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Suppression of randomly primed polymerase chain reaction products (random amplified polymorphic DNA) in heterozygous diploids

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Abstract

In a study of genetic polymorphism in the gypsy moth *Lymantria dispar* we observed the aberrant inheritance of a random amplified polymorphic DNA (RAPD) fragment designated H11-589. This fragment was present in amplification products of F_1 progeny of different crosses although it was not amplified from either parental DNA. DNA-mixing experiments revealed that the presence of DNA containing a template for another product (H11-746), amplified with the same primer, suppressed the synthesis of H11-589. The templates for both RAPD products were highly repetitive and scattered throughout the *L. dispar* genome. Southern hybridization and sequence analysis of H11-746 and H11-589 revealed an extensive sequence homology and an internal repetitive motif of 17 nucleotides present in both products. Interactions between templates for H11-746 and H11-589 are expected to occur during the polymerase chain reaction (PCR), offering an explanation for the suppression of the amplification of H11-589. The role of the internal repetitive motif and of the copy number of both templates in the suppression effect are discussed. Our results corroborate doubts regarding the suitability of the RAPD technique for quantitative genetic analysis, in particular where mixed populations are concerned.

Keywords: anomalous segregation, genetic relationships, gypsy moth, insects, molecular marker, RAPD

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Introduction

Random amplified polymorphic DNA (RAPD) markers (Welsh & McClelland 1990; Williams *et al*. 1990) are well-established genetic tools used in a variety of organisms for genomic mapping and linkage analysis, genotype fingerprinting and identification, and quantification of genetic relationships, similarities and variation. RAPDs are generated from genomic DNA by the polymerase chain reaction (PCR), primed by short, randomly constructed oligonucleotides. The advantages of this technique are the essentially unlimited number of loci that can be examined, no need for prior knowledge of DNA sequence and a small quantity of template DNA. However, reported limitations of RAPD markers include a low reproducibility (Ellsworth *et al*. 1993; Meunier & Grimont 1993; Micheli *et al*. 1994), homology of co-migrating amplification products (Cognato *et al*. 1995; Rieseberg 1996) and dominant inheritance (Williams *et al*. 1990). In addition, novel bands, not amplified from any parent, have been reported to arise in offspring of known pedigree in fungi (Ayliffe *et al*. 1994), plants (Davis *et al*. 1995), insects (Hunt & Page 1992) and primates (Riedy *et al*. 1992). Ayliffe *et al*. (1994), Davis *et al*. (1995) and Hunt & Page (1992) attributed the formation of nonparental bands to heteroduplex molecules formed between allelic RAPD products, which represent artefactual polymorphisms that confuse RAPD analysis. Another possible source of error arises when the presence or absence of a RAPD product is affected by competition effects. Williams *et al*. (1993) suggested that the formation of a given RAPD product is determined by competition for primer-binding sites in the genome. Moreover, Heun & Helentjaris (1993) observed that a

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specific fragment could be amplified in one genetic background, but not in another, because of competition from other unlinked sites. Using artificial DNA mixing experiments, Smith *et al*. (1994) demonstrated the lack of independence between RAPD products, and Halldén *et al*. (1996) proved that strong competition effects are a general feature of RAPD reactions.

During our studies on the inheritance of RAPD patterns in the gypsy moth, *Lymantria dispar* L., and their suitability for population genetic studies, we observed aberrant inheritance of certain RAPD fragments. In this report we present detailed evidence that interactions between RAPD products, which share sequence homology, result in the observation that a particular fragment might not be visible (in ethidium bromide-stained agarose gels), despite the presence of its template in the reactions. This phenomenon has important implications regarding the use of RAPDs for assessing the genetic variability of field-collected organisms.

Materials and methods

Genetic material

Material for this study was obtained from eggs of *Lymantria dispar* (Lepidoptera: Lymantriidae) collected in the field and reared in the laboratory. A total of 19 crosses were performed by isolating a male and female together as pupae and placing them in individual plastic containers. Following oviposition, adults were immediately frozen and stored at –20 °C until DNA extraction. Eggs were placed at 4 °C for 120 days. After the eggs were hatched, 20 F_1 larvae of each cross were frozen at –20 °C for genotypic analysis.

