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The utility of ISSR-primers to make difference among populations of parasitoid, *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae)

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Abstract: Various molecular techniques are available to make differences and to study the genetic divergences among species and populations, of which the most efficient ones are PCR-based markers. One of these methods, molecular fingerprinting, enables the distinction of closely related populations. ISSR-primers are universal primers that can be used for such studies. The utility of these primers was tested for three populations of *Eretmocerus mundus* Mercet (Hym, Aphelinidae), a primary parasitoid of the whiteflies. Of the 60 ISSR primers, that were checked, as many as 64 bands were obtained from 16 primers. The band sizes ranged from above 200 to 1000 bp for different primers. The rate of divergence among the bands was strong enough to make a clear difference among the studied specimens; however the intricacy of using these primers is very much for studying the divergence among several populations. Therefore, sequencing of specific regions of DNA could bring more success to find out divergence among populations of these tiny parasitoids.

Keywords: Genetic divergences**,** biocontrol, molecular techniques, whitefly parasitoid

Introduction

To date, several parasitoid species are used as biological control agents. Some of these species are cosmopolitan and have been recorded in most parts of the world. These parasitoids might appear morphologically similar, whereas, their efficiency in biological control might be different. Therefore, the exact identification of a biological control agent is a fundamental part of a successful biological control program. The genus *Eretmocerus* Haldeman is an example of a widespread parasitoid of whiteflies (Goolbsy *et al*., 1998). *Eretmocerus mundus* Mercet, is an effective parasitoid that has been commercially used for control of the sweetpotato whitefly, *Bemisia tabaci* (Gennadius) especially on vegetable crops (van Lenteren 2000). As all species of the genus *Eretmocerus* are tiny wasps, like many hymenopterans parasitoids, the differences of morphological features are not a feasible method for accurate species delineation, and requires very high expertise. In these cases, the molecular techniques can play an important role to differentiate closely related species (Landry *et al.*, 1993; Hoy *et al*., 2000, Caterino *et al*., 2000).

Various molecular techniques can resolve the discrimination of species and populations, among which the most efficient ones are PCR-based markers (Hoy 2003, Loxdale and Lushai, 1998). Molecular fingerprinting is one of these methods, that contributes to distinction of closely related populations. Several markers can be used in fingerprinting e.g. AFLP (amplified fragment length polymorphism), RAPD (randomly amplified polymorphic DNA), and ISSR (Inter Simple

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Sequence Repeat). ISSR usually produces a genomic fingerprint that is similar to that generated by AFLP-PCR. However, as simple sequence repeats (SSR), such as (CA)*n*, are distributed throughout the genomes of eukaryotes, ISSR-primers have a great potential to determine intra-genomic and inter-genomic diversity compared with other arbitrary primers (Weising *et al*., 1998). It has also an advantage over randomly amplified polymorphic DNA (RAPD) in that the longer primers allow for more stringent annealing temperatures (Wolfe and Liston, 1998). Moreover, ISSR-primers can be used as universal primers, which do not need to be adapted to individual species like in microsatellite marker. Consequently, the production of large numbers of fragments, reproducibility, and low cost are considered as advantages of the ISSR primers (Moreno *et al*., 1998). ISSR-primers have been widely used for DNA fingerprinting and assessing genetic diversity in closely related germplasm (e.g. Charters *et al*., 1996, Blair *et al*., 1999). Therefore, researchers are compelled to use this method mostly for phylogeny study in plants (e.g. Bornet and Branchard 2004, Cekic *et al.*, 2001). In contrast, they have been used for a few animal populations (Kostia *et al*., 2000; Reddy *et al*., 1999) and for population-level studies in insects (Abbot 2001, Kumar *et al*., 2001; Phillips *et al*., 2002; Chatterjee *et al*., 2004).

Although, specific molecular markers have shown differences among three *"E. mundus"* populations (Ardeh *et al.*, 2005), in larger groups this method might not show enough variation. Therefore, the demonstration of the utility of ISSR primers, as a straightforward method, for the study of diversity among populations of the tiny parasitoid, *E. mundus* was the aim of this study

Materials and Methods

Three populations of *E. mundus* were used for this study (two populations from Spain (Koppert (S1) and Biobest (S2) companies) and one from Australia (A) (collected by de Barro). The DNA-template of specimens was extracted with 50µl of 5% Chelex®-100 and 2µl of proteinase K (20 mg/ml), incubating overnight at 56 ºC followed by 10 min at 95 ºC and two min centrifugation at 14000 rmp. Initially, one population (Koppert) was tested by sixty one ISSR-markers, from UBCBL (University of Bristish Columbia Biotechnology Laboratory) in Gradient PCR amplification to find out the best primers and optimal annealing temperature for each of them. Subsequently, PCR were conducted for seven individuals of each population with the chosen primers. Each PCR reaction was carried out in a total volume of 25ml, containing 2.5µl of 10x PCR buffer (10mM tris-HCL (pH 9.0), 1.5mM $MgCl_2$ and 50mM KCl), 2.5-3.0µl DNA templates, 0.5µl of dNTP (each in a 10mM concentration), 0.7pM of primer, and 0.07µl of *Taq* DNA polymerase (5unit/µl). The PCR reactions consisted of an initial denaturation for 1 min 94 ºC, followed by 34 cycles of 45 sec at 94 ºC, 30 sec at annealing temperature for each primer, 1 min at 72 ºC, and a final 5-min extension at 72 ºC (Eppendrof).

