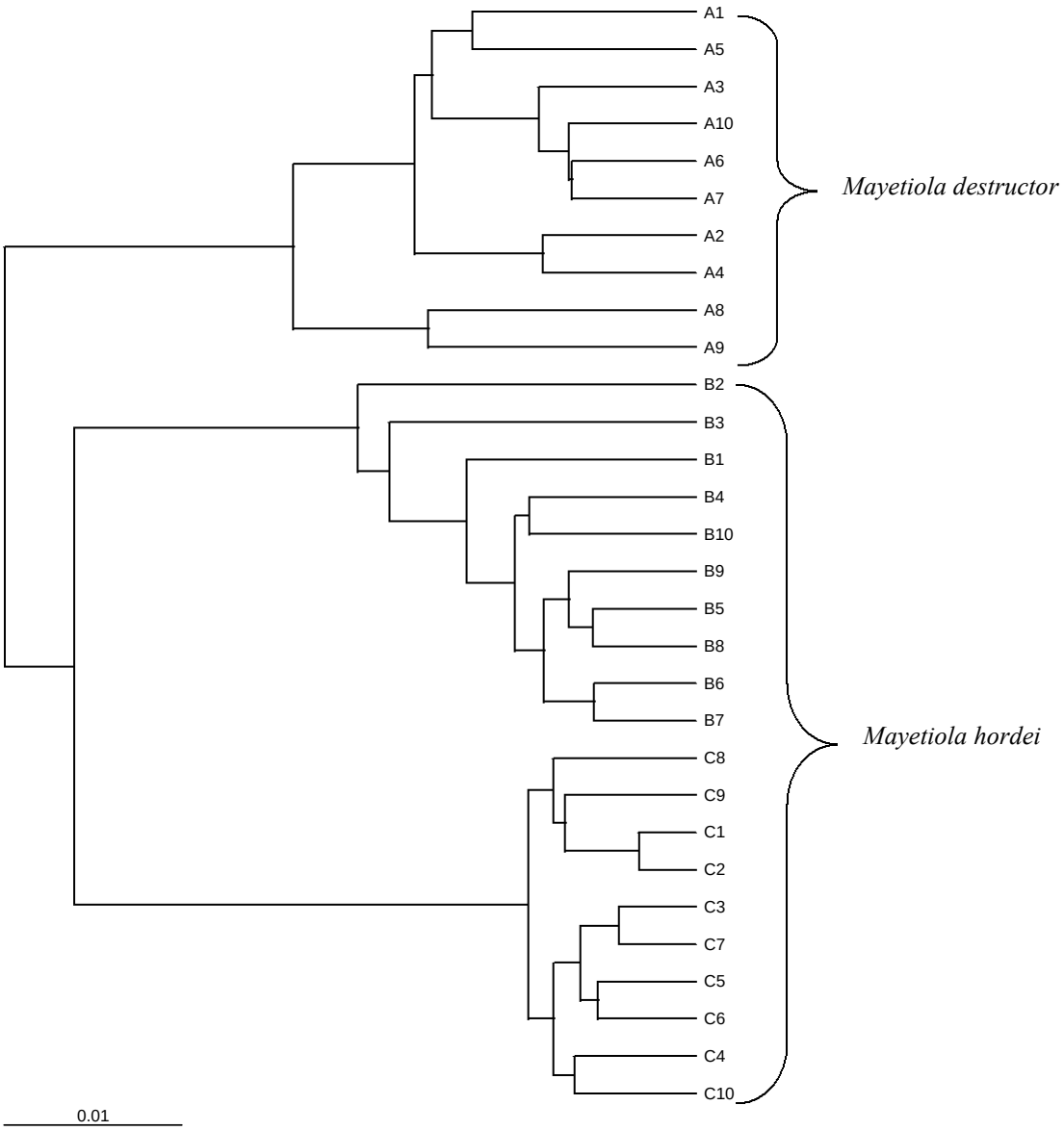

Figure 4. Dendrogram of 30 individuals of *Mayetiola* constructed with Nei and Li's formula genetic distance matrix estimated from ISSR data and clustered with the UPGMA.

three haplotypes the average genetic distance is significant. This deviation added to the important intra-haplotype variation will necessarily affect the estimation of genetic distance between the different pairwise haplotypes. Thus the mean genetic distances was estimated first without taking into account the intra-haplotype variation then on subtracting the intra-group variability by Lynch method (Table 2). Results showed that the corrected inter-haplotype mean genetic distances as well as the uncorrected ones are significant at P=0.05 and provided evidence of the high variability between the different haplotypes detected with the ISSR markers. The intra-haplotype mean genetic distances are practically the same for the three haplotype pairs. This mean varied from 0.07±0.01 to 0.084±0.013 for the uncorrected distances and from 0.046±0.015 to 0.045±0.018 for the corrected ones. In the two cases, haplotypes A and B have a high genetic divergence.

