OSVRTI REVIEWS

Prof.dr. sc. JASMINKA IGRC BARČIĆ, Prof.dr. sc. MILAN MACELJSKI, Ekološki prihvatljiva zaštita bilja od štetnika, 2001. Zrinski d.d., Čakovec

Iz tiska je izašla knjiga, sveučilišni udžbenik, naših eminentnih stručnjaka s Agronomskog fakulteta u Zagrebu iz područja biljne zaštite pod suvremenim naslovom: Ekološki prihvatljiva zaštita bilja od štetnika. Nakladnik je Zrinski d.d. Čakovec, 2001. god. Knjiga je formata 17 x 24 cm. Estetski i tehnički je na visokom nivou. Sadrži 247 stranica, dvije tablice i 173 slike shema, tehničkih prikaza i izuzetno kalitetnih originalnih slika u boji.

Knjiga uz suvremeni *Proslov* i *Uvod* sadrži 17 poglavlja, kako slijedi: *Osnovne postavke ekološki prihvatljive zaštite bilja, Karantenske mjere, Otporni kultivari, Plodored u ekološki prihvatljivoj zaštiti bilja, Agrotehničke mjere, Mehaničke mjere, Fizikalne mjere, Biološke mjere, Mjerila ekološke prihvatljivosti sredstava za zaštitu bilja, Bioinsekticidi, Biotehnička sredstva za zaštitu bilja od štetnika, Ekološki prihvatljivi zoocidi, Ekološki prihvatljiviji načini primjene sredstava za zaštitu bilja, Integrirana zaštita bilja i zaštita bilja u ekološkoj poljoprivrednoj proizvodnji, Prikaz važnijih štetnika kultura i mjera zaštite od njihova napada uz ocjenu ekološke prihvatljivosti pojedinih mjera, Prilozi i Važnija vrela podataka.*

Poljoprivredne kulture i ukrasno bilje napadaju brojne štetočinje. Gubitak u poljoprivrednoj proizvodnji u Hrvatskoj iznosi 29,2% potencijalnog prirasta, a to je 500 mulijuna USD godišnje. Tijekom Drugog svijetskog rata naglo se razvijala kemija (DDT, ditiokarbamatni fungicidi i translokacijski herbicidi). Tada je započela "era kemije". Porastom ekoloških spoznaja započinje "era ekologije". Smanjuje se potražnja kemijskih sredstava. U isto vrijeme pronalaze se novi spojevi bez "cidnog" djelovanja a poglavito biološke i biotehničke mjere suzbijanja, selekcija i uzgoj otpornih kultivara. Svijet ulazi u Treći milenij, u "eru ekologije i kakvoće". Za to je potrebno mnogo veće znanje agronoma i seljaka. Ekološki prihvatljive mjere zaštite sadrže: karantenu, uzgoj otpornih kultivara, suvremene agrotehničke mjere (zdravo sjeme i sadni materijal, čuvanje uskladištenih proizvoda), mehaničke i fizikalne mjere zaštite, primjenu biotehničkih sredstava bez "cidnog" djelovanja, dakle ekološki prihvatljivih kemijskih pripravaka. Budućnost zaštite bilja u eri ekologije može se ostvariti u integriranoj zaštiti bilja. Sve navedeno sadržaj je ove vrijedne knjige pod motom da suvremena poljoprivreda traži manje materijalnih a više intelektualnih ulaganja. Treba nam dakle mnogo više znanja i znanstvenih dostignuća. Tako obrađena tematika može biti siguran putokaz u održivu i ekološki prihvatljivu poljoprivrednu proizvodnju. Čitalac će u knjizi pronaći pravi i jedini put u suvremenu zaštitu bilja. Knjiga je namijenjena studentima Agronomskog fakulteta, agronomima, šumarima i svima onima koji se bave fitomedicinom. U njoj se nalaze nove spoznaje i znanstvena dostignuća u nas i u svijetu. Izražavamo zahvalnost i upućujemo čestitke autorima.

