Molecular markers of some tick genera in Egypt based on the internal transcribed spacer (ITS-2): 1- Ixodidae (*Boophilus and Hyalomma*)

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Kawther M. El Kammah* and Zaki A. El-Fiky**
* Zoology Department, Faculty of Agriculture, Cairo University, Giza, Egypt.
** Genetics Department, Fayoum Faculty of Agriculture, Cairo University, Fayoum, Egypt.

ABSTRACT

A method for the identification of three ixodid tick species; the cow tick (Boophilus annulatus), the camel tick (Hyalomma dromedarii) and the cattle tick (Hyalomma anatolicum excavatum) based on restriction enzyme analysis of the second internal transcribed spacer (ITS-2) in the nuclear ribosomal DNA was adopted. PCR products with MW 1375 bp, 1650 bp and 1835 bp from B. annulatus, H. dromedarii and H. anatolicum excavatum, respectively were amplified. These products were digested by the restriction enzymes Bgl II, EcoR I, Cfo I and Xmn I and the PCR-RFLP patterns of the enzyme Cfo I showed differences among the three tick species. Species-specific restriction enzyme patterns were identified for B. annulatus (500bp), H. dromedarii (750 bp) and H. anatolicum excavatum (940 bp). The PCR-RFLP patterns of the enzyme Xmn I showed species-specific restriction enzyme patterns for H. dromedarii species (1000 and 650 bp). Further studies on different ixodid and argasid tick species in Egypt are taken place.

Key words: *Ticks, Boophilus, Hyalomma, ribosomal DNA (rDNA), internal transcribed spacer-2 (ITS-2), PCR-RFLP.*

INTRODUCTION

The identification of tick species has always been based on morphological key characters of the capitulum and adjacent structures as a well-developed practice, however, ticks are impossible to identify without microscopic analysis of the characters. Separate keys must be used for larvae, nymphs and adults (Clifford *et al*., 1961; Keirans and Clifford, 1978; Durden and Keirans, 1996). In addition, capitulum and adjacent structures that is usually essential for identification may become damaged during removal of attached ticks to their hosts.

Some of these difficulties may be avoided by using keys based upon molecular genetic markers. These techniques are becoming widely used to identify arthropods, especially mosquitoes (Scott *et al*., 1993; Munstermann and Conn, 1997) and some ticks in Egypt (El Kammah and Sayed, 1999; El-Fiky *et al*., 2003).

The internal transcribed spacers (ITS) regions are very useful for distinguishing between closely related taxa (Hillis and Dixon, 1991). Once an ITS region has been successfully amplified, it can be analyzed by additional techniques (Beebe and Saul, 1995; West *et al*., 1997). Poucher *et al*. (1999)

distinguished 17 *Ixodes* tick species based on restriction enzyme analysis of the second internal transcribed spacer (ITS-2).

The present study aims to amplify the ITS-2 DNA and use the restriction enzyme analysis to determine species-specific markers among three ixodid tick species infesting farm animals in Egypt.

MATERIALS AND METHODS

Ticks identification

All tick species were identified by Parasitic Acarin Research Center (PARC), Faculty of Agriculture, Cairo University, Giza, Egypt. Engorged *B. annulatus* (Say) adults were collected from the experimental station, Faculty of Agriculture, Cairo University. The camel tick *H. dromedarii* (Koch) and the cattle tick *H. anatolicum excavatum* (Koch) adults were obtained from laboratory colonies in PARC. All engorged females were kept in an incubator regulated to 27-30^{o}C and 75% RH for oviposition. The eggs used were in the organogenetic stage (14-day-old) (El Kammah *et al*., 1982; El Kammah *et al*., 1987).

PCR amplification of the ITS-2

Total genomic DNA from examined eggs was isolated and purified according to El-Fiky (2003). The ITS-2 DNA region was amplified using conserved primers which extended from the half of the 5.8S (5'-CTGCGAGACTTGGTGTGAAT-3') to the end of the 28S rDNA genes (5'-TATGCTTAAGTTCAGCGGGT-3') (Poucher *et al*., 1999). The PCR program used was 95^{o}C for 5 min; 35 cycles at 95^{o}C for 1 min, 60^{o}C for 1 min, 72^{o}C for 2 min and one cycle at 72^{o}C for 5 min. The PCR amplification was performed in a total volume of 50 µl. The optimized conditions were 2 µl dNTPs (0.2 mM), 3 µl $MgCl_2$ (1.5 mM), 5 µl 10X reaction buffer, 2.5 µl of each primer (0.5 µM), 2 µl DNA (100 ng) and 1 µl Taq DNA polymerase (2 units) and sterile distilled water up to 50 µl (Williams *et al*., 1990).

