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Genetic differentiation at the inter- and intra-specific level of stored grain insects using a simple molecular approach (RAPD)

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Abstract

Population diversity in stored grain insects linked with geographical origin of the infesting strains may be an issue in international trade. It may be that some imported strains exhibit phenotypes that may negatively impact control strategies available in the country at the destination of imported grain lots. However, several molecular methods are routinely used to assess genetic diversity among insect species or strains in the same species. Therefore, the potential of RAPD technique for the identification of different strains based on polymorphic DNA revealing was investigated with grain insect species. The banding patterns observed from DNA extracts with six different primers were compared between several grain insect species: *Sitophilus* spp., *Oryzaephilus surinamensis, Rhyzopertha dominica, Tribolium castaneum, Ephestia kuehniella, Plodia interpunctella,* and three bruchid beetles. Then a particular study with the same molecular tools was performed for the differentiation between geographical strains in the *Sitophilus* spp. complex, with a special attention paid to *S. zeamais* strains originating from different continents (Africa, South America and Europe). The repeatability of the banding patterns was tested on: i/ extracted DNA from pool samples of different stages and generations in the same strains; ii/ Extracted DNA from individuals of the same species and strain. The differences observed in band profiles of the different species were highly significant and enabled the easy differentiation between them. It was shown frequent dissimilarities between the band profiles of geographical strains of *S. zeamais*. The potential for RAPD-PCR technique to provide useful genetic data for the discrimination up to the population level of insects found in stored products in international trade is discussed.

Key words: RAPD-PCR, genetic differentiation, grain insect pest, geographical strain, molecular technique.

Introduction

The investigation about genetic differences among populations of stored grain insect species may be used as discriminating criteria for the determination of the geographical origin, the distribution, and the migrations of these populations at the regional, national or international level (Fields and Phillips, 2002). The genetic differences among the populations of a single species are the result of the genetic variation of the founder population in response to the selection pressure resulting of novel conditions for life and reproduction encountered after migration to new environments. The difficulty to observe polymorphic DNA among species and populations of stored grain insect is mainly related to the lack of information about DNA of the major noxious species. Only the most studied species, i.e.

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Curculionidae, Bostrichidae, and Bruchidae, have some DNA sequences available in DNA databases. This issue may be overcome using the polymerase chain reaction-based randomly amplified polymorphic DNA (RAPD-PCR) which does not require previous knowledge of a DNA sequence to design primers (Williams et al., 1990; 1993). This technique has generated useful results in the differentiation between insect species and strains (Hoy, 2003). This method has a great potential for the identification of a large number of DNA polymorphisms in the genome quickly and efficiently (Haymer and McInnis, 1994). The PCR-RAPD fingerprints may be used for determining at what point in the marketing channel a commodity became infested (Dowdy and McGaughey, 1996). They may be also useful to differentiate between species with very close morphological characters e.g. at the larval stage (Hidayat et al., 1996) as well as between closely related strains of some species (Welsh et al., 1990). Despite all these advantages important questions have been raised for a practical application. One of the most important is the reproducibility of the results obtained with the same primers across a variety of protocols and laboratory conditions and the level of polymorphism that is searched (inter-specific, intra-specific, between geographical strains, etc.). Because the most frequent cause of poor quality RAPDs is poor quality DNA, it is important to purify DNA with methods that will yield pure and undegraded DNA. With the ready-to-use kits available for genomic cells and tissue DNA extraction followed by ready-to-go RAPD analysis systems the quality and reproducibility of PCR-RAPD analysis may be greatly improved. However, the reproducibility in the banding pattern discriminating among insect species or strains, when studied with several individuals in a pool or with a single individual has not been thoroughly investigated with these kits.

In the current study, we used commercial kits to determine the polymorphism among species, and geographical strains of the same species, on a pool of several individuals or on the genomic DNA extracted from a single individual. Our first goal was the choice of the more adequate primers for the differentiation between species and populations of weevil from different geographical origin. The second goal was the study of the variability factors that can affect the banding patterns obtained with different species or the different geographical populations of a single species. Our study aimed to standardize the RAPD-PCR technique in view of the identification of the origin of the infestation of stored products through the similarities with reference banding patterns of geographical strains.