Miroslav HARAPIN, Zagreb

Entomol. Croat. 2001. Vol. 5. Num. 1 – 2: 65 – 75.
ISSN 1330-6200
UDC 595.771+632.21:632.07(611)

DISTINCTION OF TWO SPECIES OF CECIDOMYIES (DIPTERA: CECIDOMYIIDAE) REVEALED BY MITOCHONDRIAL DNA GENES VARIATION

Maha MEZGHANI KHEMAKHEM[1], Hanem MAKNI[1,2] and Mohamed MARRAKCHI[1]

[1] Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie. Faculté des Sciences de Tunis. Université Tunis El Manar, 2092 Tunis. TUNISIE. e-mail : mezghani.h@planet.tn [2] Institut Supérieur de l'Animation pour la Jeunesse et la Culture de Bir Elbey. Université de Tunis

Received: February 27, 2001 – Accepted: July 10, 2001.

Mayetiola is an important pest of cereal in Tunisia, two species were identified using isoenzymatic markers. To develop a DNA diagnostic tool, we assessed mitochondrial DNA study based on Restriction Fragment Length Polymorphism in Polymerase Chain Reaction Amplified Fragments (PCR-RFLP). Specific primers were used to amplify the cytochrome b and a 3' terminal region of the 12S mt rDNA genes. Results showed that Sau3A restriction patterns can distinguish between the two morphologically similar species. Thus PCR-RFLP is a useful tool for identifying morphologically similar species of Mayetiola in large-scale pest identification.

Mitochondrial DNA, PCR-RFLP, Diptera, Cecidomyiidae, *Mayetiola*, Tunisie

MEZGHANI KHEMAKNEM, M[1]., MAKNI, H.[1,2], MARRAKCHI, M.[1], Razlučivanje dviju vrsta mušica šiškarica (Diptera: Cecidomyiidae) otkrivanjem razlika u genima za mitohondrijsku DNA. [1]Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie. Faculté des Sciences de Tunis. Université Tunis El Manar. 2092 Tunis. TUNISIE. E-mail: mezghani.h@planet.tn [2]Institut Supérieur de l'Animation pour la Jeunesse et la Culture de Bir Elbey. Université de Tunis - Entomol. Croat. 2001, Vol. 5. Num. 1-2.: 65 – 75.

Mayetiola je važan nametnik na žitaricama u Tunisu, a dvije vrste bile su određene upotrebom izoenzimskih biljega. Kako bismo razvili dijagnostički alat zasnovan na upotrebi molekule DNA, pristupili smo proučavanju mitohondrijske DNA metodom praćenja polimorfizma duljine restrikcijskih fragmenata nakon lančane reakcije polimerazom (PCR-RFLP). Kako bi se umnožio gen za citokrom b i 3' terminalno područje 12S mt rDNA bile su upotrijebljene specifične klice. Rezultati pokazuju kako se uzorak razgradnje dobiven restrikcijskim enzimom Sau3A razlikuje između dviju morfološki sličnih vrsta. Tako je ustanovljeno kako je PCR-RFLP metoda korisna za razlučivanje morfološki sličnih vrsta *Mayetiola* prilikom identifikacije ovih štetnika u velikom uzorku.

Mitohondrijska DNA, PCR-RFLP, Diptera, Cecidomyiidae, *Mayetiola*, Tunis

Introduction

Mayetiola destructor and *Mayetiola hordei* are small diptera in the family of Cecidomyiidae that cause important crop losses on cereals. *M. destructor* has a large geographic distribution. It was found in all areas where we cultivate wheat :Europe, USA, Siberia, Asia, New Zealand and they are also known to occur in North Africa. (BALACHOWSKY & MESNIL, 1935). However *M. hordei* was mentioned only in the North of Africa and in Southern Europe.

