REDIA, XCI, 2008: 55-58

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MOLECULAR IDENTIFICATION OF *SITOPHILUS* SPP. (COLEOPTERA CURCULIONIDAE) AND DETECTION OF IMMATURE STAGES IN WHEAT

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Angiolillo A., Boursier L., Pilla F., Germinara G.S., Rotundo G. – Molecular identification of *Sitophilus* spp. (Coleoptera Curculionidae) and detection of immature stages in wheat.

A polymerase chain reaction (PCR)-based assay is proposed to differentiate *Sitophilus granarius* (L.), *S. oryzae* (L.), and *S. zeamais* Motschulsky at different life stages and to detect their hidden infestation in wheat kernels. PCR amplification of genomic DNA using primers designed on the basis of the cytochrome oxidase subunit II gene sequence, followed by agarose gel electrophoresis, successfully detected the immature stages (egg, larva, pupa) and adult of each species. DNA amplification resulted in a PCR product of 462 bp for *S. granarius*, whereas *S. oryzae* and *S. zeamais* yielded a 311 bp fragment. A sequence comparison between the latter species allowed to identify a mutation that was diagnostic between *S. oryzae* and *S. zeamais* through a PCR-RFLP protocol. Digestion of DNA resulted in an uncut fragment (311 bp) for *S. oryzae*, two fragments (174 bp + 137 bp) for *S. zeamais* and 3 bands (250 bp + 160 bp + 52 bp) for *S. granarius*. Multiplex PCR reaction enabled to differentiate the three species at all life stages also when simultaneously added to and ground with a 50 g sample of wheat kernels. The reproducibility of the results and high resolution as well as the technical simplicity of the developed molecular method demonstrated its usefulness in the diagnosis of *Sitophilus* spp. and detection of their hidden infestation in wheat.

KEY WORDS: stored products weevils, differentiation, hidden infestation, PCR, PCR-RFLP.

INTRODUCTION

Sitophilus granarius (L.), *S. oryzae* (L.), and *S. zeamais* Motschulsky (the granary, rice and maize weevils, respectively) are primary pests of cereal grains worldwide. The three species develop from egg to pupa inside kernels and determine heavy post-harvest losses as well as a decrease in the nutritional quality of stored cereals. Further quality decline is caused by mycotoxigenic moulds which are frequently associated with insect-damaged kernels.

The three species share many morphological traits in the adult stage; at the immature stages they are almost indistinguishable. Nevertheless, there are significant differences at the biological level which may result in different pest statuses. For example, the rice and maize weevils are able to fly and can move from storage to field environments, whereas the granary weevil has lost its flying ability and is commonly present in storage sites.

Over the past decade, molecular-based approaches have increasingly been used to identify pests, especially where slight phenotypic variations limit traditional morphological tools. Species identity can be readily determined using DNA sequence data from any of several mitochondrial and nuclear genes (SCHEFFER, 2000, SCHEFFER & LEWIS, 2001). Nuclear ribosomal DNA and mitochondrial DNA have been widely used for taxonomic and phylogenetic studies in insects (HWANG & KIM, 1999; ROEHRDANZ, 2003; MASETTI *et al.*, 2006), and the mitochondrial region encoding cytochrome oxidase subunits I and II (COI and II) are, by far, the most commonly used sequence for quarantine application (ROEHRDANZ, 2001; SCHEFFER *et al.*, 2001; BARCENAS *et al.*, 2005). Consequently, there is an increasing number of DNA sequences available from these regions that are useful for developing polymerase chain reaction (PCR) primers. The use of PCR, which results in the rapid production of multiple copies of a specific nucleotide sequence found in DNA (MULLIS, 1990), is a powerful tool in identification studies using species-specific primers. The PCR technique makes it possible to analyze nucleotide sequences in samples that contain amounts of DNA that are either too small or too degraded to permit other types of nucleic acid analyses (GLICK & PASTERNACK, 2003). This feature of PCR allowed the development of techniques that were previously extremely time-consuming or impossible to perform. In particular, the development of PCR-based assays has provided many alternative molecular methods for species diagnosis. Among them, PCR-restriction fragment-length polymorphism (PCR-RFLP) has been successfully applied in many cases to identify otherwise cryptic taxa (CATERINO *et al.,* 2000; MASETTI *et al.,* 2006). HIDAYAT *et al.* (1996) showed that some morphological features of weevil genitalia are related to molecular markers. DNA amplification fingerprinting and nuclear ribosomal DNA amplification were successfully used to distinguish between *S. oryzae* and *S. zeamais* (PENG *et al.,* 2003).