Materials and methods

Insect materials

The different insect species used in this study were sampled in the collection of the INRA MycSA laboratory. In a first experiment, the RAPD-PCR was performed to differentiate genetic material from species with the highest occurrence in cereal grain and by products: the three grain weevils, *Sitophilus granarius* (L.), *S. oryzae* (L.) and *S. zeamais* Motschulsky; the three bruchid beetles, *Acanthoscelides obtectus* (Say), *Bruchus lentis* (L.) and *Callosobruchus maculatus* (F.); the red flour beetle, *Tribolium castaneum* (Herbst), the sowtoothed grain beetle, *Oryzaephilus surinamensis* (L.); the lesser grain borer beetle, *Rhyzopertha dominica* (F.); the Mediterranean flour moth, *Ephestia kuehniella* (Zeller); the Indian meal moth, *Plodia interpunctella* (Huebner). These species are reared in laboratory conditions for more than hundred generations after their sampling from infested products. For the grain weevils, geographical strains were also introduced in the comparison: two *S. granarius* strains referenced Sgr1 and Sgr08; three *S. oryzae* strains referenced Sor1, Sor10, Sor11; and three *S. zeamais* strains referenced Sze11, Sze12, Sze16.

In a second experiment, the differentiation potential of the RAPD-PCR kits was applied to different geographical strains of the maize weevil *S. zeamais*. Three geographical populations originating from Argentina, Ivory coast, and France have been used. These geographical

strains have been reared for more than 15 years in the laboratory on the same maize grain variety, i.e. more than hundred generations.

Storing insects for DNA preservation

The technique of storage in absolute ethanol (EtOH) was applied in all circumstances (Quicke et al., 1999). The insects were dehydrated in absolute EtOH in cryotubes. Then, EtOH was removed and replaced with fresh to reduce the dilution with endogenous water (Hoy, 2003). Prior to the use for PCR, the tubes were sealed to prevent the evaporation of EtOH and the dehydrated insects were stored in a refrigerator at 4 °C. This technique is safe for the preservation of insect DNA for more than two years at least (Quicke et al., 1999).

Insect DNA isolation (extraction)

In the first experiment on different species of beetles, the DNA extraction was carried out on a pool of five individuals. For the Lepidoptera species, a single larva was used. For the second study of the variability factors a part of the RAPD-PCR analyses were carried out on DNA extracted from a single individual. In this particular case, five replicates were compared to assess the stability level of the RAPD profiles on DNA extracted from different individuals. For DNA extracted on a group of five individuals, the RAPD analysis was carried out on five DNA samples from the same extract. Prior to DNA extraction, insects were removed from EtOH and leave to dry on sterile absorbent paper for. Then, the protocol of DNeasy® kit (Qiagen S.A., Courtaboeuf, France) was used. In brief, the insects were introduced in a 1.5 mL Eppendorfä tube in which 180 µL PBS 1x solution were added. Insect sample was homogenized using an electric homogenizer with a disposable microtube-pestle. Then, 4 µL R-Nase A(100 mg mL^{-1}) were added and the tubes were incubated at room temperature during 2 min. To the R-Nase A treated lysate, 20 µL of protein precipitation solution (proteinase K 20 mg mL^{-1}) and 200 µL buffer AL were added followed by a thorough vortexing. The tubes were then incubated in a water bath at 70 °C for 10 min.

Then, 200 µL EtOH (96 to 100 %) were added and tubes were thoroughly mixed by vortexing. The mixture was transferred into a mini spin column placed in a 2 mL collection tube. The tubes were centrifuged 1 min at 6,000 g. The mini spin column was placed in a new 2 mL collection tube and 500 µL washing buffer 1 were added before a centrifugation for 1 min at 6,000 g. Then, the mini spin column was placed in a new 2 mL collection tube. 500 µL washing buffer 2 were added and the tube was centrifuged for 3 min at 20,000 g to dry the membrane.

For the DNA elution step, after the mini spin column was transferred with caution in a 1.5 mL new tube, 200 µL eluting buffer were added directly onto the membrane before an incubation at room temperature for 1 min followed by a centrifugation for 1 min at 6,000 g. This elution step was repeated once in the same conditions. The tubes with re-hydrated DNA were stored at 4 °C in a refrigerator before PCR.

