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# MOLECULAR MARKERS DISTINGUISHING ENCYRTID (*Hymenoptera: Encyrtidae*) PARASITOIDS ATTACKING SOFT SCALE INSECTS (*Hemiptera: Coccidae*)

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## ABSTRACT

Reciprocal molecular markers were developed to distinguish the closely related parasitoid genera *Encyrtus* Latreille, *Metaphycus* Mercet and *Paraceraptrocerus* Girault (Hymenoptera : Encyrtidae). These parasitoids are widely used in the biological control of soft scales and based upon morphology, it is extremely difficult to distinguish them. DNA extraction and RAPD-PCR were used to assess the amount of genetic variation within and among species at this locus. From parsimony analysis, populations of *Encyrtus infelix* (Embleton), *Metaphycus anneckei* Guerrieri & Noyes and *Paraceraptrocerus africanus* Giralut were separated. Specific DNA markers of *E. infelix*, *P. africanus* and *M. anneckei* at *M.W.* of (475.07, 429.60, 311.37*bp*) and (473.33*bp*), (700.0, 400.0 and 270.59*bp*) and, (918.79, 814.45, 771.44*bp*) for (AC07), (AE02), (B10) and (B07) primers, respectively were used. Based upon the sequence variation among species, the present study appeared a simple molecular assay to rapidly and unambiguously distinguish *E. infelix*, *P. africanus* and *M. anneckei* species.

**Keywords:** Encyrtidae , molecular markers, parasitoids and soft scale.

## INTRODUCTION

Soft scale insects (Hemiptera: Coccidae) feed on the phloem of the host plant and were found on stems, leaves and green twigs where they are associated with veins. Damage due to feeding by an individual scale is small. However, large populations result in yellowing, defoliation, reduction in fruit set and loss in plant vigor (Hamon and Williams, 1984). The control of soft scale insects by traditional chemical means has become a problem because of ground water contamination and pest resistance, which makes it necessary to develop alternative means of control. Encyrtids ( Hymenoptera : Encyrtidae) are common parasitoids of soft scales. These parasitoids include members of the genera *Encyrtus* Latreille, *Metaphycus* Mercet and *Paraceraptrocerus* Girault which have been used with success in several biocontrol programs ( Ben-Dov and Hodgson, 1997). *Encyrtus*, *Metaphycus* and *Paraceraptrocerus* have became the most successful natural enemies in biological control after their use to control the greenhouse soft scale insects especially *Saissetia oleae* Olivier and *Saissetia coffeae* (Walker) (Hemiptera: Coccidae) (Abd-Rabou, 2001a). There are documented behavioral differences among strains of *Encyrtus*, *Metaphycus* and *Paraceraptrocerus* . The strains showed consistent differences in percent parasitization and other life-history characteristics as compared with commercially reared strains of

*Encyrtus*, *Metaphycus* and *Paraceraptrocerus*. However, at the biochemical and molecular levels there is a surprising lack of detectable genetic differences among strains of encyrtid parasitoids which unable to detect sufficient genetic differences for the development of molecular markers to clearly distinguish the strains. The taxonomic problem of distinguishing *Encyrtus*, *Metaphycus* and *Paraceraptrocerus* will not be resolved solely by using classic morphological techniques.

Understanding the systematic of agricultural pests is important for the development on effective control and management programs (Parrella and Keil 1984, Rossman and Miller 1996). Species identity can be readily determined using DNA markers data from any of several Genomes and genes (Scheffer 2000, Scheffer and Lewis 2001). However, this method is somewhat time-consuming and expensive for those not routinely involved with DNA sequencing. However, DNA polymerase chain reaction (PCR) is a less expensive and rapid molecular method. The size and number of PCR products obtained using multiprimer sets, the multiplex PCR method, can be used for distinguishing several species (Portillo et al. 1996, Roehrdanz 2003).

Rather, it has been suggested that morphometrics combined with other methods will be necessary to overcome these difficulties. Here, this study review the morphological characters currently used to distinguish *E. inflelix* , *M. anneckei* and *P. africanus* . Then the amount of genetic variation within and among species, using a molecular assay that rapidly distinguishes the species was characterized in this work. This study investigated the genetic variation using RAPD-PCR data from several DNA genomes of *Encyrtus infelix*, *Encyrtus chilinurus*, and *Metaphycus anneckei* that occurred in the same crops and chose a particular gene that was most useful for identification. This work examined whether the RAPD-PCR method using multiprimer sets can be used to rapidly differentiate between *Encyrtus infelix*, *Encyrtus chilinurus*, and *Metaphycus anneckei*.