For DNA-mixing experiments, *L. monacha* (Lepidoptera: Lymantriidae) and *Ephestia kuehniella* (Lepidoptera: Pyralidae) isolates were included in the analysis, as well as a fragment from pUC19 that was restricted with *Pvu*II and ligated to double-stranded adapters containing RAPD primer-binding sites.

Extraction and purification of DNA

DNA was isolated from insect tissue using a modified CTAB protocol with an additional polyethylene glycol precipitation (Reineke *et al*. 1998). DNA for dot-blot hybridization was purified in caesium chloride (CsCl) gradients with bisbenzimide (Karlovsky & de Cock 1991). For DNA-mixing experiments, the respective DNA was further purified using GeneClean KitII (BIO 101) according to the manufacturer's instructions. DNA was quantified spectrophotometrically at 260 nm using GeneQuant II (Pharmacia) and diluted to a final concentration of 100 ng/μL.

PCR conditions

RAPD reactions were carried out in a final volume of 25 μL containing 10 ng of genomic DNA, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.5 mg/mL of bovine serum albumin (BSA), 0.625 units of *Taq* DNA polymerase (MBI Fermentas) and 0.2 μM of primer in buffer consisting of 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.8% Nonidet-P40. For the present study, primer OPH11 (CTTCCGCAGT, Operon) was used. Amplifications were performed in a MJ Research PTC-100 thermal cycler programmed for: 2 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, with a final extension at 72 °C for 4 min. A 13-μL aliquot of each PCR sample was separated on a 1.2% agarose gel at 11–13 V/cm for 150 min in TBE buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M boric acid, 2 mM EDTA). The gel was stained with ethidium bromide (0.5 μg/mL) and DNA was visualized under UV light. Negative controls lacking template DNA were included in all experiments. In addition, DNA from each individual was amplified in duplicate to assess reproducibility.

For DNA-mixing experiments, template DNA from two sources were mixed in different proportions (3:1, 2:1, 1:1, 1:2, and 1:3) prior to PCR analysis. In addition, each DNA was amplified separately under identical reaction conditions as a control.

Reamplification of putative RAPD fragments was conducted with DNA fragments of 450–700 bp excised from an agarose gel. Agarose was removed using the GeneClean KitII (Bio 101). Conditions for the PCR were as described above.

Cloning and sequencing of PCR products

Polymorphic RAPD products H11-746 and H11-589 were excised from an agarose gel, purified using the GeneClean KitII and cloned into the *Sma*I site of pUC18 using the SureClone™ ligation kit (Pharmacia) according to the manufacturer's instructions. Sequences of the cloned RAPDs H11-746 and H11-589 were determined using primers similar to the M13 forward and reverse primers and the SequiTherm™ Cycle Sequencing Kit (Epicentre Technologies).

Probe labelling, Southern analysis and dot-blot hybridization

Hybridization of RAPD products and genomic *L. dispar* DNA was conducted using cloned RAPD fragments H11-746 and H11-589 as probes labelled either with [γ^{33}P]- or [α^{32}P]-dATP.

For Southern hybridization, RAPD products and 20 μg of genomic DNA digested with different enzymes were separated on agarose gels and transferred to Hybond-N+

membrane (Amersham) following the manufacturer's instructions. The copy number of templates for H11-746 and H11-589 was estimated by dot-blotting 5 μg to 100 ng of *L. dispar*, DNA purified in CsCl gradients, to Hybond-N+ membrane using a Minifold II apparatus (Schleicher & Schuell). The copy number of genomic sequences homologous to RAPD products was determined by using cloned PCR products, in amounts corresponding to 1–100 copies per genome, as standards on dot-blots.

After hybridization of Southern blots and dot-blots to the radioactively labelled probes, membranes were washed at high stringency and exposed to a phospho-imaging screen that was subsequently scanned using a Bio-image Analyser (BAS-1000, Fuji Photo Film). For reprobing, blots were stripped by boiling twice for 30 min in 0.5% SDS and checked for complete removal of the probe by exposing them to a phospho-imaging screen, as described above.

DNA sequences

DNA sequences were deposited in the EMBL database under Accession nos AJ001672 (H11-746) and AJ001673 (H11-589).

