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Structure of worldwide populations of *Lasioderma serricorne* (Coleoptera: Anobiidae) as revealed by amplified fragment length polymorphism profiles

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Abstract

The cigarette beetle *Lasioderma serricorne* (Fabricius) is the most widespread and destructive pest of stored tobacco. The capability to differentiate between populations from different geographic origins would enable researchers to better understand how insect dispersal through transportation affects the infestation of stored tobacco. Using amplified fragment length polymorphism (AFLP), DNA polymorphisms were assessed in 16 populations of *L. serricorne* collected from 15 countries. The dendrograms constructed from profile distance matrices revealed well-supported colony clusters. There was no clear clustering as a function of the geographic origin of the samples. The results suggest extensive insect dispersal among geographical regions due to movement of infested commodities worldwide. This first AFLP population study of a stored-product insect demonstrates the potential of AFLP for distinguishing *L. serricorne* populations.

Keywords: AFLP, stored-product insects, *Lasioderma serricorne*, population structure

Introduction

The cigarette beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae) is the most widespread and destructive pest of stored tobacco (Ashworth, 1993; Ryan, 1995). Damage to tobacco is caused by the insect larvae that eat stored leaves and contaminate the product with excreta and body oils. Infestations may occur during on-farm storage, tobacco shipments, in the warehouses, factories or the retail chain (Ryan, 1995). Besides tobacco, *L. serricorne* also infests a wide range of other stored commodities such as grains, rice, pasta and beans (Howe, 1957; Ryan, 1995). Therefore, this insect is of considerable economic importance.

Lasioderma serricorne is a cosmopolitan stored product pest and identifying the origins of infestations in stored products is difficult, owing to the complexity of supply chains and the number of points where cross-infestations between commodities can occur. Therefore, population movements of stored-product insects are poorly documented. Actually, infestations in the tobacco supply chain may have already started in the field, as suggested by Carvalho *et al*. (2000).

Since morphological features of insects are often poorly distinctive, molecular biology techniques have proved to be a valuable tool for the characterization of such organisms (Loxdale, 2001). These techniques may help track the dispersal routes of stored-product insect pests in agricultural commodities and determine at what point the product becomes infested (Dowdy & McGaughey, 1996).

In recent years, DNA fingerprinting techniques have been increasingly used for the identification of insect populations and species (for a review, see Loxdale & Lushai, 1998). Numerous recent papers have focused on the genetic variation of insect populations in relation to their geographical origin (Reineke *et al*., 1999; Cervera *et al*., 2000; Katiyar *et al*., 2000). Although there is considerable literature on the genetic structure of model insects such as the fruit

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Table 1. Origin of *Lasioderma serricorne* colonies studied.

Colony	Country of collection	Year collected	Reared at[1]
L3N	India	1999	Our facilities
L4M	Switzerland	2000	Our facilities
CSL10	Australia (NSW)	1995	CSL
CSL12	Canada	1996	CSL
CSL30	USA	1996	CSL
CSL32	France	1996	CSL
CSL34	Japan	1996	CSL
CSL36	Colombia	1996	CSL
CSL49	Brazil	1997	CSL
CSL52	Panama	1997	CSL
CSL58	Honduras	1997	CSL
CSL59	Guatemala	1997	CSL
CSL65	Italy	1997	CSL
CSL70	Philippines	1997	CSL
CSL73	India	1997	CSL
CSL79	Zimbabwe	1998	CSL

[1] CSL: Central Science Laboratory, Department for Environment, Food and Rural Affairs (DEFRA), Sand Hutton, York, UK; our facilities: Philip Morris International Research and Development, Neuchâtel, Switzerland.

fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), very little information is available on the genetic make-up of *L. serricorne* (Smith & Brower, 1974). The cigarette beetle is a small insect (adults are 2.0–3.7 mm long and weigh 0.5–4.4 mg) from which only nanograms of genomic DNA are recoverable. Considering the lack of published information on the *L. serricorne* genome, we selected a widely-used DNA fingerprinting technique, which does not require previous DNA sequence knowledge: the amplified fragment length polymorphism (AFLP[1]) approach (Vos *et al*., 1995). Typically, the method allows the specific co-amplification of 50–100 restriction fragments. The amplified products are visualized on sequencing polyacrylamide gels using radioactive labelling, silver-staining technique, or by using fluorescence-based capillary electrophoresis. Recently, Dresler-Nurmi *et al*. (2000) reported that fungal strain clusters revealed by fluorescently labelled and silver stained AFLP patterns were similar. These authors also showed that fluorescent AFLP pattern detection by capillary electrophoresis facilitated surveying with a computerized analysis system after recomposition into a virtual gel picture. Likewise, Ticknor *et al*. (2001) developed a computational routine that identified AFLP fluorescent fragments on gels to allow for very large sample analyses of AFLP profiles in an automated fashion.