RAPD-PCR primers

The choice of discriminating primers was done among the six primers included in the "Ready-To-Go RAPD analysis kit®" from Amersham Biosciences (GE Healthcare Europe GmbH, Orsay, France). Each kit contains 2.5 mmol of each of the following primers:

P1 GGTGCGGGAA
P2 GTTTCGCTCC
P3 GTAGACCCGT
P4 AAGAGCCCGT
P5 AACGCGCAAC
P6 CCCGTCAGCA

After the first experiment with the six primers, only P3 and P5 were used in the other experiments. This choice was based on favourable characteristics of the banding patterns observed: a limited number of major bands for RAPD analysis with these two primers and a high potential of differentiation observed up to the strain level with the species for which several populations were compared.

PCR protocol

In each tube of RAPD analysis with a micro-bead the following products were included: 5 µL of a single RAPD primer (25 pmol); 2 µL of insect DNA extract (yield of the extraction assessed to 5 to 50 ng per mg of insect body weight); bi-distilled water added to total of 25 µL. Two different *Escherichia coli* DNAs were used as standard DNA "extract" control for each RAPD analysis run (in each electrophoresis gel). The tube content was gently mixed by vortexing or by repeatedly pipeting up and down. A brief centrifugation is needed to seat the mix in the bottom of the tube. The samples were placed in the thermal cycler using the following PCR conditions: one cycle 95 °C, 5 min; 45 cycles 95 °C, 1 min; one cycle 36 °C, 1 min; one cycle 72 °C, 2 min.

Gel analysis

The electrophoresis revealing the banding pattern of the RAPD-PCR amplified DNA was carried out on a 2 % agarose gel using 1x TBE buffer containing 0.5 µg mL^{-1} ethidium dibromide. In each well of the gel, 5 µL of PCR amplified product and 1 µL of 6x tracking buffer were loaded. The electrophorese is performed until good separation of RAPD bands and the setting depended of the capacity of the electrophoresis system (e.g. 150 V for 3 h).

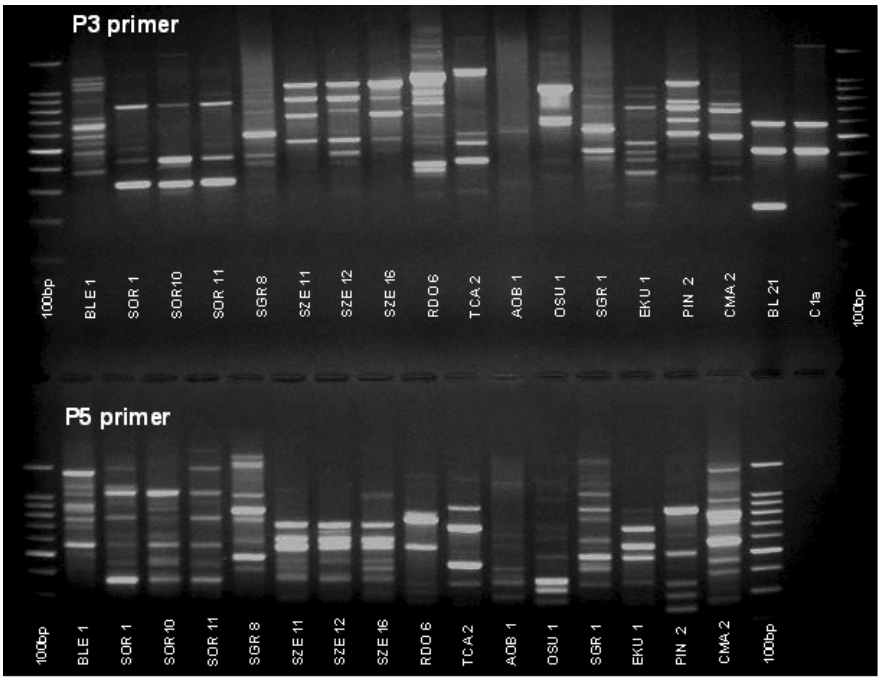
Variability factors investigated from banding pattern interpretation