Results

Suppression of RAPD bands in progeny of Lymantria dispar *crosses*

As part of our research to generate genetic markers in the gypsy moth *L. dispar* using the RAPD technique, we examined the inheritance of RAPD fragments generated with different primers (results to be presented elsewhere). Primer OPH11 detected two characteristic and distinct RAPD bands with sizes of 746 bp and 589 bp (designated H11-746 and H11-589, respectively). In the analysis of the inheritance of these products among F_1 progeny of 19 crosses, several crosses showed unexpected results. For example, in one cross, RAPD band H11-589 was not amplified from either of the parental DNAs but was present in 11 out of a total of 20 F_1 progeny (Fig. 1). The reproducibility of this nonparental band was confirmed in three replicate analyses.

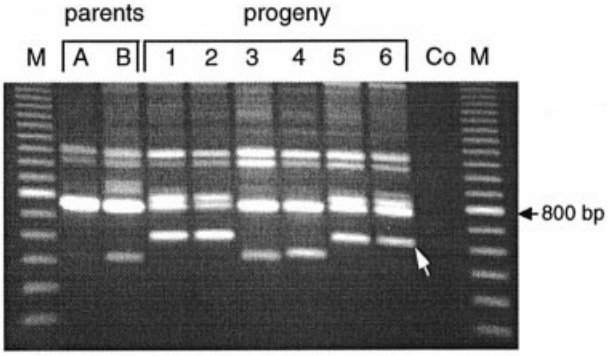

Fig. 1 Random amplified polymorphic DNA (RAPD) fragments of *Lymantria dispar* parents (lanes A and B) and their F_1 progeny (lanes 1–6). Note the presence of a 589-bp fragment in progeny lanes 1, 2, 5 and 6 (arrow). Co, control without template DNA; M, 100-bp ladder.

Characterization of RAPD products H11-746 and H11-589

To study the relationship of RAPD products H11-746 and H11-589 with each other and with the other RAPD products, both fragments were isolated from PCR products of DNA from *L. dispar* individuals A1 and N1, respectively, and cloned into the *Sma*I site of pUC18.

Southern blot analysis was carried out on a blotted RAPD gel using cloned RAPD products H11-746 and H11-589 as probes. Both the 746-bp and 589-bp bands were detected by each probe, indicating a sequence similarity between these two fragments (results not shown). Sequence analysis revealed that the fragments shared a region of 542 bp with 99.8% bases identical (Fig. 2). In addition to the homologous area, H11-746 contained an insertion of 204 bp and H11-589 an insertion of 48 bp. Neither insertion occurred in the other product.

Analysis of genomic equivalents of RAPD products H11-746 and H11-589

The organization and copy numbers of genomic sequences corresponding to PCR products H11-746 and H11-589 were determined using Southern blotting and dot-blot hybridization. No distinct patterns were seen after Southern hybridization of digested genomic DNA with cloned H11-746 and H11-589 as probes (data not shown) indicating that a large number of the respective sequences are scattered throughout the *L. dispar* genome.

Dot-blot hybridization confirmed that genomic sequences homologous to H11-746 and H11-589 belong to repetitive elements. From the comparison of signal intensities of cloned H11-746 and H11-589 DNAs, used as standards, and signals produced by 3 μg of genomic DNA, the number of copies of each sequence per haploid genome was estimated to be more than 100 (data not shown).

DNA-mixing experiments

Our observations that: (i) RAPD products H11-746 and H11-589 did not segregate as expected in crossing experiments; and (ii) the H11-589 fragment was present in some of the progeny although it was not amplified from DNA of either parent, suggested that an interaction between these two amplification products occurred during PCR.