AFLP has been successfully applied to the study of genetic variation of insect populations (Reineke *et al*., 1999; Behura *et al*., 2000; Cervera *et al*., 2000; Forneck *et al*., 2000; Katiyar *et al*., 2000; Parsons & Shaw, 2001; Miller *et al*., 2002), as well as in linkage-mapping studies of insects (Heckel *et al*., 1999; Hawthorne, 2001; Tan *et al*., 2001; Zhong *et al*., 2004). In the present study AFLP markers have been used to estimate differences between insects of 16 *L. serricorne* colonies from 15 countries on five continents to provide information on the level of genetic variability within and between populations of this cosmopolitan insect pest. In particular, the capability to differentiate the genotypes of cigarette beetle populations is important for a better understanding of how insect movements affect the infestation of stored tobacco.

[1]AFLP is a trademark of and is licensed by Keygene N.V., Wageningen, the Netherlands.

Materials and methods

Insect colonies

Sixteen populations of *L. serricorne* either currently reared in our laboratory or reared at the Central Science Laboratory of the UK Department for Environment, Food and Rural Affairs (DEFRA) (Central Science Laboratory (CSL), Sand Hutton, UK) were studied (table 1). They all originated from insects collected in tobacco stemmeries, warehouses and factories on behalf of the subgroup on Pest and Sanitation Management in Stored Tobacco of CORESTA (Centre de Coopération pour les Recherches Scientifiques relatives au Tabac, http://www.coresta.org/). All colonies were built up in the late 1990s and no conspicuous morphological polymorphisms were observed. All colonies had been routinely reared on whole wheat flour : yeast extract (95 : 5 w/w) and maintained at 26°C and 65–70% relative humidity, with a photoperiod of 15 : 9 (L : D) for the whole insect development (approximately 1.5 months per generation). Colonies were maintained by regular transfer of 300–600 emerging adults into fresh jars of flour diet.

Genomic DNA isolation

For each colony, five living female beetles from one rearing jar were randomly selected for DNA extraction in August 2000. Genomic DNA was isolated from individual beetles using the NucleoSpin™ Plant DNA kit (Macherey-Nagel, Düren, Germany) with modifications as follows. Each insect was put into a microtube with two 4-mm diameter glass beads and 80 µl buffer C1, 10 µl RNase (10 g l^{-1}), 10 µl proteinase K (10 g l^{-1}), 0.25 µl PCR mineral oil and 0.25 µl antifoam reagent DX (Qiagen, Hilden, Germany) were added. The tubes were shaken for 20 min at 400 rpm, then incubated at 65°C for 90 min. A 100-µl volume of buffer C4 was added and the tubes gently shaken prior to addition of 65 µl ethanol (100%). For DNA purification, 200 µl was transferred from the tubes into a well of a microscreen plate (Millipore, Bedford, Massachusetts) prepared as described below. The plate was centrifuged for 3 min at $200 \times g$ and the filtrate discarded. Washing of the DNA bound to the matrix was performed by adding 250 µl C5 buffer followed by 2 min centrifugation at $200 \times g$. This washing step was repeated once and completed by a final centrifugation step at $1000 \times g$ for 2 min. The matrix was finally allowed to dry completely at 70°C for 30 min. To recover the DNA bound to the matrix, 30 µl CE buffer were added, and the plate was centrifuged at $1000 \times g$ for 2 min. The filtrate was collected and precipitation of DNA was carried out by adding 60 µl of 100% isopropanol. The DNA was subsequently pelleted by centrifugation at $3000 \times g$ for 20 min, dried at room temperature before being resuspended in 30 µl TE buffer. The quality of the DNA was evaluated by 0.8% agarose gel electrophoresis and ethidium bromide staining. DNA concentration was measured with an ImageMaster imaging system (Amersham Pharmacia Biotech, Uppsala, Sweden) using a high MW DNA mass ladder (GibcoBRL, Gaithersburg, Maryland) as a standard.

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Table 2. Primer combinations used for AFLP amplifications.