The similarities from one generation to another in the same strain were studied on the different geographical populations of *S. zeamais*. Additionally, the differences related to the sex were revealed through the comparison of bands obtained with the two parents issued from the F1 progeny (considered as F2 generation) and that were used to obtain inbreed lines for F3 generation. The variability in banding pattern with these F3 inbreed lines was observed at the individual level. The RAPD-PCR analysis was carried out on DNA extracted from different stages of *S. zeamais* of the same four "continental" strains (South-American, African and the two European populations from Portugal and France) used in all species comparison. Thus, the similarity of RAPD-PCR profiles from DNA extracted from individual larva, pupa and adult sampled in the same generation of *S. zeamais* strain ref.11 was checked. Finally, the stability of the banding patterns observed on successive generations of different *S. zeamais* strains was studied as well as the polymorphism related to the sex from individual DNA extract.

Results

Differentiation between species

On the RAPD products observed in the 11 different species which were studied none was identical to another (Figure 1). Each species has its own profile that is specific and allows a clear discrimination with another species (Table 1). For the three species of the Bruchidae Family, *Bruchus lentis*, *Acanthoscelides obtectus* and *Callosobruchus maculatus*, only one RAPD-PCR amplified products with P3 primer is common to the three species (approximately 680 bp). All other fragments amplified either with P3 or P5 primers are differently distributed (Table 1). For the geographical strains of the three weevil species (*S. oryzae*, *S. granarius*, and *S. zeamais*), the specific combination of the amplified fragments for each strain enabled the differentiation between populations of *S. zeamais* originating from different continents. For *S. oryzae* and *S. granarius* strains originating from different geographical locations in France, the profiles were very similar either with P3 or P5 primer (Figure 1 and Table 1). In this last situation, the differentiation between the origin of "domestic" populations originating from different areas of the same country seems more difficult, even if some dissimilarities were detected.



Legend of species: Ble: *Bruchus lentis*; Sor: *Sitophilus oryzae*; Sgr: *S. granarius*; Sze: *S. zeamais*; Rdo: *Rhyzopertha dominica*; Tca: *Tribolium castaneum*; Aob: *Acanthoscelides obtectus*; Osu: *Oryzaephilus surinamensis*; Eku: *Ephestia kuehniella*; Pin: *Plodia interpunctella*; Cma: *Callosobruchus maculatus* (the species initials are followed by the reference of the studied strain). *E. coli* controls: strains BL21 and C1a

Figure 1. RAPD-PCR profiles from the amplification with arbitrary P3 primer (upper profiles) and P5 primer (lower profiles) of fragments of genomic DNA of different species or strains of certain species of stored-product insects.

Differentiation between geographical populations of *S. zeamais*

The profiles observed with P3 primer applied to the 8 geographical populations of *S. zeamais* are discriminative for almost all the eight populations (Figure 2, left part). However, with P5 primer, the profiles of the three strains: Sze 10, Sze 11 and Sze 12 are very similar (Figure 2, right part). If the reproducibility of these similarities is confirmed, they should be related to the genetic proximity of these three populations.

Reproducibility of the differentiation between geographical populations

The study of the differences in the RAPD mediated profiles of the four geographical strains of *S. zeamais* (originating from Argentina, Ivory Coast, Portugal and France) with P3 primer confirms the possibility of an easy discrimination between these strains (Figure 3). Especially the profile of the Portugese strain that was collected in a pasta factory in 2004 appeared very different of the profiles of the two other strains which were reared in consanguinity for more than 15 years for their sampling as a wild strain. The difference is still accentuated with the profiles obtained with P5 primer (Figure 4).

Variability of profiles from individual DNA extraction on different stages of *S. zeamais*

With single specimen DNA extracts, the comparison of the profiles obtained with larva, pupa and adult of *S. zeamais*, strain 11, showed very similar patterns (Figure 5). However, if all the band are more or less visible in all the profiles on any stage, the level amplification of some bands is not uniform (see L3 replicate 2 and 3). Additionally, as expected, the profiles of adult male and female seems different (see Ad replicate 3).