Mayetiola hordei (Kieffer, 1913) live in galls they cause on the lower part of stems of barley, and *Mayetiola destructor* (Say, 1817) reproduces mainly on wheat but it can also sometimes be found on barley, where it does not produce gall.

The two phytophageous species can be difficult to differentiate morphologically using characteristics identified by GAGNÉ et al., (1991) namely the shape of the female post abdomen, the structure of male terminalia and the number of spicule covering the puparium.

An effective pest management programs of cecidomyies need an accurate and reliable method of diagnosing of these two species in order to understand their evolution and their interaction with the host plants.

Molecular approaches such as isoenzymatic analyses have been also used to reveal markers of taxonomic value for these two species (MAKNI et al., 2000). However this technique has the disadvantage to require refined storage conditions, as specimens must be maintained below –70°C to preserve allozyme activity.

Advances in molecular biology, in particular the comparison of nucleotide sequences of specific DNA regions amplified by Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) analysis, may help to characterize these two closely related species. In fact these techniques have been used to resolve taxonomic problems in anopheles (KOEKEMOER et al., 1998) and phlebotomines sandflies (ARANSAY et al., 1999).

Mitochondrial DNA has been recognized to be useful for assessing the relationships between species (SIMON, 1994) and between many insect species as phlebotomines sandflies (READY et al., 1997; ARANSAY et al., 1999), mites (NAVAJAS et al ., 1997), butterflies (TAYLOR et al., 1993; BROWER, 1994; WILLIS et al.,1992; ARIAS & SHEPPARD, 1996) blackflies (TANG et al., 1995), drosophile (DESALLES, 1992; RUTTKAY et al., 1992) and mosquitoes (CACCONE et al., 19996).

The popularity of mtDNA for such studies has developed in part because the genetics of this molecule differs from nuclear DNA in several ways that offer practical advantages in the collection and analysis of data, (i) it is maternally inherited; (ii) the genome is haploid; (iii) it does not undergo recombination; (iv) it exhibits relatively rapid sequence evolution; (v) the genetic organisation of the entire mitochondrial genome is known for a diverse array of species (BROWN et al., 1982; SOLIGNAC et al., 1983;WILSON et al ., 1985).

We had surveyed mitochondrial DNA sequences and site variation in the cytochrome b (Cytb) and the 3' region mitochondrial RNA 12S coding DNA (12S mt-rDNA).

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The objective of our study is to develop a simple and reliable method for distinguishing the two species of the Cecidomyiidae family by means of PCR-RFLP technique.