Primer pair designation	Fluorescent dye	Primer sequence (5′→3′)	PMS
Preamp.		E+**A**	
		M+**C**	
AB	FAM	E+**AAC**	27
	–	M+**CT**	
AY	HEX	E+**ACT**	20
	–	M+**CA**	
BB	FAM	E+**ACC**	30
	–	M+**CA**	
BG	TET	E+**AGA**	42
	–	M+**CC**	
BY	HEX	E+**ATT**	43
	–	M+**CT**	
CB	FAM	E+**ATG**	18
	–	M+**CT**	
CG	TET	E+**ACA**	29
	–	M+**CG**	
CY	HEX	E+**AGT**	19
	–	M+**CG**	
DG	TET	E+**ATC**	23
	–	M+**CC**	
Total			251

Selective nucleotides in primer sequences are in boldface. E (for *Eco*RI site+adapter)=GACTGCGTACCAATTC. M (for *Mse*I site+adapter)=GATGAGTCCTGAGTAA. The number of polymorphic markers scored (PMS) is given for each primer pair.

The microscreen plates referred to above were prepared as follows. We mixed thoroughly 1.5 g diatomaceous earth 97.5% SiO_2 (Sigma chemicals, St Missouri Louis) with 25 ml H_2O and 50 µl HCl (32%). Ethanol (100%) was added to a final volume of 40 ml. The mixture was allowed to settle and the supernatant was discarded. The washed diatomaceous earth was resuspended in 40 ml H_2O and 200-µl aliquots were distributed into each well of the plate. After 1–2 h the plate was centrifuged for 1 min at 100 × *g* and the filtrate was discarded. Finally, 50 µl C5 buffer were added to each well prior to a final centrifugation step at 200 × *g* for 3 min. This matrix plate was used within 24 h.

AFLP reactions and marker scoring

AFLP analysis was performed using a modified protocol of Vos *et al*. (1995) with incorporation of fluorescent-labelled primers in the polymerase chain reaction (PCR) and detection by capillary electrophoresis with a genetic analyser. Adapter and primer sequences are given in table 2. Briefly, the AFLP Core Reagent Kit™ (Life Technologies, GibcoBRL, Gaithersburg, Maryland) was used for the first steps: 10–100 ng of genomic DNA were digested with 2.5 U *Eco*RI and 2.5 U *Mse*I and the digestion was checked for completion on agarose gel. Double-stranded adaptors were ligated to the restriction fragments according to the manufacturer's instructions. This was followed by preamplification of DNA fragments according to procedure III of Vos & Kuiper (1998) in a 9600 GeneAmp PCR system (Applied Biosystems, Foster City, California). We used primers with one nucleotide extension (E+A and M+C, 0.5 µM each, see table 2) and 5 µl of diluted ligation product (1 : 10) in Ready-To-Go™ PCR bead tubes of 0.2 ml (Amersham Pharmacia Biotech, Uppsala, Sweden) with a final volume of 25 µl.

Selective amplifications were performed subsequently on a 1 : 20 dilution of the pre-amplified fragments. Fluorescent-labelled primers with three selective nucleotides on one side (E+3, 0.1 µM) and unlabelled primers with two selective nucleotides on the other side (M+2, 0.5 µM) were used. The amplification procedure was performed as described by Vos & Kuiper (1998) with hot start.

Aliquots from the AFLP amplifications were analysed with an ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, California). The capillary was filled with denaturing polymer POP-4 (Applied Biosystems). A 2-µl volume of PCR products and 0.8 µl of GeneScan internal size standard (TAMRA-500, Applied Biosystems) were added to 9 µl of deionized formamide. The samples were denatured for 2 min at 90°C, cooled down to 4°C and transferred into the sample tray. Samples were injected at 15 kV for 5 s onto a 47-cm capillary (36 cm to detector) and electrophoresed at 15 kV for 24 min at 60°C. Fluorescent dyes attached to DNA were excited by laser and detected using filter C of ABI 310. The GeneScan analysis software (Applied Biosystems) was used to determine PCR fragment sizes by comparison with internal size standards.

The detection and scoring of markers was performed in a semi-automated fashion using Genotyper 2.5 (Applied Biosystems) after certain parameters had been defined as follows. Scale factors for the normalization of the chromatograms were calculated on the basis of the sum of signal. Chromatograms with scale factor <0.4 (i.e. sum of signal $<40\%$ of average chromatogram) were discarded and the samples were rerun when possible. Categories (corresponding to 'markers') were created based on a peak scaled-height cut-off of 100 (arbitrary fluorescence units). Overlapping categories were skipped and chromatograms were labelled for absence or presence of peak within each category (+/−0.5 bp). Labelled peaks present in all chromatograms generated with one primer pair were discarded, thus monomorphic markers were not considered for further evaluation.