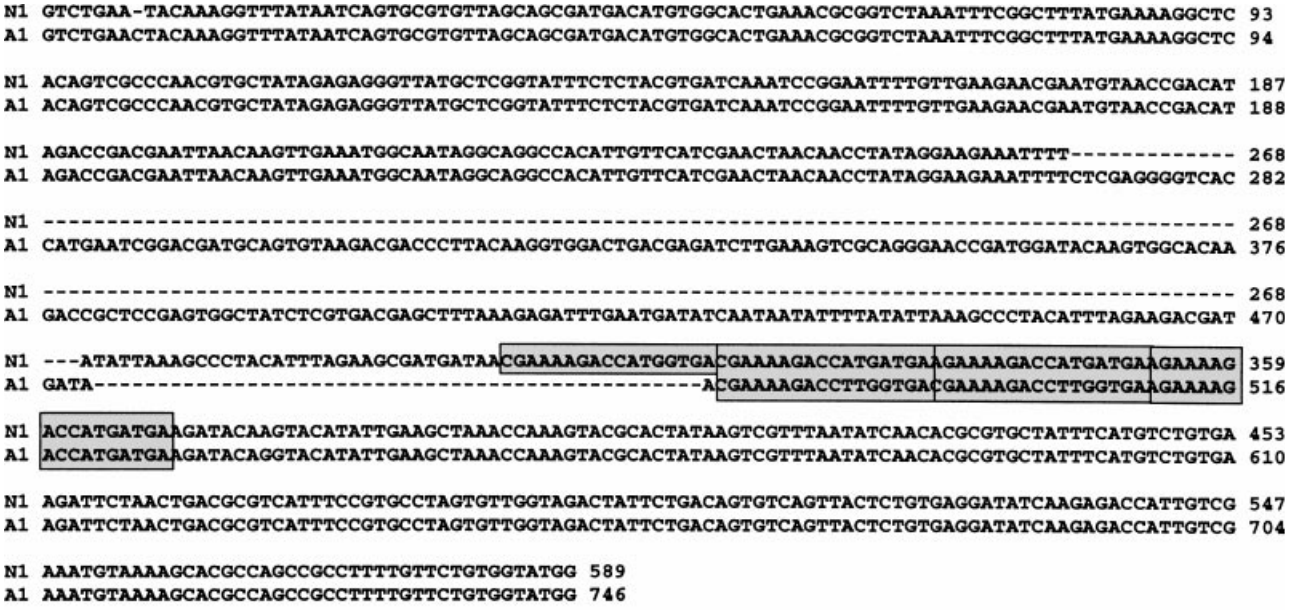

Fig. 2 Nucleotide sequences of random amplified polymorphic DNA (RAPD) products H11-746 and H11-589, generated from *Lymantria dispar* individuals A1 and N1, respectively. Tandem repetitions are indicated by shaded boxes.

We hypothesized that amplification of the H11-589 fragment was suppressed by the presence of the H11-746 fragment in the reaction. To verify this hypothesis, PCR experiments using mixed genomic *L. dispar* DNAs, mixed cloned fragments and mixed primer OPH11 products from different genomic backgrounds, as templates, were conducted.

If one template DNA produced fragment H11-746 and the other template DNA led to the amplification of fragment H11-589 with the same primer, one would expect both fragments to occur as products of PCR performed with a 1:1 mixture of both templates. However, after mixing genomic DNA from one *L. dispar* parent (which itself produced the H11-746 band only) with DNAs that each produced fragment H11-589 when amplified separately, only the H11-746 fragment was observed in an ethidium bromide-stained agarose gel (Fig. 3A). Similar results were obtained when mixing the cloned fragments H11-746 and H11-589 prior to PCR: H11-589 was amplified less efficiently than H11-746 when the template for H11-589 was present as 50% (Fig. 3B, lane 1), 66% (Fig. 3B, lane 2) or even 75% (Fig. 3B, lane 5) of the total DNA. These results indicate that the presence of DNA containing a template for the H11-746 product suppresses the synthesis of the H11-589 fragment. To verify this hypothesis, template for H11-746 was replaced in the template-mixing competitive PCR with DNAs from three unrelated loci (Fig. 3C). RAPD product H11-589 was extracted from an agarose gel and was mixed 1:1, prior to PCR, with a 900-bp OPH11 product from *Ephestia kuehniella* and an 850-bp fragment from *L. monacha*, respectively. In both cases, H11-589 was amplified normally (Fig. 3C, lanes 1 and 2). The same result was obtained when the mixture contained a fragment from pUC19, cut with *Pvu*II and ligated to adapters containing binding sites for primer OPH11 (Fig. 3C, lane 3). When the unrelated sequences were mixed with the H11-589 fragment at ratios different from the 1:1 ratio shown (Fig. 3C), band intensities of the respective fragments increased or decreased with regard to their proportion in the template mixture (data not shown).

Reamplification of suppressed RAPD product H11-589

The observations of the DNA-mixing experiments imply that the formation of the H11-589 product may also be suppressed in genomic DNA from a single animal, if it contains the respective template together with 'suppressing' sequences. The presence of a template for H11-589 in such a DNA sample cannot be confirmed by mixing experiments. However, it is possible that the H11-589 product is formed in small quantities that are insufficient for visual detection on ethidium bromide-stained agarose gels but are adequate as a template for reamplification. Therefore, we extracted putative DNA from an area of an electrophoretic gel corresponding to the position of the 589-bp fragment and used the extract as template for a second PCR. Figure 4 shows that the 589-bp fragment was recovered from both reamplified samples, although it was not visible in the product of the first PCR with either DNA. This indicates that although sequence information necessary for the amplification of the H11-589 RAPD fragment was present in both templates, its amplification was suppressed.