Restriction enzyme analysis

The amplification products were purified with QIA quick PCR Purification Kit (QIAGEN). The purified amplicons were subjected to one of four restriction enzymes to identify enzymes that produced species-specific fragment patterns. These enzymes included *Bgl II*, *EcoR I*, *Cfo I* and *Xmn I* (Promega). Restriction enzyme analysis of amplicons for each species was performed in a total volume of 20 µl by using 1 µl of each enzyme, 4 µl of purified amplicon, 2 µl of 10x restriction buffer and 13 µl sterile deionized water. The reaction mixture was pipetted into a microfuge tube and then incubated at 37^{o}C for three hours and followed by 15 minutes at 65^{o}C.

Gel electrophoresis

ITS-2 amplified DNA and the restricted fragments were separated on 1.5% containing 0.5µg ml^{-1} ethidium bromide in 1x TBE buffer at 100 V / 2 hours. The molecular weight standard used was the 1 kb DNA ladder (Promega). Migration distances of ITS-2 amplicons and restricted fragments were measured for molecular weight calculations.

RESULTS AND DISCUSSION

Amplification of the ITS-2

The ITS-2 products were directly amplified using conserved primers extended from the half of the 5.8S to end of the 28S rDNA genes. Amplified PCR products of about 1375 bp, 1660 bp and 1835 bp in size were obtained from *B. annulatus, H. dromedarii* and *H. anatolicum excavatum*, respectively (Fig. 1a). However, it was previously found that amplifying the ITS-2 for

the genus *Ixodes* was 900 bp using the same primers (Poucher *et al.*, 1999).

The different sizes of the amplified products did not overlap among the three species used, indicating presence of species-specific marker. The size of the overlapped ITS amplified product among certain mosqueto species was not useful as a diagnostic character (West *et al.*, 1997).

PCR-RFLP analysis

Although a 6-base pair recognition restriction enzymes (*Bgl II*, *EcoR I*) were unable to digest ITS DNA (Fig. 1b), a 4-base pair recognition restriction enzyme (*Cfo I*) was able to produced species-specific patterns for all species examined (Fig. 1c). On the other hand, the 10-base pair recognition restriction enzyme (*Xmn I*) was arbitrarily able to produce species-specific patterns for *H. dromedarii* species (Fig. 1d).

For the Ixodes, no single enzyme produced species-specific patterns was found and double digestion with *Msp I* and *Cfo I* could produce distinct banding patterns for the genus Ixodes (Poucher *et al.*, 1999). In case of mosquitoes, the ITS-2 product digested with a single enzyme *Msp I* and were able to resolve distinct patterns for 10 cryptic species in the *Anopheles punctulatus* (Beebe and Saul, 1995).

This procedure used herein is rapid and results in adequate resolution and size estimation of most bands obtained. In conclusion, it is determined that the PCR-RFLP of the ITS-2 can be used for the identification of two ixodid tick genera when a 4-base pair recognition restriction enzyme (Cfo I) is used.