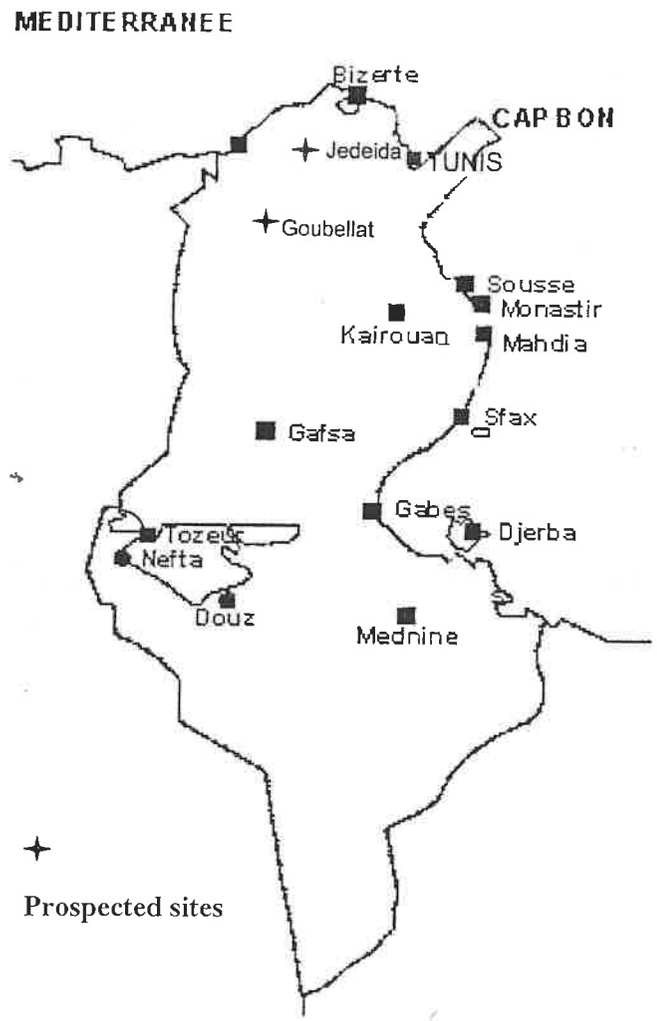

Fig. 1: Tunisian map showing location of prospected sites. *Mayetiola destructor* samples were collected from Jedeida. *Mayetiola hordei* samples were collected from Goubellat.

Materials and methods

Samples

Mayetiola species were collected during the period from December 1998 to June 1999 from two different sites: *M. destructor* were collected from Jedeida (Northeast of Tunisia) and *M. hordei* were collected from Goubellat (Center of Tunisia) (Fig. 1). The infested plants of each field were kept in separate screened cages and placed into a greenhouse where conditions were favorable for adult emergence (12: 12 h Photoperiod, 18°C-20°C Temperature and 60% relative humidity). Emergence was checked every day and Mayetiola adults were stored in 1.5 ml microcentrifuge tubes at -70°C.

DNA extraction

DNA was isolated from single fly using the standard procedure described in COEN et al (1982). Insect was gently homogenized in a 1,5 ml microfuge tube containing 400 µl of 10 mM Tris, 60 mM NaCl, 5% sucrose, 10 mM EDTA pH 7.5. 400µl of 0.3 M Tris, 0.1 M EDTA, 5% sucrose pH 9 were then added and samples were grinded.

After incubation at 65°C for 30 min, 30µl of 8M potassium acetate was added and the mixture kept on ice for 45 min. The precipate was spun down for 5 min and the supernatant discarded.

The pellet was resuspended in 400 µl of 0,1X SSC (150µl mM NaCl, 15 mM Sodium Citrate), 0,2 diethyl pyrocarbonate (freshly mixed) and left to stand at room temperature for 30 min; three volumes of ethanol were then added and the mixture left at room temperature for further 5 min and the DNA was spun down for 10 min at 13000g and washed with 70% ethanol.

Residual ethanol was removed by drying the precipate in a speedvac for 30 min and the DNA was dissolved in 50µl of sterile water.

Amplification

Two segments of the mitochondrial DNA were amplified the DNA corresponding to the 3' region of 12S mitochondrial rDNA and the cytochrome b DNA using insect primers described in Simon et al 1994 (Table I). Amplification reactions were performed in 10 X Taq DNA polymerase buffer; 3 mM MgCl2; 0,1 mM dNTP; 0.7 µM primers and 1 µl of DNA (50 ng). The PCR were run using 1U of Taq polymerase (Appligen) in a final volume 50µl.

Thirty-five cycles were run in a Braun thermocycler as follows: denaturation at 94°C for 1 minute, annealing at 48°C for 1 minute and extension for 1'30.

Restriction fragment analysis

PCR products were purified on QiaQuick PCR columns (Qiagen) and 10µl were used in digestion with several restriction enzymes (Amersham). Restricted DNA was analyzed on vertical polyacrylamide gel electrophoresis (PAGE) or on 1.5% agarose gel.

Digestions were done using 10µl of purified PCR product, 1U enzyme, 1X buffer and water to bring the volume to 20µl in 0,5 µl tubes. Samples were incubated at 37°C for 3-16 h, followed by 65°C for 10 min and held at 4°C.