A binary matrix reflecting presence (1) or absence (0) of peak for each marker and in each sample was generated.

AFLP data analysis

The relationships between individual insects were calculated from the binary matrix of markers by using the Nei & Li coefficient of similarity (S_{ij}; Nei & Li, 1979). The data were then converted to a dissimilarity (distance) matrix ($D=1-S_{ij}$) for constructing two individual-based dendrograms. The first tree was generated following the unweighted pair-group method of arithmetic averages (UPGMA; Sneath & Sokal, 1973) by using SAS 8.02 (Statistical Analysis System Institute Inc., Cary, North Carolina). Secondly, a neighbour-joining tree (Saitou & Nei, 1987) was constructed using PHYLIP version 3.57c (Felsenstein, 1993).

Results and Discussion

AFLP markers and polymorphism

An initial survey of a small number of adults with primer combinations of 2+2 selective nucleotides at their 3′-end resulted in complex peak patterns, which could not be

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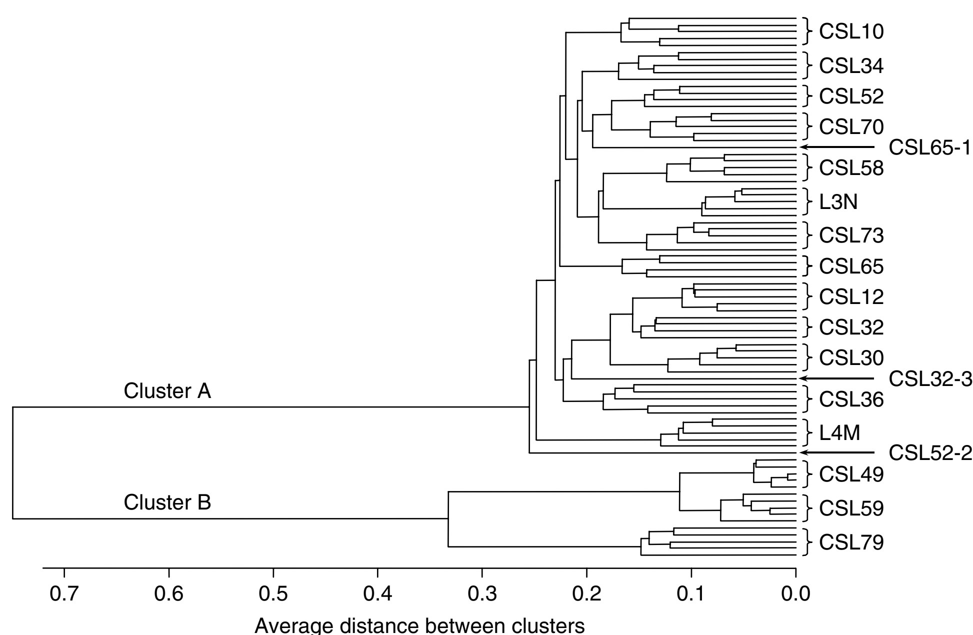

Fig. 1. UPGMA dendrogram computed from Nei & Li's (1979) genetic distances between AFLP profiles of individual beetles of *Lasioderma serricorne* (16 colonies). Distances were calculated from data of 251 polymorphic markers obtained with nine selective primer pairs (table 2). The colony name is reported for all insects within the same colony. Insects that did not group with their colony counterparts are indicated separately. Clusters are discussed in the text.

reliably distinguished using Applied Biosystems' Genotyper software. Conversely, primer combinations containing 3+3 selective nucleotides yielded too few fragments for enough polymorphic markers to be scored (data not shown), as was also reported by Miller *et al*. (2002) for a freshwater beetle, *Psephenus montanus* Brown & Murvosh (Coleoptera: Psephenidae). These observations clearly emphasize the need of an initial screening of different primer types to determine combinations with the greatest value for genotypic analysis of the respective organism, as previously pointed out for insects by Reineke *et al*. (1999).

Consequently, nine primer pairs with 3+2 selective nucleotides were used for the selective amplification of preamplified DNA restriction fragments of 80 adult beetles from 16 colonies (table 1). Similarly, Miller *et al*. (2002) used 2+3 selective nucleotides for *P. montanus*. The scoring and comparison of chromatogram peaks generated by all individual insects revealed a total of 251 polymorphic markers. Each primer pair yielded 18 to 43 polymorphic markers (table 2), with fragment sizes ranging from 60 to 350 bp. Another 59 markers were detected in all insect profiles (monomorphic) and were therefore discarded. Monomorphic markers accounted for 19% of all the markers detected, which compares with the 11% monomorphic AFLP markers reported for populations of the gypsy moth (Reineke *et al*., 1999).