Table 1. Approximate size of the major bands of RAPD-PCR profiles observed with P3 and P5 arbitrary primers for the different species and strains of stored product insect pests. [e.g.: S.or = *Sitophilus oryzae*; st… = laboratory strain reference; species of the same family or strains of the same species are presented in contiguous columns: *S. oryzae*, *S. granarius*, *S. zeamais* and species of the *Bruchidae* family]. For the different species names, see in text. (unit: number of base-pairs).

Species	S.or			S.gr		S.ze			R.do	T.ca	0.su	E.ku	P.in	B.le	A.ob	C.ma
	st1	st10	st11	st08	st1	st11	st12	st16	st6	st2	st1	st1	st2	sty	st1	st2
P3 pr.	820	820	-	1,200	1,200	1,050	1,050	1,050	1,200	1,250	925	750	980	1,220	-	-
	750	-	-	750	750	880	880	880	920	560	650	490	800	1,150	-	-
	620	-	-	600	600	650	-	650	800	500	620	425	730	1,050	-	1,050
	440	440	440	-	-	550	550	550	380	410	450	390	620	-	-	880
	320	320	320	-	-	-	480	480	-	-	400	325	540	-	800	800
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	750
	-	-	-	-	-	-	-	-	-	-	-	-	-	680	680	680
	-	-	-	-	-	-	-	-	-	-	-	-	-	600	600	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	510
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	420	420
	-	-	-	-	-	-	-	-	-	-	-	-	-	390	-	390
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	300	300
P5 pr.	-	-	1,420	1,600	1,600	1,080	-	-	810	1,250	800	670	825	1,400	-	1,400
	1,200	-	-	1,500	1,500	-	-	1,000	780	840	350	540	780	1,200	-	-
	1,050	1,050	1,050	-	1,150	750	750	750	540	730	310	460	490	1,100	-	-
	760	760	760	1,000	1,000	-	630	-	-	420	270	350	340	900	-	900
	570	570	570	810	810	600	600	600	-	330	-	-	290	-	-	800
	360	360	360	760	760	550	550	550	-	-	-	-	250	750	-	750
	-	-	-	-	600	460	-	460	-	-	-	-	-	-	700	-
	-	-	-	-	550	380	380	380	-	-	-	-	-	-	600	-
	-	-	-	470	470	-	300	300	-	-	-	-	-	-	580	-
	-	-	-	-	400	-	-	-	-	-	-	-	-	560	-	560
	-	-	-	-	340	-	-	-	-	-	-	-	-	-	450	450
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	400
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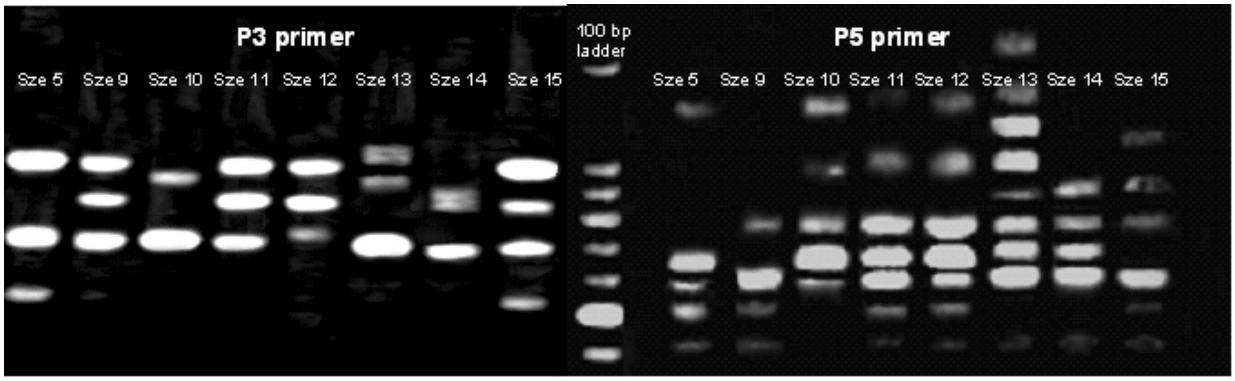

Figure 2. Agarose gel showing RAPD products polymorphism in eight geographical strains of *S. zeamais* sampled in different geographical locations of the three continents: America, Africa and Europe (different numbers following the species initials correspond to different geographical origins). The primer P3 products are on the left and P5 on the right; the 100 base pairs ladder is common.