The entire digestion product was loaded onto a 12% gel [8ml 30% acrylamid (29g acrylamid, 1g bisacrylamid), 8ml H2O, 4ml TBE 5X, 35µl TEMED and 160µl ammonium persulfate]. Gels were run in a 1X TBE buffer at constant 100V for two hours. Gels were stained for 15 min with ethidium bromide (10µl in 50 ml of TBE 1X). For Cytochrome b, digested products were run on a 1.5% agarose gel.

Results

The 12S mt-rDNA

PCR with primer12Sbi and 12Sai (Table I) amplified a single DNA fragment with an approximate length of 400 pb for the two species of *Mayetiola*.

In order to search for species-specific patterns, PCR products are digested with restriction enzymes.

The enzyme EcoRV produced the same restriction patterns composed of two fragments of about 200 pb) in the *M. hordei* and *M. destructor* amplicons which co-migrated on a 12% acrylamid gel and appeared as a single band (Fig 2).

However polymorphic profiles were observed when the digestions were carried out with Sau3A which recognized two sites in M. destructor amplified products and gave three fragments of 190 pb, 160 pb and 45pb respectively, while M. hordei had a single Sau3 A recognition site that produces two fragments of 230 pb and 185 pb (Fig 2).

The Cytochrome b

The Cytochrome b gene was amplified by means of primer CB1 and TRs (Table I). The amplified fragment is about 800pb. For RFLP analysis, the PCR products obtained for both species were digested by different enzymes (Fig.3).

The digestion by Taq I yielded the same profile formed by two fragments of about 400 pb for both species of Cecidomyies.

The restriction enzyme DraI has one site on the Cytochrome b amplified fragment from the two species which produces two bands of 633 pb and 230 pb (Fig 3).
The enzyme AluI produced for M. destructor and M. hordei the same restriction patterns composed of two fragments of 427 pb and 350 pb (Fig 3).

Although TaqI, Dra I and AluI did not reveal any differences between the two species, digestion of the Cytochrome b amplicon with Sau3A has led to a diagnostic pattern composed of two fragments of 553 pb and 249 pb for M. hordei amplified fragment. The M. destructor amplicon seems not to have a recognition site for this enzyme or maybe it has a site undigested by this enzyme (Fig 3).

In order to confirm the absence of Sau3A site on the Cytochrome b amplified fragment of M. destructor, digestion was carried out using pUC18 plasmids (GibcoBRL). The pUC18 vector has five Sau 3A recognition sites that produce five fragments.

After Sau 3A digestion, the RFLP patterns showed besides the five expected bands an additional fragment of 800 pb that corresponds to the uncut PCR products (Fig 4).