Early detection of hidden weevil infestation is critical to decide the best commercial destination of a cereal stock and to develop effective management programs. Physical and chemical methods commonly used to detect hidden insect infestations in cereals are rather subjective and slow;

– Received 26 November 2007 Accepted 5 May 2008 Published 1 December 2008

moreover, they require the use of highly skilled and oftentimes costly technicians. Immunological methods have also been developed but provided uncertain diagnoses of earlier developmental stages (QUINN *et al.*, 1992; SCHATZKI *et al.*, 1993; KITTO *et al.*, 1994; ROTUNDO *et al.*, 2000). Molecular techniques could represent a very powerful alternative also for detecting hidden infestation in cereals. The purpose of this study was to develop a simple and quick molecular approach based on PCR-RFLP to differentiate *S. granarius*, *S. oryzae*, and *S. zeamais* at immature stages and to evaluate its usefulness in detecting hidden infestation in wheat.

MATERIALS AND METHODS

INSECTS

Adults of *S. granarius*, *S. oryzae*, and *S. zeamais* were collected from a wild population of each species in traditional storage near Campobasso (central Italy) and reared on wheat kernels for two generations at 25 ± 2°C, 60 ± 5% R.H., and L12:D12 photoperiod in glass jars (diameter 15 x 15 cm) closed by a nylon mesh. Ten pairs of adults of each species were exposed to starch pieces (3 g) placed in a Petri dish (diameter 3 cm) for 5 days. The pieces were transferred to a 100 µm sieve and dissolved using distilled water. Eggs retained by the sieve were collected by a humidified camel hair paint brush (CRAVEDI & SCHETTINO, 2001). Larvae of different instars and pupae were collected from infested kernels and maintained at 4°C for 2 days. All insects were stored at -80°C until DNA extraction.

KERNEL SAMPLE PREPARATION

To exclude living insect infestations, whole wheat kernels were incubated in glass jars at 25 ± 2°C and 60 ± 5% R.H. for 2 months. The kernels were then washed with distilled water to remove possible insect fragments from the surface. Kernel samples (50 g) added with either one egg, or I-IV instar larva, or pupa, or adult of one of the three species, or of the three species together were ground in a standard household blender at low speed for 30 s. Uninfested kernel samples were used as controls. Each sample was replicated 3 times. From each ground sample, six different aliquots of 35 mg each were used for DNA extraction.

DNA EXTRACTION

The commercial DNeasy Tissue Kit (Qiagen, Milan, Italy) was used, according to manufacturer's instructions, to extract total genomic DNA from:

- one egg, or I-IV instar larva, or pupa, or adult of one of the three species;
- one egg, or I-IV instar larva, or pupa, or adult of each species;
- ground kernels infested with either one egg, or I-IV instar larva, or pupa, or adult of one of the three species;
- ground kernels infested with either one egg, or I-IV instar larva, or pupa, or adult of each species;
- uninfested ground kernels.

Except for adults, which were ground with liquid nitrogen in a mortar before the lysis phase, all the above-mentioned samples were directly placed in the lysis buffer.

PRIMER DESIGN

Two sets of primers were used for PCR amplification of a DNA region encoding the COII gene. Primers were designed, using the Primer3 program (ROZEN & SKALETSKY, 2000), on the sequences reported in the literature for *S. granarius* (M83970), *S. oryzae* (AY014880) and *S. zeamais* (AY014881) available in the GenBank database.

DNA AMPLIFICATION

The PCR mix contained 2 mM magnesium chloride, 200 µM of each dNTP, 0.4 µM of the primer pair A or B, 5 ng of template DNA and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in a final 25 µl volume. For both primer pairs, the thermal profile was 95°C for 10 min, 35 cycles of 94ºC for 45 s, 59ºC for 45 s and 72ºC for 45 s, with a final extension at 72°C for 10 min, using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA).