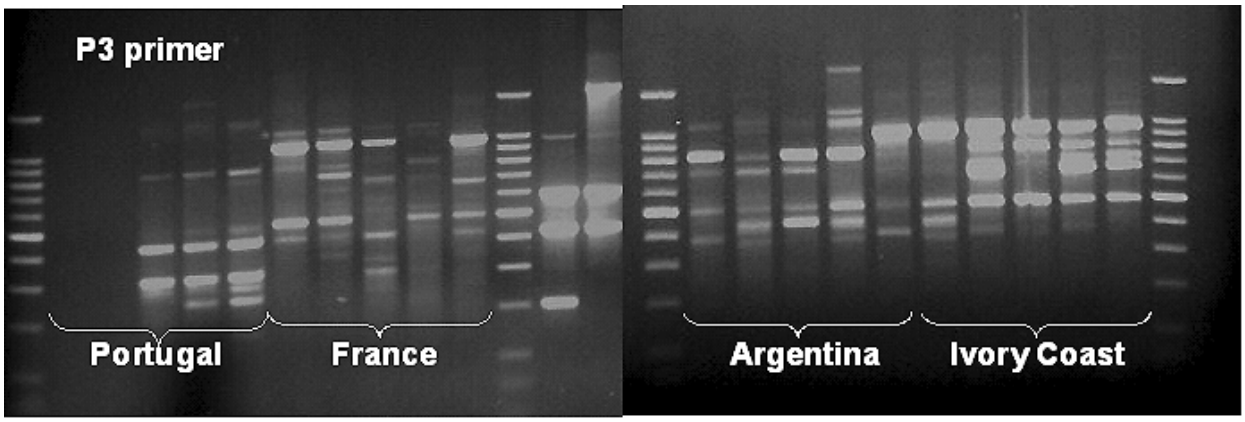

Figure 3. Agarose gel showing RAPD products obtained with arbitrary primer P3 in PCR applied to four geographical populations of *S. zeamais* collected in Argentina, Ivory Coast, France, and Portugal (the last one more recently than the three others). Each reaction is repeated 5 times (the two first profiles of the Portugese strain are lacking).

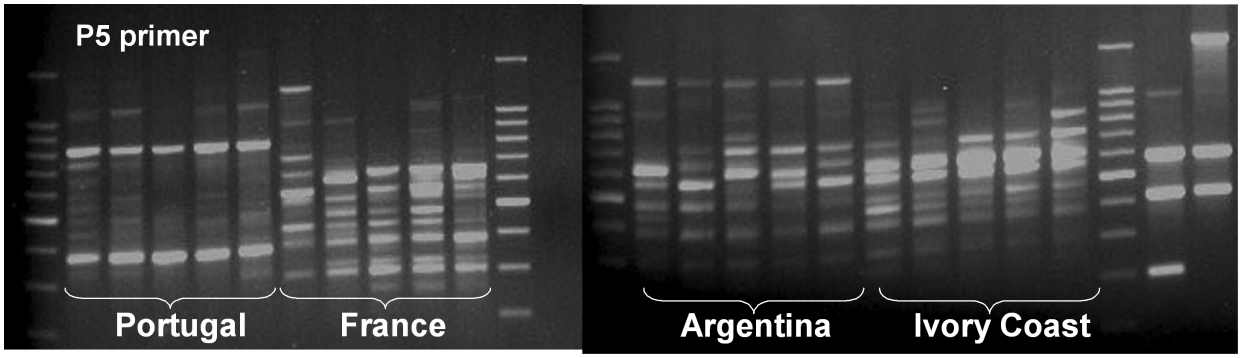

Figure 4. Agarose gel showing RAPD products obtained with arbitrary primer P5 in PCR applied to four geographical populations of *S. zeamais* collected in Argentina, Ivory Coast, France, and Portugal (the last one more recently than the three others). Each reaction is repeated 5 times on the same DNA extract.