DISCUSSION

Previous results provided by isoenzyme and mitochondrial markers showed weak molecular variation

Table 2. Significance test of the mean genetic distances between and within haplotypes (n= number of haplotype pairwise; Df= Degrees of freedom, S: significant at *P*=0.05; The Z test were used when Df 30).

Average (n)		Standard deviation (Df)	Test	
			t	z
Intra-haplotypes distance				
A	0.033 (10)	0.014 (9	7.454 (S)	-
B	0.026 (10)	0.010 (9	8.222 (S)	-
C	0.017 (10)	0.004 (9)	13.440 (S)	-
Inter-haplotypes distances				
A/B	0.084 (100)	0.013 (99)	-	64.615 (S)
A/C	0.071 (100)	0.011 (99)	-	64.545 (S)
B/C	0.070 (100)	0.011 (99)	-	63.636 (S)\
Corrected inter-haplotyp distances				
A/B	0.054 (100)	0.018 (99)	-	30.000 (S)
A/C	0.046 (100)	0.015 (99)	-	30.667 (S)
B/C	0.048 (100)	0.013 (99)	-	36.923 (S)

Table 3. Intra-haplotype genetic distance variation Nei and Li (1979).

Haplotype	m	s	CV
A	0.033	0.014	42.42%
B	0.026	0.010	38.46%
C	0.017	0.004	23.53%

M, Mean distance; s, standard deviation; CV, coefficient of variation expressed in %.

in the two species of *Mayetiola* (Makni et al., 2000b; Mezghani et al., 2002b). As ISSR markers have the potential to detect intra-specific and inter -specific variation (Zietkiewicz et al., 1994), we tested 14 primers to assess the genetic variability within and between thirty individuals belonging to the three haplotypes of *Mayetiola*. Among these primers, only ten revealed polymorphic and unambiguously scorable bands. While smear or no amplified products were observed with the other primers. In addition, DNA profiles obtained revealed three primers producing diagnostic markers that distinguished the B haplotype from the two others haplotypes of *Mayetiola.*

The number of polymorphic DNA bands produced by this technique is higher than those observed in other insect species such as aphids or mosquitoes (Abbot, 2001). Moreover the overall polymorphism exhibited by the ISSR procedure is rather high in comparison with the diversity reported using RAPD markers in *Mayetiola* (Naber et al., 2000). The present work provides evidence that ISSRs appear very effective to assess molecular polymorphism and phylogenetic relationships in *Mayetiola* species. Genetic diversity estimated at intra-haplotype level revealed considerable amount of genetic variability within the three haplotypes and the highest coefficients of variation were recorded for A and B haplotypes. The phenogram typology is in agreement with those obtained on the basis of mitochondrial and ribosomal sequences (Mezghani et al., 2002b) where the two haplotypes of *M. hordei* clustered together while the A haplotype of *M. destructor* clustered in another group. Data established by ISSR provide evidence of a high genetic diversity that could be exploited at intra and inter-specific level. Indeed genetic distances were significant between and within studied taxa of *Mayetiola.* Thus, these inter and intra-species polymorphism obtained by ISSR markers signifies the power of this technique in diversity analysis especially between closely related species.

It is also important to note that genetic distances obtained by ISSR are relatively higher than those obtained by mitochondrial markers. This could be explained by the evolutionary forces and molecular mechanisms influencing the evolution of satellite DNA which differs from those governing the mitochondrial variability. Indeed unequal crossing over, slippage replication and various mechanism of gene amplification are considered to influence the evolution of microsatellite DNA sequences (Smith, 1976; Walsh, 1987; Strand et al., 1993; Charlesworth et al., 1994; Stephan and Cho, 1994).

In summary, ISSR amplification is an interesting and quick way to evaluate SSR abundance, for example before screening of repetitive elements to elaborate SSR enriched library. Non-anchored and anchored ISSR are

good tools to reveal genetic diversity within and between the two species of *Mayetiola* and to study their genetic relationships.

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