To simultaneously genotype the three species at each life stage, a multiplex PCR reaction using both A and B primer pairs in single test tubes was done using the above-mentioned conditions. PCR products were analyzed in 1% agarose gels in TAE buffer, containing 0.5 µg/ml ethidium bromide, and visualized under short-wave ultraviolet light.

PCR - RFLP

To identify a restriction enzyme that could discriminate between *S. oryzae* and *S. zeamais*, the sequences drawn from Genbank were aligned with the Multialin program (CORPET, 1988) and analysed by the REMAP program (RICE *et al.,* 2000). The *Bfa*I enzyme, whose recognition sequence was detected in *S. zeamais*, was selected for restriction. Following the recommendations of the enzyme supplier (Biolabs, Ipswich, MA), amplicons (5 µl) were digested in a total volume of 20 µl with 15 U of *Bfa*I endonuclease and left overnight at 37°C. The restriction product was visualized under UV light after electrophoresis on 2% agarose gels and staining with ethidium bromide.

RESULTS AND DISCUSSION

The kit used for DNA extraction produced enough high-quality nucleic acid from every sample. The DNA was used to set up a PCR method amplifying the COII, a gene from mitochondrial DNA chosen because of its highly conserved regions. For this gene *S. oryzae* and *S. zeamais* present a higher degree of homology (88%) than that between *S. granarius* and each of the other two species (82%). Consequently, areas of minimal difference were chosen to design PCR primers to amplify *S. oryzae* and *S. zeamais* DNA, whereas areas of maximal difference were used for *S. granarius*. Once the optimal PCR conditions were established, amplifications were always successful and consistently resulted in a single band of expected size: a PCR product of 462 bp for *S. granarius* and a fragment of 311 bp for both *S. oryzae* and *S. zeamais* (fig. I, lines 1, 2, 10, 11). The sequences of the three species were also amplified together using multiplex PCR in a single test tube, resulting in advantages in time and costs. To discriminate between the PCR products obtained from *S. oryzae* and *S. zeamais,* a PCR-RFLP protocol was developed. In fact, the *S. zeamais* sequence has a target site for *Bfa*I endonuclease, which is absent in the *S. oryzae* sequence. Two fragments (174 bp + 137 bp) are therefore produced through *Bfa*I restriction of *S. zeamais* amplicon, whereas the same treatment does not affect the *S. oryzae* amplicon (311 bp) (fig. II). The amplified sequence of