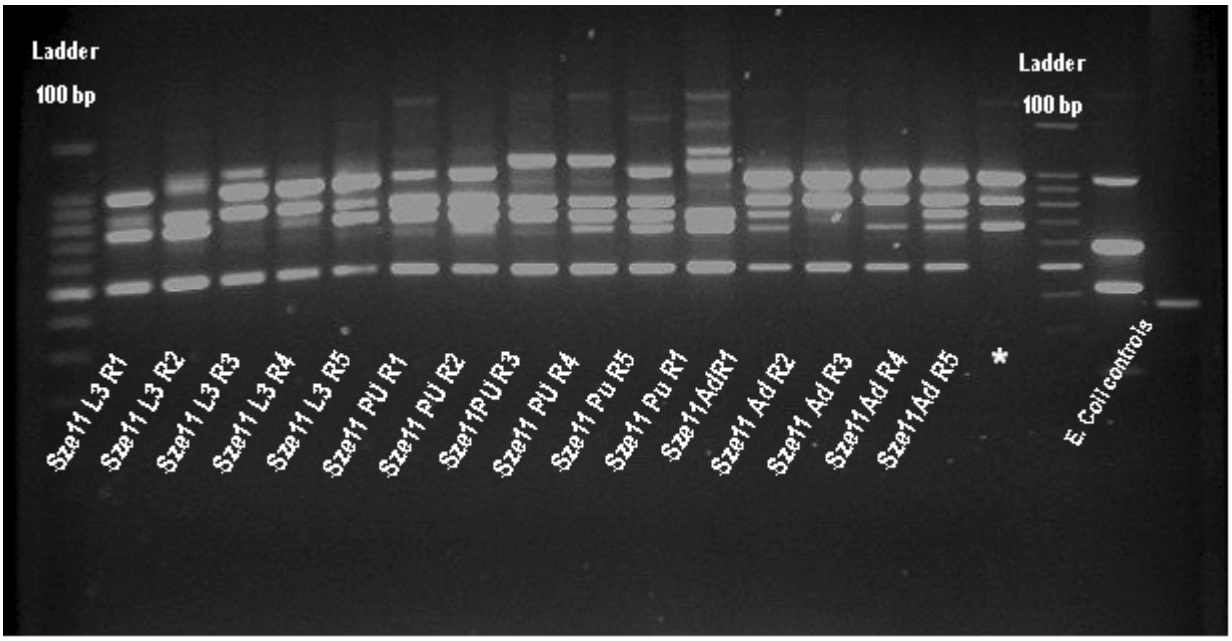

Figure 5. Agarose gel showing the reproducibility of the RAPD products obtained on the same strain of *S. zeamais* on extracts from three different development stages: larva (L3), pupa (Pu), and adult (Ad). Each reaction is repeated (Ri) on 5 different DNA extracts from a single individual.

Variability from one generation to another

From the banding patterns in RAPD products observed with different generations of three different geographical strains ("continental strains"), it was observed that the specific profiles allowing the differentiation between these geographical strains were stable along the generations, when DNA was extracted from 5 individual (generations G0, G1 and G3) (Figure 6). However, when DNA was extracted on a single individual, as it is the case with the parents (male and female) of the G3 generation (referenced G2 male or female on Figure 6), the banding pattern showed some different bands between the two sexes.

Discussion

It was showed that RAPD-PCR may detect small differences in the genomes of stored product insects (Coleoptera or Lepidoptera), either at the species or the intra species level, such as between geographical populations of a cosmopolitan species. It was shown that this "fingerprinting" can be easily carried out on individual insects and that the banding profiles were little dependant of the development stage. Moreover, this technique has already been recommended for the differentiation between the two species of the grain weevils (*S. oryzae* and *S. zeamais*) at the larval stage when morphological criteria are lacking (Hidayat et al., 1996). However, the male and female have not exactly the same pattern with this technique and this difference become visible during the pupal stage. This technique seems especially useful for the discrimination between biotypes among a species since RAPD-PCR pattern are correlated with the evolutionary adaptation of sub-populations when these are introduced in new environments or in stressing physical conditions (Espinasa and Borowsky, 1998).