## MATERIALS AND METHODS

**1. Insect materials:**

Infested plants with the soft scale insects were examined in the field, using a pocket lens. Leaves were collected and placed separately in paper bags for further examination in the laboratory. Identification of the soft scale was done by examining its adult in Canada balsam, according to Abd-Rabou (2003). Materials were also kept in a well-ventilated container until the emergence of any parasitoids. Identification of parasitoids was done by examining their mounted adults in Hoyer's medium (Noyes, 1982).

**2. DNA Extraction:**

DNA extraction and PCR amplification were similar to those reported in Scheffer (2000). Fresh adults were preserved in 95-100% ethanol and stored at -80° C until DNA extraction. From each specimen, DNA was extracted using the insect protocol recommended by Qiagen (Valencia, CA).

**3. RAPD-PCR:**

RAPD-PCR was carried out according to Williams *et al.*, (1990) , Welsh and McClelland (1990) however the primers used were 10 mer oligncleotide; nine primers were selected as potentially useful and the codes and sequences of the used primers are shown in Table (1). PCR reactions were optimized and mixtures (25µl total volume) were composed of dNTPs (200µM), $MgCl_2$ (1.5mM), 1x buffer, primer (0.2µM), DNA (50ng), and two units of Taq DNA polymerase. Amplification was carried out in a thermo Cycler programmed for 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min and elongation at 72°C for 1 min. The 40th cycle was followed by an extended primer extension step at 72°C for 4 min and then the sample was held at 4°C until electrophoresis.

**4. Data Analysis:**

The obtained data of RAPD analysis was entered in a computer file as binary matrices where (0) stands for the absence of a band and in each individual sample. Similarity coefficients were calculated according to dice matrix (Nei and Li, 1979 ; Rohlf, 1993). Parents were grouped by cluster analysis with the similarity matrix and unweighted pair group method based on arithmetic mean (UPGMA).

**Table 1: Name and sequences of primers used with RAPD molecular markers.**

| Nucleotide sequence 5' to 3' | RAPD Primer Name |
|---|---|
| 5'-CTCAAGCGCA-3'. | OPERON – AE 07 |
| 5'-TGAGGGCCGT-3'. | OPERON – AE 02 |
| 5'-CACGAACCTC -3'. | OPERON - AF 13 |
| 5'-GTGCCCGATC -3'. | OPERON – AC 07 |
| 5'-GGTGACGCAG-3'. | OPERON -B07 |
| 5'-CTGCTGGGAC-3'. | OPERON -B10 |
| 5'-GTTTCGCTCC-3'. | OPERON -B01 |
| 5'-TGCGCCCTTC-3'. | OPERON -B05 |
| 5'-AGGGAACGAG-3'. | OPERON -B17 |

## RESULTS AND DISCUSSION

**1. Morphological characters of Parasitoids :**

The parasitoids *Encyrtus inflelix* (Embleton), *Metaphycus anneckei* Guerrieri & Noyes and *Paraceraptrocerus africanus* Giralut ( Hymenoptera : Encyrtidae) were collected from Alexandria, Qena and El-Arish associated with *Ceroplastes floridensis* Comstock on *Citrus* sp., *Pulvinaria. tenuivalvata* (Newstead) on *Saccharum officinarum and Saissetia oleae* (Oliver) (Hemiptera : Coccidae) on *Olea* sp., respectively. *E. inflelix* diagnosed by Mesopleuron large and without a femoral groove. Forewing normal at least very nearly reaching apex of gaster, scutellum with a group of coarse, long, dark setae arranged in a more or less combact bundle. Antenna with scape