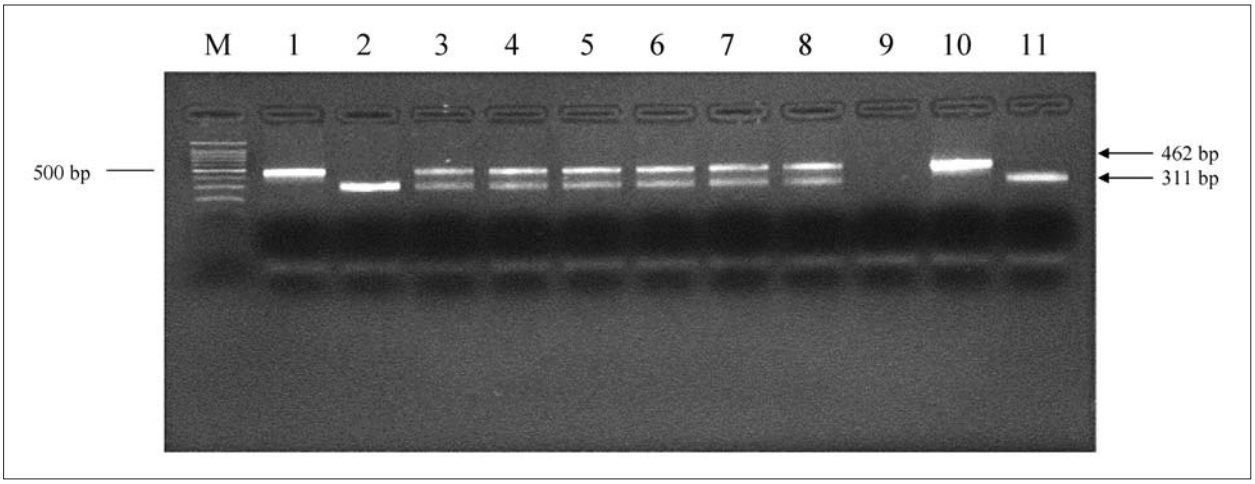

Fig. I – DNA amplicons of estimated length from a II instar larva of *S. granarius* (lanes 1/10, primer pair A) and *S. oryzae-S. zeamais* (lanes 2/11, primer pair B); Lanes 3-8: multiplex PCR (primer pairs A+B) of insect DNA purified from wheat kernels ground with one II instar larva of the three species (each lane refers to a 35 mg aliquot randomly taken from 50 g of ground kernels); Lane 9: negative control (uninfested kernels); M = 100 bp markers.

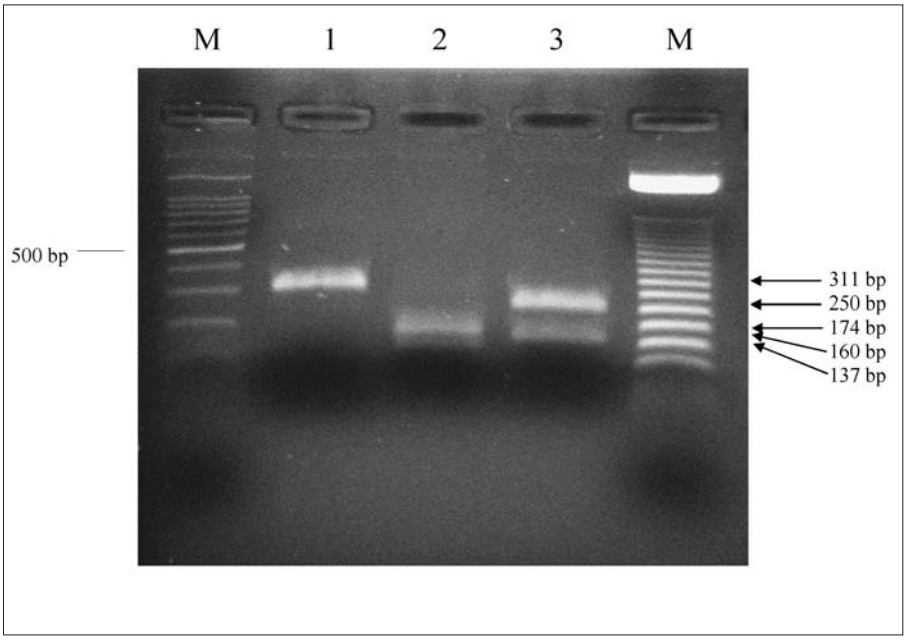

Fig. II – RFLP profiles of DNA from 35 mg aliquots randomly taken from 50 g of wheat kernels ground with one egg of *S. oryzae* (lane 1, 311 bp), or *S. zeamais* (lane 2, 174+137 bp), or *S. granarius* (lane 3, 250+160+52 bp; the latter is located under the bromophenol blue band); M=100 bp markers (right); 50 bp markers (left).

S. *granarius* contained two *Bfa*I motifs and, after digestion, three fragments were produced (250 bp + 160 bp + 52 bp) (fig. II). For all samples, the restriction pattern obtained was consistent with the predicted results. The presence or absence of the restriction sites allowed rapid identification of each of the three species when independently or simultaneously amplified at any life stage.

Using the established method, experiments have been performed to detect insect DNA in kernel samples added, before grinding, of one egg, or I-IV instar larva, or pupa, or adult of one of the three species or of the three species together. fig. I (lines 3-8) shows insect DNA amplification in 35 mg aliquots of a wheat kernel sample (50 g) ground with one II instar larva each of *S. granarius*, S. *oryzae*, and *S. zeamais*. Fig. II reports the differentiation of the three species when one egg of each was added to a wheat kernel sample. On the whole, PCR-RFLP was able to detect and identify insect DNA in minute aliquots from 50 g of wheat kernels containing single life stages of the three *Sitophilus* species.