Further investigation will be started to test the correspondence between these differences and the geographical origin, at least at the continental level. The next step will be the constitution of a collection of a strain database of DNA fingerprints allowing the comparison of genetic proximity in

geographical strains for the major insect pest species of grain entering the international market channels. The identification of genetic variability in pest populations found in food products may be especially important in international trade by providing an accurate information about the origin of the insect populations. These results are encouraging the conception of derived detection tools allowing the identification of the geographical origin of the strains of insect pests discovered in stored food products trade, especially within cereal grain shipments and the marketing channels of processed cereal foods. Thus, the examination would not only protect from an undesirable introduction of pest insects but also would indicate the potential of noxiousness related to the identified geographical origin of the invasive species or strain.

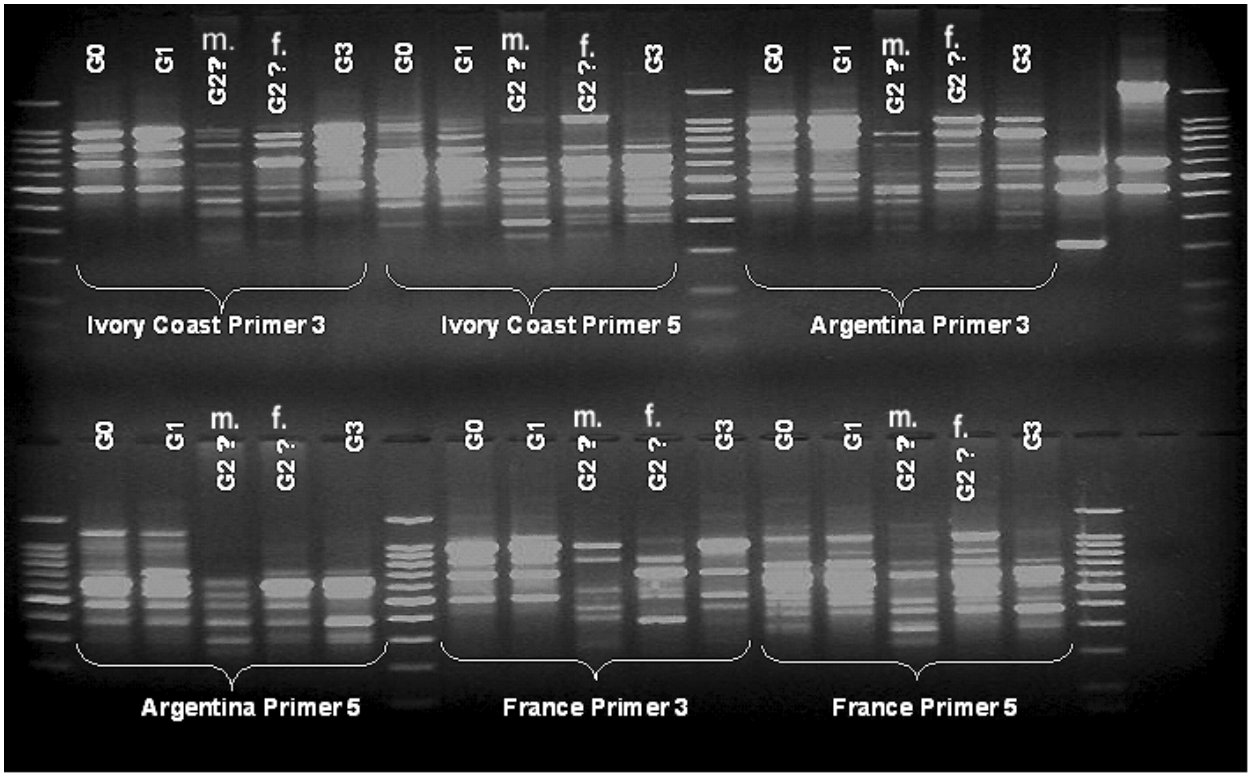

Figure 6. Variation of agarose gel profiles of RAPD products obtained with the two primers P3 and P5 from different generations (referenced G0, G1, and G3) of three geographical populations of *S. zeamais* (DNA extracted from 5 individuals). Additionally, the parents of the G3 (reference G2 male or female) are compared with profiles carried out on individual genomic DNA extract.

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