longer than the basal three funicle segments combined. Also *M. anneckei* diagnosed by head at least 4X as wide as frontovertex; notaular lines incomplete, not reaching more than halfway across mesoscutum; scape at most 2.7X as long as broad;clava with apical sensorial slightly oblique and transverse,4/5 as wide as clava; forewing hyaline and uniformly infuscate male torulus without associated pores. *P. africanus* diagnosed by mesoscutum large and without a femoral groove. Hypopygium not reaching more than two-third a long gaster, scale tending to be subrectangular the flattened part of upper edge more than one-half as long as the straight part of the lower edge. The morphological characters among the three species under consideration are very similar. The difference among them was forewing shortened, clearly not reaching apex of gaster , hypogygium not reaching more than two-thirds along gaster in case of *P. africanus*. While, hypogygium reaching apex of gaster in case of *E. inflelix* , *M. anneckei* . The difference between two species of later genera was forewing with postmarginal vein not longer than stigmal vein (*M. anneckei* ) and forewing with postmarginal vein longer than stigmal vein (*E. inflelix*) ( Abd-Rabou, 2001a).

**2. Genetic relationship using random amplified polymorphic DNA analysis (RAPD-PCR):**

**a. RAPD-PCR analysis.**

Genetic variability among different genotypes of three insects based on RAPD-PCR analysis was studied. Screening six random primers with three genotypes of insect resulted in six primers that produce different polymorphic bands and amplified DNA fragments as shown in Tables (2 ) and Fig.(1). Six primers used in this study generated a total of 68 amplification products, among which 50 were found to be polymorphic; this resulted in 82.21% polymorphism. All the primers produced polymorphic amplification products, however, the extent of percent polymorphism varied with each primer (50.0 to 100%).

**Table 2: Number of amplified fragments and specific markers of three insect genotypes based on RAPD-PCR analysis with six primers.**

| | | AC 07 | AE 02 | AE 07 | AF 13 | B10 | B07 |
|---|---|---|---|---|---|---|---|
| Genotypes | *TAF | 14.0 | 7.0 | 10.0 | 16.0 | 9.0 | 12.0 |
| | MB | 3.0 | 3.0 | 5.0 | 1.0 | 0.0 | 6.0 |
| | PB | 11.0 | 4.0 | 5.0 | 15.0 | 9.0 | 6.0 |
| | PB% | 78.57 | 57.14 | 50.0 | 93.75 | 100.0 | 50.0 |
| *Encyrtus infelix* | *AF* | 3.0 | 0.0 | 5.0 | 7.0 | 2.0 | 2.0 |
| | *SM* | 0.0 | 1.0 | 0.0 | 0.0 | 1.0 | 1.0 |
| *M. anneckei* | *AF* | 5.0 | 3.0 | 3.0 | 3.0 | 3.0 | 1.0 |
| | *SM* | 3.0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.0 |
| *P. africanus* | *AF* | 5.0 | 1.0 | 2.0 | 6.0 | 6.0 | 2.0 |
| | *SM* | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.0 |
| Total | *AF* | 13.0 | 4.0 | 10.0 | 16.0 | 11.0 | 5.0 |
| | *SM* | 3.0 | 1.0 | 0.0 | 0.0 | 3.0 | 3.0 |

***TAF=Total amplified fragment, Mb= Monomorphic bands, PB=Polymorphic bands. AF=amplified fragment, SM=Specific marker,**