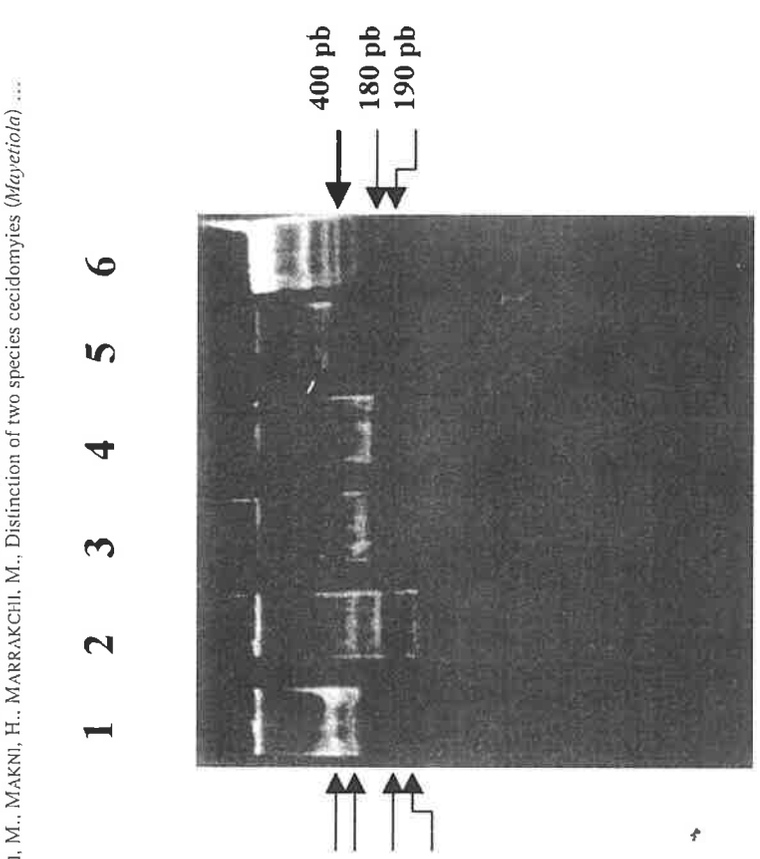

Figure 2: Acrylamid gel (12%) of RFLP patterns of PCR-amplified 12S mt-rDNA digested by Sau3A and EcoRV. Lane 1 digestion of *Mayetiola hordei* amplicon by Sau3A, lane 2 digestion of *Mayetiola destructor* amplicon by Sau3A. Lane 3 digestion of *Mayetiola hordei* amplified fragment by EcoRV, lane 4 digestion of *Mayetiola destructor* amplified fragment by EcoRV, lane 5 PCR product of 12S mt-rDNA, lane 6 1Kb DNA size marker.

Table 1. Polymerase chain reaction primers

Amplicon	Forvard primer	Sequence 5' – 3'	Rever-se primer	Sequence 5' – 3'	Location[a]
12S mt-RNA	12Sbi[b]	AAGAGCGACGGGCGAT	12Sai	ACTAGGATTAGATACCCTAT	14214-14588
Cytochrome b	CB1[c]	TATGTTTTACCTTGAGGACAAATATC	TRs	AAATTCTATCTTATGTTTTCAAAAC	10933-11683

a *Drosophila yakuba* mtDNA map (Simon et al. 1994) – b Designed by Kocher et al. 1989 – c Designed by Jermiin et al. 1994

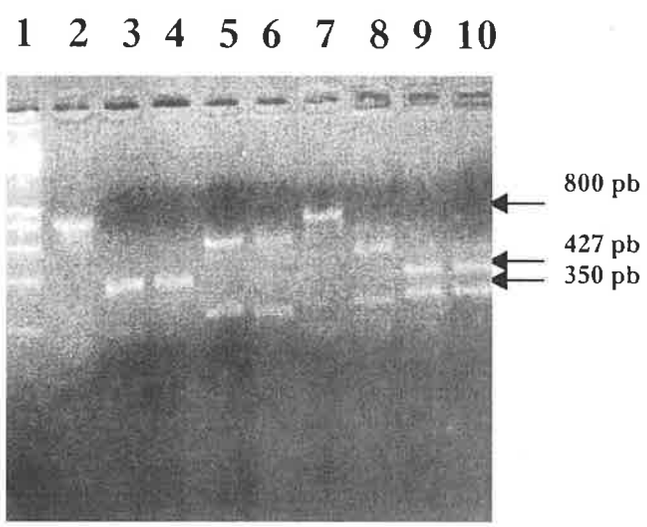

Figure 3: Cytochrome b amplified products following digestion with restriction endonuclease (1,5% agarose gel): TaqI, DraI, Sau3A and AluI. Lane 1: 1Kb DNA size marker; lane 2: undigested amplicon; lane 3: *M. destructor* Cytb digested by TaqI: lane 4: *M. hordei* Cytb digested by TaqI; lane 5: *M. destructor* Cytb digested by DraI; lane 6: *M. hordei* Cytb digested by DraI; lane 7: *M. destructor* digested by Sau3A; lane8: *M. hordei* Cytb digested by Sau3A; lane 9: *M. destructor* Cytb digested by AluI; lane 10: *M. hordei* Cytb digested by AluI.