In the present study, a high-throughput procedure has been developed for generating AFLP profiles of insects, from the extraction of DNA to the matrix of polymorphic markers. Furthermore, it has been shown that capillary electrophoresis of amplification fragments and computing of chromatogram data made it possible to generate fingerprints of *L. serricorne* adult beetles effectively, as suggested in a previous study (Dresler-Nurmi *et al*., 2000). Combining capillary electrophoresis and direct scoring of peaks in AFLP chromatograms alleviates the need for reconstructing an intermediary virtual gel picture, which should facilitate the analysis of very large insect samples in the future.

Population structuring and colony clusters

Genetic distances derived from Nei & Li's similarity indexes between individuals were calculated from the 251-marker matrix. Both trees constructed following the UPGMA and neighbour-joining (NJ) methods revealed that insects originating from a same colony grouped together in almost all cases. In the UPGMA tree (fig. 1), only three beetles referred to as CSL32-3, CSL52-2, and CSL65-1 did not cluster with the other four insects of their colony. The neighbour-joining tree revealed that only one beetle (CSL 65-1) clustered away from its colony counterparts (data not shown). Hence, discrete clusters of insects were revealed at the colony level. Moreover, both trees revealed the same two major colony clusters (A and B). Cluster A consisted of all colonies except CSL49, CSL59 and CSL79, the latter three colonies forming cluster B.

The cigarette beetle, *L. serricorne*, can complete its life cycle for several generations inside the packaged tobacco leaf, which is usually stored for 1–2 years prior to manufacturing (Ryan, 1995). Thus, gene flow among distant populations may be expected to be lower than that of highly mobile field insect pests. In the present study, the 16 colonies of cigarette beetles collected from all over the world formed two discrete groups. There was no clear clustering related to geographical origin since the colonies included in well-supported clusters were from very different geographic sampling points. Consequently, the results suggest extensive insect transportation among geographical regions due to movement of infested plant material worldwide. In addition, the cigarette beetle infests a

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wide range of other stored products (Howe, 1957; Ryan, 1995), which can widen its distribution and, thereby, contribute to population mixing and to a higher gene flow. Interestingly, the level of genetic differentiation between colonies was not markedly different from the variability reported in a previous study of field insects collected worldwide (Reineke *et al*., 1999). This is in accordance with a mobility possibly higher than expected for a stored-product beetle.

It is noteworthy that the origin of an infestation is extremely difficult to track, owing to the complexity of the supply chain of tobacco and the number of points such as ports where cross-infestations with other commodities can occur. Indeed, the countries given in table 1 are the countries in which the samples were collected and sent from, not necessarily the country where the infestation started. The tobacco may already have been infested when it entered the country where the sample was collected. In order to study the actual geographic origin of cigarette beetle populations, samples should be taken in those commodity batches where an infestation is detected in the first place. In parallel, insects could be trapped and collected on tobacco farms, as described by Carvalho *et al*. (2000), and also at local stemmeries where tobacco strips (lamina) are separated from the stems (midribs). Investigating the genotypes of such insect samples would indicate whether the gene flow is limited to the ports and warehouses, where insect populations from different origins may come together more easily, or if cigarette beetles can easily travel between distant farms.

Specific colony attributes, including resistance to insecticides such as phosphine (Savvidou *et al*., 2003) and methoprene (Benezet & Helms, 1994), have been described in *L. serricorne*. It would be of interest to evaluate if phenotypic features of our colonies could possibly be linked to the strongly supported clusters revealed in our study. However, the limited number of AFLP markers used here (with respect to the entire genome) is unlikely to account for differences in the very limited number of genes thought to be involved in resistance (Savvidou *et al*., 2003).

Developing a molecular tool for the positive identification of the origin of infestations or of insecticide resistance was beyond the scope of the present study. This being said, such an approach may benefit from fingerprinting mixed DNA extracted from pools of 5–10 insects. This could possibly decrease the differences recorded between profiles within a colony (intra-colony variability). Comparing pool profiles may thus lead to the identification of reliable colony- or biotype-specific markers, as reported for dipteran pests of rice in India (Behura *et al*., 2000) and for another stored-product beetle, *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) (Schlipalius *et al*., 2002).

In conclusion, this first AFLP population study of a stored-product insect demonstrates the potential of a computer-integrated AFLP approach for distinguishing *L. serricorne* populations and better understanding their population structure and movements in the future. Two major insect colony clusters were revealed, yet these were apparently not linked to the geographic location of the original sampling point.

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