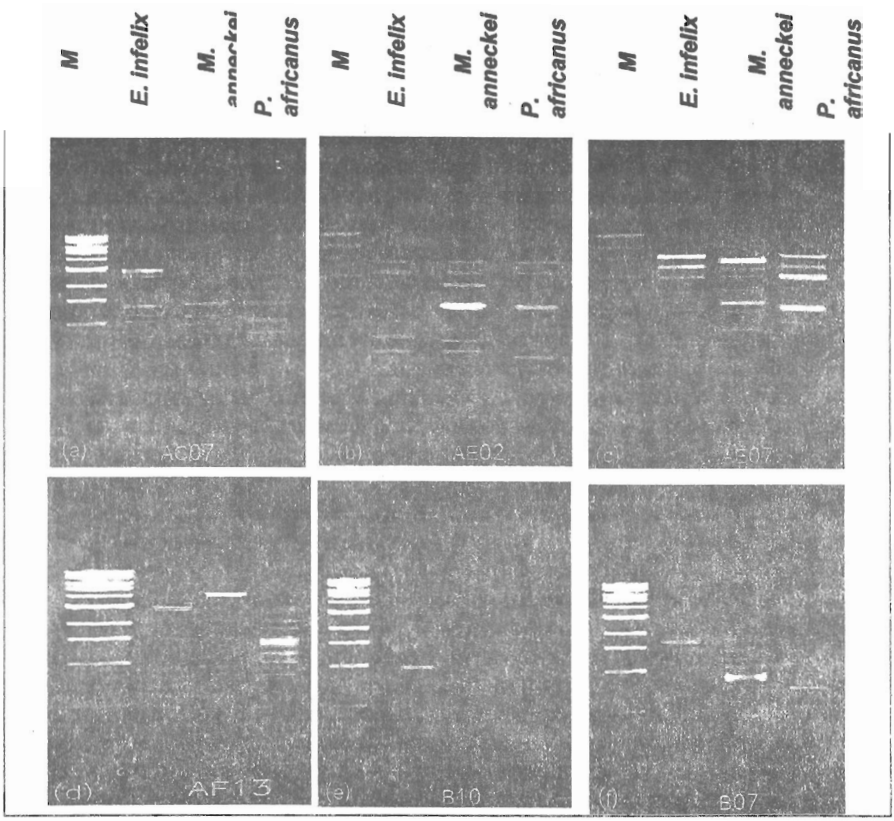


**Fig. 1: DNA polymorphism of three insect genotypes using RAPD-PCR with six primers.**
**Note: E= *Encyrtus inflelix*, M= *Metaphycus anneckei*, P= *Paraceraptrocerus africanus***

The PCR products of primer *(AC07)* analysis of there products are illustrated in Fig.1a and Table (2). This primer produced 3-5 bands for the studied genotypes, as well as, three monomorphic bands in all genotypes. Three positive specific markers were found in *M. anneckei* with *M.W.* of 475.07, 429.60 and 311.37*bp*, respectively.

PCR products of primer *(AE02)* ranged from one band in *P. africanus and M. anneckei* to absent in *Encyrtus infelix* (Tables 2 and Fig.1b). This primer produced three monomorphic bands in all genotypes; Some genotypes had some specific bands and could be used to distinguish among them; for instance *Encyrtus infelix* has one positive specific marker at molecular weight of 473.33*bp*.

PCR products of primer *(AE07)* ranged from two bands in *P. africanus* to five bands in *Encyrtus infelix* (Tables 2 and Fig.1c). This primer produced five monomorphic bands in all genotypes; the other bands were polymorphic as they were present in some genotypes and absent in the other.

In addition, PCR products of primer *(AF13)* analysis of three products are illustrated in Fig.1d and Table (2). This primer produced 3-7 bands for the studied genotypes; as well as, only one monomorphic band in all genotypes. These primers don't produce any specific marker of bands in all genotypes.

PCR products of primer *(B*10) analysis of three products are illustrated in Fig.1e and Table (2). This primer produced 2-6 bands for the studied genotypes and don't produce any monomorphic bands in all genotypes. Some genotypes had some specific bands and could be used to distinguish among then; for instance *Encyrtus infelix, M. anneckei* and *P. africanus* had three positive specific markers having the following molecular weights; 700.0, 400.0 and 270.59*bp*, respectively.

PCR products of primer *(B*07) ranged from only one band in *M. anneckei* and two bands in *Encyrtus infelix* and *P. africanus* (Tables 2 and Fig.1f). This primer produced six monomorphic bands in all genotypes; some genotypes had some specific bands and could be used to distinguish among them; for instance *Encyrtus infelix, M. anneckei* and *P. africanus* had three positive specific marker at having the following molecular weight of 918.79, 814.45, 771.44*bp*, respectively.

RAPD-PCR analysis was performed using primers to obtain markers to assist screening of some aforementioned traits in insects. Primers *(AC07)*, *(AE02)*, *(B*10) and *(B*07) produced RAPD fragments with different lengths which could be used as markers to assist selection for some of the morphological and taxonomical related traits between different genus and species in insects (*Encyrtus infelix, M. anneckei* and *P. africanus*) used in this study.