The reamplification of DNA extracted from a gel portion containing no visible RAPD products resulted in the formation of an additional 500-bp fragment, which was not present in amplification products of the first PCR (Fig. 4). Southern blot and sequence analysis of this fragment revealed no homology to RAPD products H11-746 or H11-589 (data not shown). However, during our studies on the inheritance of RAPD fragments in *L. dispar*, PCR products of some individuals exhibited a band of the same size. We hypothesize that the 500-bp fragment is amplified inefficiently, e.g. because of forming secondary structures and thus preventing primer binding. If other RAPD products are formed rapidly, PCR resources may be exhausted before the 500-bp band is amplified to a level that is visible on ethidium bromide-stained agarose gels.

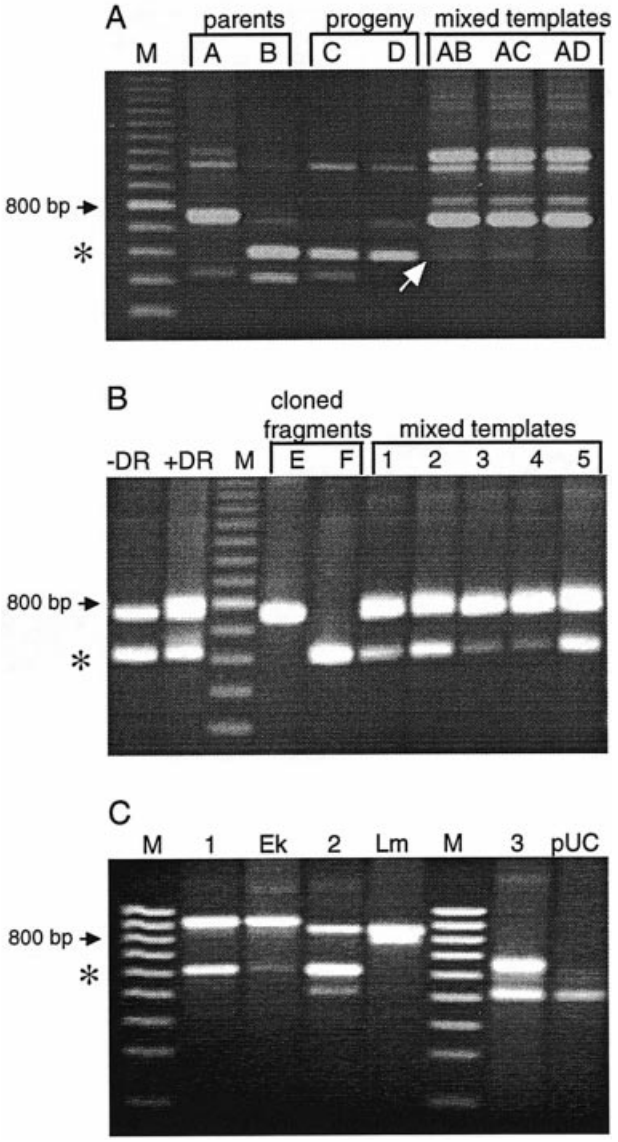

Fig. 3 Amplification products of competitive polymerase chain reaction (PCR) using, as templates: (A) mixed genomic *Lymantria dispar* DNAs; (B) mixed cloned PCR products; and (C) mixed primer OPH11 products from different genomic backgrounds. (A) random amplified polymorphic DNA (RAPD) products of *L. dispar* parents (lanes A and B), their progeny (lanes C and D) and the results of mixing parent–parent (lane AB) and parent—progeny (lanes AC and AD) template DNAs 1:1 prior to PCR. Note the absence of the 589-bp fragment (lanes AB, AC and AD) after template mixing (arrow). (B) RAPD products of cloned fragments H11-746 (lane E), H11-589 (lane F) and of both templates mixed 1:1 (lane 1), 1:2 (lane 2), 2:1 (lane 3), 3:1 (lane 4) and 1:3 (lane 5) prior to PCR. The doublet pattern observed in the longer PCR product of mixed templates (lanes 1–5) is a consequence of hybrid formation. It can be generated by denaturation (3 min at 94 °C) and renaturation (5 min at 36 °C) of separately amplified products mixed after PCR. Lane –DR, a mixture of separately amplified PCR products before denaturation/renaturation. Lane +DR, the same mixture after denaturation/renaturation. (C) Individual RAPD products of *Ephestia kuehniella* (Ek), *L. monacha* (Lm) and of a fragment isolated from pUC19 and ligated to adapters containing primer OPH11 binding sites (pUC). Lanes 1, 2 and 3 show amplification products of the *E. kuehniella*, *L. monacha* and pUC19 fragment, respectively, mixed with a H11-589 fragment 1:1 prior to PCR. Note the successful amplification of H11-589 in all mixtures. Lane M, 100-bp ladder. The asterisk in (A), (B) and (C) indicates the position of the H11-589 fragment in the different PCR products.

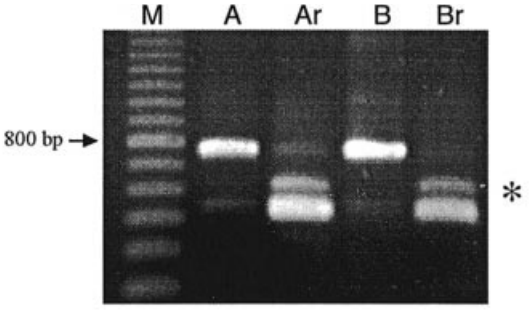

Fig. 4 Reamplification of suppressed random amplified polymorphic DNA (RAPD) products. Aliquots of RAPD products generated from template DNAs A115 (lane A) and 436 (lane B) and products obtained after extraction and reamplification of putative RAPD products from template DNAs A115 (lane Ar) and 436 (lane Br). Note the presence of an additional 589-bp fragment in the reamplified samples (asterisk). M, 100-bp ladder.