Discussion

Restriction Fragment Length Polymorphism of the amplified 12S mt-rDNA and the cytochrome b sequences has proved to be an accurate and simple method for the typing of the two *Mayetiola* species.

By digesting the amplified 12S mt-rDNA with Sau3A we were able to discriminate the two species of *Mayetiola*. Besides the digestion by Sau3A of the Cytochrome b amplified sequence of *M. destructor* and *M. hordei* had given a diagnostic pattern.

The results presented here confirm the species status of *M. h*ordei by discriminating it from *M. destructor* and a more extensive study using samples of a wide geographical distribution may allow us to confirm the maintenance of species-specific patterns.

We have shown that the molecular approach is useful for solving taxonomic problems when diagnostic morphological traits are scarce.

Although species may be usually identified by morphological characteristics, it is important to confirm by molecular means if slight morphological differences are reliable for distinguishing them.

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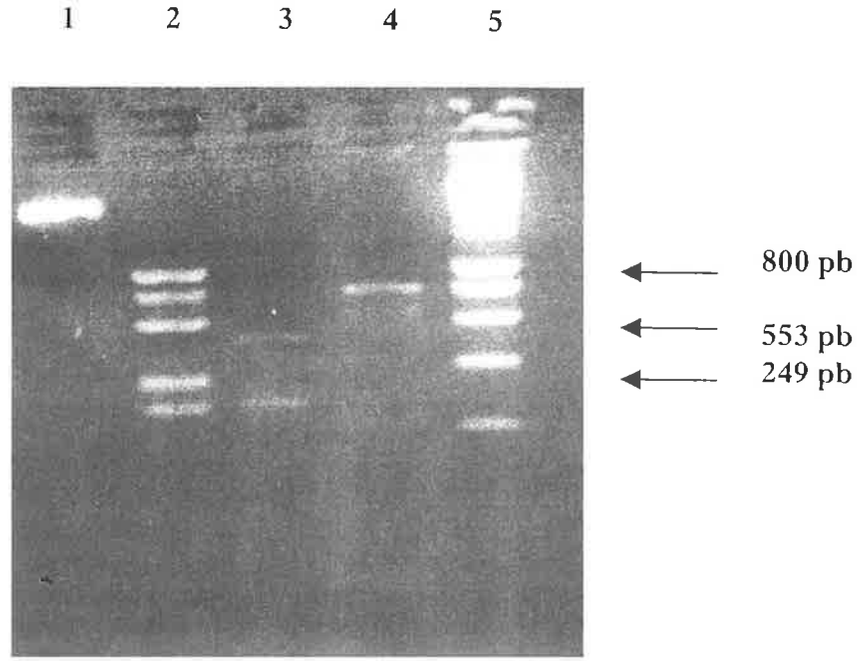

Figure 4: Sau3A digestion patterns of *M. destructor* Cytochrome b and pUC18. Lane1: undigested pUC18; lane 2: pUC18 and *M. destructor* Cytb digested by Sau3A; lane 3: *M. hordei* Cytb digested by Sau3A; lane 4: undigested Cytb amplicon; lane 5: 1Kb DNA size marker.

Acknowledgments

This work was supported by grant N° 97/F0908 from the Comité Mixte Franco-Tunisien pour la coopération Universitaire, by l'Institut français de coopération auprès de l'ambassade de France en Tunisie and the Direction Générale de la Recherche Scientifique et à la Technique au Ministère e de l'enseignement Supérieur du secrétariat d'Etat à la Recherche Scientifique et à la Technologie.We are grateful for the helpful discussions and collaboration from all those involved in this project for their comments or help in the laboratory. Particular thanks must go to Nicole Pasteur (Laboratoire Génétique et Environnement, CNRS, URA 327, Université de Montpellier II).

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