**b. Nei's similarity coefficient.**

Similarity indices and two consensus were developed on the basis of the scorable banding patterns of the three insect genotypes using six RAPD primers as shown in Table (3). Two most closely related genotypes; *Encyrtus infelix* and *M. anneckei* with highest genetic distances (0.66)were found. On the other hand, two most closely related genotypes; *P. africanus* and with low genetic distances (0.52)were found.

**Table 3: Genetics distance for three insects based on Nei's Coefficient obtained from six RAPD-PCR markers.**

| Genotypes | *Encyrtus infelix* | *M. anneckei* | *P. africanus* |
|---|---|---|---|
| *Encyrtus infelix* | 1.0 | | |
| *M. anneckei* | 0.66 | 1.0 | |
| *P. africanus* | 0.54 | 0.52 | 1.0 |

**c. Cluster analysis.**

Nei's genetic distance (Fig.2) showed that the genetic distances for each genotype combination was ranged from 0.52 to 0.66, as well as, the

studied genotypes formed two main clusters. The first main cluster separated at genetic similarity of 0.52 and created two clusters, the first subcluster included *Encyrtus infelix* and *M. anneckei* at genetic similarity of 0.66; In addition, second main cluster was separated at genetic similarity of 0.52 and created one subcluster, included an individual cultivar of *P. africanus* at genetic similarity of 0.52.

The aforementioned results confirmed that RAPD profiling is a powerful method for identification and molecular classification which agreed with Portillo *et al.* (1996) and Roehrdanz (2003).

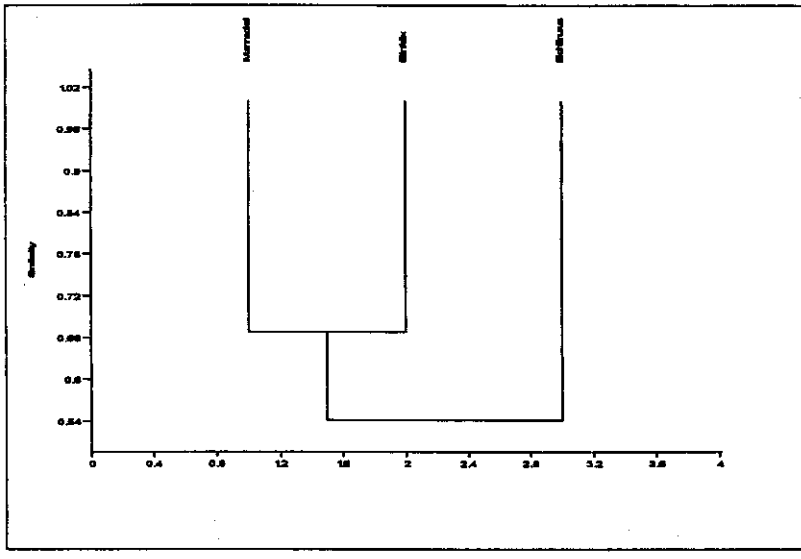

**Fig. 2: Dendogram obtained from UPGMA cluster based on RAPD data from three insect genotypes.**

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## العلامات الجزئية لتمييز طفليليات الأنسرتيد التى تهاجم الحشرات القشرية الرخوة

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تم عمل علامات جزئية لتمييز أجناس طفليليات الأنسرتيد أنسرتس و ميتافيكس و باراسيرابتيروس. نظرا لما تتميز به هذه الطفيليات من دور حيوى فى المكافحة البيولوجية للحشرات القشرية الرخوة و التى من الصعب تمييزها بالطرق المورفولوجية التقليدية. لذا تم فى هذا البحث أستخلاص الحامض النووى و عمل رابيد .

: تم فصل المجاميع الحرية للأنواع الاتية

أنسرتس و ميتافيكس و باراسيرابتيروس وقد أتضح أن الوزن الجزئيى للحامض النووى لكل نوع هو على النحو التالى:

(475.07, 429.60, 311.37*bp*) and (473.33*bp*), (700.0, 400.0 and 270.59*bp*) and, (918.79, 814.45, 771.44*bp*) for (AC07), (AE02), (B10) and (B07)

على الترتيب.

قام بتحكيم البحث

أ.د / خليفة عبد المقصود زايد — كلية الزراعة – جامعة المنصورة

أ.د / فتحى عبد العدل — مركز البحوث الزراعية