The PCR products were run on 2% agarose gels in 1X TBE buffer and stained with ethidium bromide. Electrophoresis was performed at voltage 70 mA until the bromphenol dye marker had migrated approximately ¾ the length of the gel, then the gel was photographed by UV light using a GDS8000 Gel Documentation System (UVP, Inc., Upland, CA). The band size was estimated using a 100 bp ladder (NEB, Beverly, Massachusetts, USA).

Results

ISSR Analyses of *E. mundus* Populations

About 60 simple-sequence repeat (SSR) primers (801 to 861) of the UBC kit were initially surveyed for amplification of the DNA templates. Of these, the PCR products from 16 primers gave clear scorable bands in all three populations (Fig. 1A). The other primers either did not amplify bands or the bands were not reproducible in final testing for different populations (Fig. 1B).

Among the ISSRs used, dinucleotide repeats such as (CA)*n* with different anchors yielded most of the good amplification patterns. These were followed by (TC)*n*, (TG)*n*, (AC)*n*, (GA)*n*

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and (CT)*n* SSRs (Table 1). The band sizes ranged from above 200 to 1000 bp for different primers, where within some specimens slight variation in sizes were visible (Fig. 1A).

Table 1 The primer code numbers and number of bands produced for three populations of *Eretmocerus mundus*.

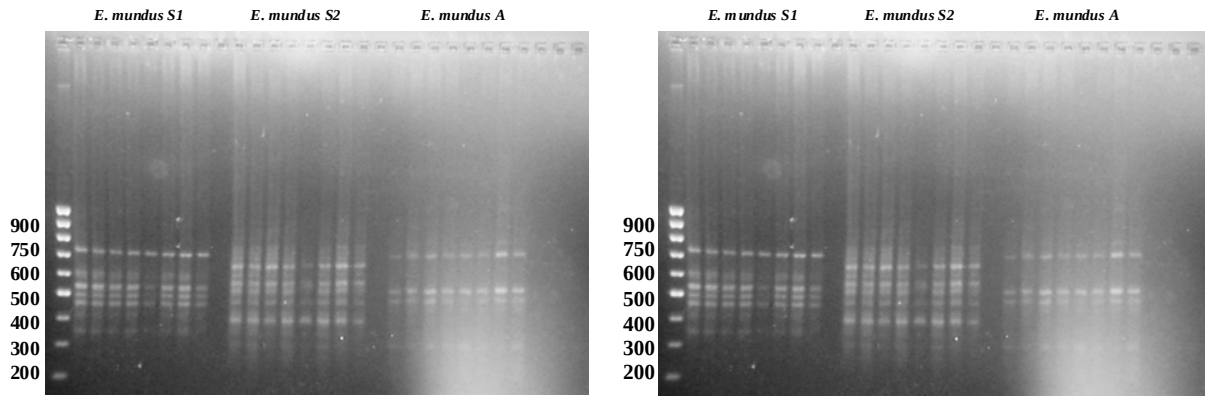
no	No of primer	Sequence	Annealing temperature	Number of bands
1	808	(AG)8-C	39	7
2	810	(GA)8-T	49	3
3	815	(CT)8-G	46	3
4	816	(CA)8-T	47	4
5	817	(CA)8-A	46	6
6	818	(CA)8-G	47	5
7	822	(TC)8-A	47	3
8	823	(TC)8-C	46	4
9	824	(TC)8-G	47	2
10	826	(AC)8-C	49	5
11	829	(TG)8-C	54	5
12	830	(TG)8-G	47	4
13	846	(CA)8-*RT	47	4
14	847	(CA)8-*RC	47	4
15	855	(AC)8-**YT	37	3
16	858	(TG)8-*RT	49	2

* Purine (Guanine/Adenine)
** Pyrimidine (Cytosine/Thymine)

Discussion

Since ISSR-PCR DNA fingerprinting method targets random SSR or microsatellites, it has been used extensively to uncover species level divergences and is also a good tool for distinguishing geographic populations. In this method banding patterns are usually consistent because the anchors serve to fix the annealing of the primer to a single position of the target site, thus resulting in a low level of slippage during amplification (Zietkiewicz *et al*., 1994, reviewed in Karp and Edwards 1997). Still, slight variation in size is not uncommon to see at hyper mutable microsatellite sites (Zietkiewicz *et al*., 1994, de Leon *et al*., 2005). Such slight variations in sizes were visible in the results too (Fig. 1 A). Meanwhile, the result also shows the utility of the primers containing CA-repeat motif with a high number of bands (23 bands) (Table 1). In general, this di-nucleotide repeat is normally found in greater frequency in intergenic regions and introns than in exons in arthropoda (Toth *et al*., 2000).