Discussion

Fragments amplified simultaneously during randomly primed PCR compete for primer, nucleotides and polymerase. Differences in the efficiency of the amplification between fragments determine which fragments are amplified to a sufficient extent to be visible on agarose gels. A competition for primer and nucleotides among RAPD products amplified with different efficiencies is expected to lead to a mutual dependence of the yields:

the more efficiently a particular fragment is amplified, the fewer resources that remain for competing, slowly synthesized products. Therefore, competition for substrates is expected to magnify the difference in yield among fragments amplified with different efficiencies.

The interdependence of the amplification of RAPD products was demonstrated by Smith *et al*. (1994), Heun & Helentjaris (1993) and Halldén *et al*. (1996). Smith *et al*. (1994) have shown that a particular RAPD band occurs in the reaction products only when a band from an unrelated locus is absent. Our study presents evidence for suppression of a RAPD product by DNA containing a template for a related product.

Heun & Helentjaris (1993) stated, in their study on the inheritance of RAPD fragments in F_1 hybrids of corn, that only 'minor' fragments tend to occur irreproducibly owing to competition with more 'intense' fragments. They suggest considering only three or four of the most intense fragments for linkage analysis as a measure for preventing artefacts caused by competition between RAPD products. Our results have shown that even major RAPD products can be obscured by interference with other products, even when the interfering template is present in a smaller amount. The fact that a major RAPD product is amplified to a very limited extent under suppressing conditions proves that competition for PCR resources between single-copy sequences cannot account for this phenomenon. However, if the 'winning' fragment was present in many copies at the start of the reaction, its amplification would proceed with an advantage that would lead to the exhaustion of PCR resources before the suppressed fragment reached a detectable amount. Indeed, Southern and dot-blot analysis of genomic *Lymantria dispar* DNA have shown that sequences homologous to both H11-746 and H11-589 fragments are highly repetitive and scattered throughout the *L. dispar* genome. Furthermore, results of DNA-mixing experiments have proved that the suppression of fragment H11-589 by H11-746 depends on the amount of template for H11-746 in the PCR reactions. Because the template for H11-746 is a multicopy sequence, a segregation of copy number will occur in offspring provided some of the loci are heterozygous. However, the detectability of the H11-589 band depends on the sensitivity of the staining method used. For a given detection limit, offspring with a copy number of H11-746 higher than a certain value will not produce a detectable amount of H11-589 while progeny with a lower copy number will produce both bands (Fig. 1).