The developed PCR-based methodology is promising for routine identification of hidden insect infestation in cereal grains, although insects from different geographical regions should be tested to establish the broad applicability of the PCR assay. However, the success of primer sets drawn from DNA sequences of Chinese populations and applied to the populations of central Italy is promising, indeed.

This molecular method offers several advantages, such as relatively low cost and high reproducibility, specificity and reliability; moreover, it does not require specialized skills or sophisticated molecular biology equipment. Further studies will be aimed at setting up a quantitative PCR methodology to quantify the level of infestation.

RIASSUNTO

DIFFERENZIAZIONE DI SITOPHILUS *SPP. (COLEOPTERA CURCULIONIDAE) ED INDIVIDUAZIONE DEGLI STADI GIOVANILI IN FRUMENTO MEDIANTE ANALISI MOLECOLARE*

In questo lavoro viene proposto un metodo basato sulla reazione a catena della polimerasi (PCR) al fine di differenziare *Sitophilus granarius* (L.), *S. oryzae* (L.) e *S. zeamais* Motschulsky nei diversi stadi di sviluppo e per rilevare la presenza di infestazioni latenti in cariossidi di frumento. L'amplificazione del DNA genomico, seguita da elettroforesi su gel d'agarosio, è stata effettuata usando primers disegnati sulla base della sequenza del gene per la citocromo ossidasi, subunità II (COII) ed ha permesso di evidenziare sia gli stadi giovanili (uovo, larva, pupa) che gli adulti di ciascuna specie. Il prodotto PCR derivante dall'amplificazione del DNA è risultato delle dimensioni di 462 bp per *S. granarius* mentre da *S. oryzae* e *S. zeamais* si è ottenuto un frammento di 311 bp. Un confronto della sequenza nucleotidica delle ultime due specie ha consentito di identificare la presenza di un polimorfismo che è stato usato per differenziarle, tramite la messa a punto di un protocollo PCR-RFLP. La digestione del DNA ha prodotto un frammento non tagliato di 311 bp per *S. oryzae,* due frammenti rispettivamente di 174 bp e 137 bp per *S. zeamais* e tre bande di 250 bp, 160 bp e 52 bp per *S. granarius.* L'uso di una reazione di PCR multipla ha consentito di differenziare le tre specie ad ogni stadio di sviluppo anche quando singoli individui di ciascuna delle tre specie sono stati aggiunti contemporaneamente ad un campione di 50 g di cariossidi di frumento. La riproducibilità dei risultati, l'alta risoluzione e la semplicità di esecuzione del metodo molecolare messo a punto suggeriscono una sua possibile utilizzazione pratica per differenziare gli stadi giovanili delle tre specie di *Sitophilus* e per rilevarne le infestazioni latenti nel frumento.

REFERENCES

BARCENAS N.M., UNRUH T.R., NEVEN L.G., 2005 – *DNA Diagnostics to Identify Internal Feeders (Lepidoptera: Tortricidae) of Pome Fruits of Quarantine Importance.* – J. Econ. Entomol., 98: 299-306.

CATERINO M.S., CHO S., SPERLING F.A.H., 2000 – *The current state of insect molecular systematics: a thriving tower of Babel.* - Annu. Rev. Entomol., 45: 1-54.

CORPET F., 1988 – *Multiple sequence alignment with hierarchical clustering.* - Nucl. Acid Res. 16: 10881-10890.

CRAVEDI P., SCHETTINO M., 2001 – *Temperature letali per insetti della pasta.* In: Atti 5° Congresso Italiano di Scienza e Tecnologia degli Alimenti, Porretta S. Ed., Chiriotti Editori, Pinerolo, Italia, pp. 43-50.

GLICK B.R., PASTERNAK J.J., 2003 – *Molecular biotechnology: principles and applications of recombinant DNA.* - ASM Press, Washington, D.C., XIX + 760 pp.