Figure 1 The bands patterns of two ISSR primers (808 and 817) from three *E. mundus* populations*, (A: scorable bands, B: not reproducible bands).



A **B**

E. mundus (S1) = Koppert, *E. mundus* (S2) = Biobest and *E. mundus* (A) = Australia specimens.

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An advantage of this method is that the same ISSR primer can be rapidly applied across several different taxa (e.g. plants, insects, fungi, and even bacteria) without prior knowledge of DNA sequences (Zietkiewicz *et al*., 1994). In some cases, these primers readily distinguished closely related species (*Encarsia diaspidicola* (Silvestri) and *En. berlesei* (Howard)) (de Leon *et al.*, 2010). The results showed that these primers can also be used to discriminate the populations of *Eretmocerus* species, although, a lot of variation would not be expected within species.

For analysis of the data, the gel bands had to be scored as either, presence (1) or absence (0). For that, the data recording should follow the three principles: 1) Only the easily recognizable bands can be recorded, and the obscure bands are excluded; 2) the bands that cannot be precisely identified should be excluded; and 3) the bands with the same mobility but different intensity should not be treated as the same bands (Weising *et al*., 2005).

To go on those steps and to confirm the reproducibility of the bands, enough DNA-templates were needed to find an optimal annealing temperature for several ISSR primers. Moreover, different band size variations could also be seen in populations of closely related species. For example the *Gontocerus tuberculifemur* (Ogloblin) (Hym., Mymaridae) populations showed some, while the population of *G. fasciatus* Girault showed extensive variations (de Leon *et al*., 2010). *Eretmocerus* includes small size parasitoids, therefore getting enough DNA template for making several PCR was a challenge in the study, and after some PCR reaction the DNA extraction had to be repeated using another specimen. In the meantime, PCR reaction with some of ISSR primers did not amplify. Therefore, it is better to use ISSR primers for genetic characterization of some large sized insects (e.g. *Bombyx mori* by Reddy *et al*., 1999). However, when efficient DNA extraction kits are available this problem may be overcome. Whereas, in other methods, which are based on DNA sequencing, there are more chances to find out differences by doing a few PCRs. In this condition very small amounts of DNA template would suffice to find out differences between populations. Therefore, it is concluded that specific DNA sequencing regions (like mitochondrial and ribosomal regions) could bring more success to find out divergence among population and species of *Eretmocerus* and other small size parasitoids.

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کارآیی آغازگرهای ISSR برای مشخص کردن تفاوت‌های بین جمعیت های پارازیتوئید، *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae)

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چکیده: برای مطالعه تنوع و تفرق بین گونه‌ای و جمعیتی، روش‌های مختلف مولکولی را می‌توان به‌کار برد که از بین آنها نشانگرهای مبتنی بر چرخه PCR متقن قابل استنادتر هستند. انگشت‌نگاری مولکولی یکی از این روش‌هاست که به‌کمک آن می‌توان جمعیت‌های نزدیک به هم را متمایز نمود. از جمله روش‌های انگشت‌نگاری مولکولی، استفاده از آغازگرهای عمومی ISSR می‌باشد که برای چنین مطالعاتی مورد استفاده قرار می‌گیرد. در این تحقیق امکان بهره گرفتن از این آغازگرها برای مطالعه تنوع و تفرق بین جمعیت‌های زنبور *Eretmocerus mundus*، که پارازیتوئید سفیدبالکها می‌باشند، مورد بررسی قرار گرفته است. شانزده آغازگر از بین ۶۰ آغازگر ISSR مورد استفاده، باندهای واضح تولید کردند که در مجموع ۶۴ باند را شامل می‌شد. اندازه این باندها بین ۲۰۰ تا ۱۰۰۰ bp برای آغازگرهای مختلف بود. اختلافات بین باندها برای تمایز افراد سه جمعیت به‌قدر کافی واضح بود. با این حال تمایز حاصله برای مطالعه تنوع جمعیتی بین جمعیت‌ها به‌قدر کافی قابل استناد نبود. بر این اساس به‌نظر می‌رسد که استفاده از روش‌های مولکولی مبتنی بر توالی‌یابی DNA برای مطالعه تنوع جمعیتی این زنبورهای پارازیتوئید، که از جثه کوچکی برخوردارند، با موفقیت بیشتری همراه خواهد بود.

واژگان کلیدی: تفرق ژنتیکی،کنترل بیولوژیکی، روش‌های مولکولی و پارازیتوئید سفیدبالک

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