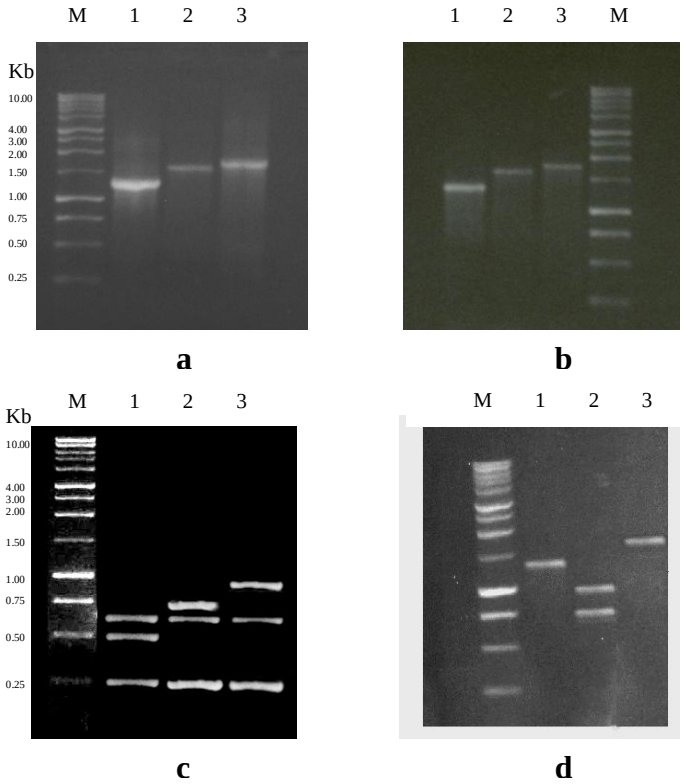

Fig. (1): Agarose gel in TAE buffer stained with ethidium bromide showing: (a) ITS-2 rDNA of tick species; (b) digested with Bgl II or EcoR I ; (c) digested with Cfo I; (d) digested with Xmn I. (M, 1 kb DNA ladder; Lane 1, B. annulatus; Lane 2, H. dromedarii; Lane 3, H. anatolicum excavatum).

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الملخص العربي

استخدام التتابعات النوتيدية الواقعة بين وحدات الريبوزوم (ITS-2) فى تحديد الواسمات الجزيئية لبعض أنواع القراد المنتشر فى مصر: ١- فصيلة القراد الجامد (بووفيلس، هيالوما)

كـــوثر م القمـــــاح* و زكـــى أ. الفقـــــى**

*قسم الحيوان الزراعى – كلية الزراعة – جامعة القاهرة

**قسم الوراثـــة – كلية الزراعة – جامعة القاهرة – فرع القيوم

تم إكثار الحمض النووي الريبوزى الخاص بالتتابعات النوتيدية الواقعة بين وحدات الريبوزوم (ITS-2) و تفريده على بيئة الأجاروز لثلاثة أنواع من القراد المتطفل على الحيوانات فى مصر : قراد البقر *Boophilus annulatus* ، قراد الجمال *Hyalomma* dromedarii و قراد المواشى *H. anatolicum excavatum* والتي تم الحصول عليها من مركز أبحاث أكاروس الحيوان – كلية الزراعة – جامعة القاهرة. وباستخدام التفريد الكهربي لنواتج تفاعل البلمرة المتسلسل PCR تبين وجود حزمة واحدة لكل نوع من القراد المستخدم، وكانت الأوزان الجزيئية لهذه الحزم ١٣٧٥ زوج قواعد لقراد *B. annulatus* ، و١٦٥٠ زوج قواعد لقراد *H. dromedarii* و ١٨٣٥ زوج قواعد لقراد *H. anatolicum excavatum*. ونظرا لعدم تداخل بين هذه الأوزان الجزيئية فانه يمكن استخدامها كواسمات جزيئية مميزة لهذه الأنواع .

وعند معاملة هذه الحزم بأربعة إنزيمات قطع متخصصة (Restriction endonuclases) – هي *Bgl II, EcoR I, Cfo I, Xmn I* وبتفريدها كهربيا اتضح عدم وجود أماكن تعرف لكلا الإنزيمين *EcoR I* ، *Bgl II* فى الأنواع الثلاثة المستخدمة. أما الإنزيم *Cfo I* فقد أظهر ثلاثة حزم لكل نوع من أنواع القراد المستخدم. وأمكن استخدام الحزمة ذات الوزن الجزيئى ٥٠٠ زوج قواعد كواسم جزيئى لقرادالبقر *B. annulatus* ، والحزمة ذات الوزن الجزيئى ٧٥٠ زوج قواعد كواسم جزيئى لقرادالجمال *H. dromedarii* و الحزمة ذات الوزن الجزيئى ٩٤٠ زوج قواعد كواسم جزيئى لقراد المواشى *H. anatolicum excavatum*. وأظهر الإنزيم *Xmn I* حزمتين ذوات أوزان جزيئية ١٠٠٠ و٦٥٠ زوج قواعد لنوع القراد *H. dromedarii* فقط مما يدل على عدم وجود أماكن تعرف Recognition sites لهذا الإنزيم في كلا نوعي القراد (قراد البقر وقراد المواشى).

ومن النتائج المتحصل عليها أستنتج أنه يمكن استخدام التتابعات النوتيدية الواقعة بين وحدات الريبوزوم (ITS-2) وطريقة (PCR-RFLP) باستخدام انزيم *Cfo I* فى تصنيف هذه الأنواع من القراد.