HIDAYAT P., PHILLIPS T.W., FFRENCH-CONSTANT R.H., 1996 – *Molecular and morphological characters discriminate* S. oryzae *and* S. zeamais *(Coleoptera: Curculionidae) and confirm reproductive isolation.* - Ann. Entomol. Soc. Am., 89: 645-652.

HWANG U.-W., KIM W., 1999 – *General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics.* - Kor. J. Parasitol., 37: 215-228.

KITTO G.B., QUINN F.A., BURKHOLDER W., 1994 – *Development of immunoassays for quantitative detection of insects in stored products.* In: Proceedings of the 6th International Working Conference on Stored-product Protection, Volume 1, Highley E., Wright E.J., Banks H.J. & Champ B.R. Ed., CAB International, Wallingford, Oxon, pp. 415-420.

MASETTI A., LUCHETTI A., MANTOVANI B., BURGIO G., 2006 – *Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Assays to Distinguish* Liriomyza huidobrensis *(Diptera: Agromyzidae) from Associated Species on Lettuce Cropping Systems in Italy.* - J. Econ. Entomol., 99: 1268-1272.

MULLIS K.B., 1990 – *The unusual origin of the polymerase chain reaction.* - Sci Am., 262: 56-65.

PENG W.K., LIN H.C., CHEN C.N., WANG C.H., 2003 – *DNA identification of two laboratory colonies of the weevil,* Sitophilus oryzae *(L.) and* S. zeamais *Motschulsky (Coleoptera: Curculionidae) in Taiwan.* - J. Stored Prod. Res., 39: 225-235.

QUINN F.A., BURKHOLDER W., KITTO G.B., 1992 – *Immunological technique for measuring insect contamination of grain.* - J. Econ. Entomol., 85: 1463-1470.

RICE P., LONGDEN I., BLEASBY A., 2000 – *«EMBOSS: The European Molecular Biology Open Software Suite».* - Trends Genet., 16: 276-277.

ROEHRDANZ R. L., 2001 – *Genetic Differentiation of Southeastern Boll Weevil and Thurberia Weevil Populations of* Anthonomus grandis *(Coleoptera: Curculionidae) Using Mitochondrial DNA.* - Ann. Entomol. Soc. Am., 94: 928-935.

ROEHRDANZ R. L., 2003 – *Multiplex Polymerase Chain Reaction Method for Differentiating Western and Northern Corn Rootworm Larvae (Coleoptera: Chrysomelidae).* - J. Econ. Entomol., 96: 669- 672.

ROTUNDO G., GERMINARA G.S., DE CRISTOFARO A., 2000 – *Immuno-osmophoretic technique for detecting* Sitophilus granarius *(L.) infestation in wheat.* - J. Stored Prod. Res., 36: 153-160.

ROZEN S., SKALETSKY H.J., 2000 – *Primer3 on the WWW for general users and for biologist programmers.* In: Bioinformatics Methods and Protocols: Methods in Molecular Biology, Krawetz S. & Misener S. Ed., Humana Press, Totowa, NJ., pp. 365-386.

SCHATZKI T.F., WILSON E.K., KITTO G.B., BEHRENS P., HELLER I., 1993 – *Determination of hidden* Sitophilus granarius *(Coleoptera: Curculionidae) in wheat by miosin ELISA.* - J. Econ. Entomol., 86: 1584-1589.

SCHEFFER S.J., 2000 – *Molecular evidence of cryptic species within* Liriomyza huidobrensis *(Diptera: Agromyzidae).* - J. Econ. Entomol., 93: 1146-1151.

SCHEFFER S.J., LEWIS M.L., 2001 – *Two nuclear genes confirm mitochondrial evidence of cryptic species within* Liriomyza huidobrensis *(Diptera: Agromyzidae).* - Ann. Entomol. Soc. Am., 94: 648-653.

SCHEFFER S.J., WIJESEKARA A., VISSER D., HALLETT R.H., 2001 – *Polymerase Chain Reaction-Restriction Fragment-Length Polymorphism Method to Distinguish* Liriomyza huidobrensis *from* L. langei *(Diptera: Agromyzidae) Applied to Three Recent Leafminer Invasions.* - J. Econ. Entomol., 94: 1177-1182.