Using completely homozygous material for artificial DNA-mixing experiments, Halldén *et al*. (1996) have proved that competition in RAPD assays is not a specific feature of a certain set of PCR conditions and is independent of the complexity of the template DNA used. Furthermore, the authors argued that the actual DNA sequence is more important for successful amplification of a given RAPD product rather than the sequence copy number. This hypothesis is consistent with our observations on the DNA sequence of the two interacting RAPD products. Because fragments H11-746 and H11-589 share an extensive sequence homology, their templates are expected to interact during PCR by forming hybrids during the annealing step. In order to account for the suppression phenomenon, these hybrids must be a good template for the amplification of one of the bands (H11-746) but a bad template for the amplification of the other fragment (H11-589). This situation is different from simple competition because not only the excess of one product over another, but also the relative efficiency of its amplification, will grow during PCR. Combined with the exponential nature of PCR, this process can prevent particular DNA sequences from amplification. What kind of interactions between templates can account for the suppression of H11-589 amplification? The products share long, perfect homologies at both ends, differing only by insertions of 204 bp in H11-746 and 48 bp in H11-589. Interestingly, three copies of a 17-bp sequence repeated in tandem are present in the homologous area of both products and an additional copy is present in H11-589 as part of the insertion (Fig. 2). We speculate that heteroduplexes between different copies of the repetitive sequence in both templates can be formed in the course of PCR, leading to a suppression of the amplification of H11-589. Interactions between molecules of the same PCR product are known to occur during the final stages of PCR, limiting the extent of the reaction (Ruano *et al*. 1991). Interactions between co-amplified PCR products were found to inhibit the annealing of primers in a competitive PCR, leading to the derivation of a kinetic model of DNA amplification (Suzuki & Giovannoni 1996). However, in this model the authors assumed that each template reanneals only to its homologous complement and so does not inhibit the priming of the other template for amplification. In the case of extensive sequence homologies, interactions between different products are expected to compete with primer annealing, reducing the efficiency of amplification. Because H11-589 contains four copies of a repetitive sequence that occurs only three times in H11-746, both reannealing of H11-589 products with themselves and hybridization of H11-589 with H11-746 is kinetically favourable compared to reannealing of H11-746 products with themselves.

We hypothesize that the copy number of a homologous repetitive sequence within competing PCR products effects the proportion of the products at the end of the reaction by two mechanisms: (i) by slowing down amplification of the product with a higher number of the repetitive unit owing to a faster reannealing; and (ii) by hybridization of both products mediated by common repetitive elements.

In summary, we have confirmed and extended previous observations about the nature and occurrence of competitive and suppressive effects between RAPD products and have demonstrated a possible mechanism that could account for such a phenomenon. As the occurrence of competition and suppression in RAPD assays can affect both the determination of genetic distances between species and varieties as well as the result of paternity analysis, our results corroborate doubts regarding the suitability of the RAPD technique for quantitative genetic analysis, in particular when mixed populations are concerned. Halldén *et al*. (1996) present a thorough discussion of the effects of errors, caused by competition, in the genomic analysis of sexual diploids. The authors suggest that artificial template-mixing methods provide an effective and economical means for the identification of interacting fragments. Problematic bands can also be recognized by performing crossing experiments. However, when working with sexual, diploid organisms it is not known whether the respective genomes are homo- or heterozygous for the alleles involved. Hansen *et al*. (1997) further showed that a RAPD band segregating correctly in one genotype can be affected by competition in another genotype combination owing to crossover events occurring between the markers. In addition, crossing experiments as an approach for checking suppression and competition are not applicable when RAPDs are used for assessing genetic variability of organisms collected in the field.

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This research forms part of the PhD thesis of Annette Reineke on the use of molecular markers for genetic analysis of geographical populations of the gypsy moth, *Lymantria dispar*. She is now a postdoctoral fellow at the University of Adelaide, Department of Applied and Molecular Ecology, studying the molecular basis of host–parasitoid interactions. Petr Karlovsky is head of the Molecular Biology Laboratory of the Institute of Phytomedicine, University of Hohenheim. His research focuses on the molecular biology of plant pathogenic fungi and their secondary metabolites. Claus Zebitz is professor of Entomology with main research interests in insect–plant relationships, population genetics and ecosystem analysis.
