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DETECTION OF DNA POLYMORPHISMS IN PREDATORY COCCINELLIDS USING POLYMERASE CHAIN REACTION AND ARBITRARY PRIMERS (RAPD-PCR)

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DNA polymorphisms were identified in some Coccinellid predators that are being tested as biological control agents against aphids and other insects in North America. The technique employs a variation of the Polymerase Chain Reaction (PCR), called RAPD-PCR, that uses single arbitrarily selected primers to amplify a random group of genomic sequences. Using this technique it was possible to distinguish among laboratory reared colonies of diverse geographic origin. Several colonies each, of three species were examined (*Coccinella septempunctata, Hippodamia variegata,* and *Propylea quatuordecimpunctata*). It was also possible to distinguish *C. septempunctata* from a closely related species *C. transversoguttata biinterrupta.* The technique promises to be a very useful source of markers for maintaining colonies and tracking genes in biological control projects and in identifying species and immature stages of insects.

KEY-WORDS: RAPD-PCR, aphids, biological control, genetic markers, Coleoptera, Coccinellidae.

Various species of Coccinellids (lady beetles) are a group of predators that have been the object of collection expeditions, laboratory propagation, and test releases to the field. While primarily directed towards aphid control in cereal crops, lady beetles are generalist predators that have been released in orchards and even home gardens. Genetic markers are needed, both to detect potential mixing of laboratory reared colonies and follow the survivability of genes from strains that have been intentionally released.

The random amplified polymorphic DNA (RAPD) procedure combines the polymerase chain reaction (PCR) and single relatively short primers to uncover DNA sequence polymorphisms in the absence of specific sequence information (Welsh & McClelland, 1990 ; Williams *et al.,* 1990). The resulting collection of amplification products can be separated by electrophoresis to generate a "fingerprint" of genetic variation that can be useful for strain identification (Welsh *et al.,* 1991b). RAPD analysis has found considerable favor with plant geneticists (e.g. Welsh *et al.,* 1991a ; Arnold *et al.,* 1991 ; Roy *et al.,* 1992).

RAPD markers have been used to try to identify species, strains, "biotypes", and geographic variation of insects. Among the insect groups examined are aphids and their

parasitoids (Black *et al.*, 1992 ; Puterka *et al.*, 1993 ; Roehrdanz *et al.*, 1993), grasshoppers (Chapco *et al.*, 1992), mosquitoes (Ballinger-Crabtree *et al.*, 1992), microhymenopteran parasitoids of Lepidoptera (Landry *et al.*, 1993), and whiteflies (Perring *et al.*, 1993).

This paper demonstrates the extension of the technique in distinguishing strains of Coccinellid beetles of diverse geographic origin. The species and strains described here were collected from several areas within the original distribution of the Russian wheat aphid (*Diuraphis noxia*). They have been reared in the laboratory and released in widely scattered fields in the United States and Canada in an effort to control the Russian wheat aphid (Flanders *et al.*, 1991). The technique may have applicability for other biological control agents or insect populations in general.

MATERIALS AND METHODS

Insects were taken from colonies maintained at the USDA-APHIS National Biological Control Laboratory in Niles, MI, USA. These colonies originated from Europe, the Middle East, Asia, South America, and North America, most from areas where wild populations of the Russian wheat aphid occur (table 1). Most of the strains passed through at least one generation of quarantine in Europe (USDA-ARS European Parasite Laboratory, Behoust, France, recently moved to Montpellier, France) followed by another generation at the USDA Quarantine Laboratory in Newark, NJ, USA. Egg masses were shipped to Niles to start the colonies there. The number of individuals used to start the Niles colonies ranged from 20 to about 900 depending on the strain. A notable exception was the Delaware strain of CS which originated as about 18,000 adults captured in the field. We do not have information on how many individuals of each strain were collected and brought into quarantine in Europe. Each colony at Niles was maintained in isolation from other colonies of the same species. Although all of the samples were reared at the Niles, MI Laboratory, some of the samples were initially sent to W. Steiner, USDA-ARS, BCIRL, Columbia, MO. These were frozen and a portion shipped frozen to Fargo. The remaining samples were shipped live, directly from Niles, MI to Fargo, ND. They were frozen and stored at − 80 C. Fifteen strains representing four different species were examined. The species and their abbreviation are : *Coccinella septempunctata* 5 strains, *C. transversoguttata biinterrupta, Hippodamia variegata* 6 strains, and *Propylea quatuordecimpunctata* 3 strains.

Total DNA from individual insects was prepared as in Boyce *et al.* (1989). The number of insects used for each species was : *C. septempunctata* = 26 (F = 6, 5 each other strains), *C. transversoguttata biinterrupta* = 4, *H. variegata* = 24 (4 each strain), *P. quatuordecimpunctata* = 14 (F & U = 5 each, C = 4). The insects were dropped into liquid nitrogen prior to crushing. Homogenization was in 1.5 ml micro tubes using plastic pestles (Kontes Corp.). 1-2 μl of the DNA sample was used per amplification reaction. PCR reactions were carried out in 50 μl reaction volumes. 5 μl of 10X reaction buffer (Perkin-Elmer), 6 μl 25 mM $MgCl_2$, 1 μl each of four dNTPs, 0.5 μl Taq polymerase (2.5 inits), and 0.5-1.0 μl (= 0.075-0.15 μg) of primer were used per reaction. Each reaction was overlaid with one drop of mineral oil. Pipetting of components was done using pipet tips with filters to reduce the possibility of aerosol contamination by extraneous DNA in the pipet cylinder. Amplifications were carried out in a programmable thermal cycler (Perkin-Elmer) using the following modification of the program described by Black *et al.* (1992) : 1) 92 C for 30 sec, 2) 35 C for 1 min, 3) 5 min ramp up to 72 C, 4) 72 C for 2 min, 5) cycle to step 1, 45 times, 6) 72 C for 7 min, 7) 5 C hold until samples retrieved. The slow ramp from annealing to extension temperature improves the amplification from insect extracts (W. C. Black IV, personal communication ; Roehrdanz *et al.*, 1993). Other PCR

TABLE 1
Geographic Strains of Lady Beetles

Species	Collection	Strain	Origin
Coccinella septempuncta			
Coccinella septempuncta	EPL8939	M	Moldavia
Coccinella septempuncta	EPL8953	F	France
Coccinella septempuncta	—	D	Delaware (USA)
Coccinella septempuncta	T90030	S	Syria
Coccinella septempuncta	EPL89103	U	Crimea (USSR)
C. transversoguttata biinterrupta			
C. transversoguttata biinterrupta	EPL8988	K	Kirghiz (USSR)
Hippodamia variegata			
Hippodamia variegata	BIRL89113	C	Canada
Hippodamia variegata	EPL8954	F	France
Hippodamia variegata	EPL8989	K	Kirghiz (USSR)
Hippodamia variegata	T90015	M	Morocco
Hippodamia variegata	EPL8941	U	Moldavia (USSR)
Hippodamia variegata	PSRF9002	CH	Chile
Propylea quatuordecimpunctata			
Propylea quatuordecimpunctata	BIRL89112	C	Canada
Propylea quatuordecimpunctata	EPL8853	F	France
Propylea quatuordecimpunctata	EPL8940	U	Moldavia (USSR)

program modifications have also been reported to either enhance the yield of RAPDs or reduce the running time (Yu & Pauls, 1992). The PCR products (10 µl) were run on 1.5 % TBE agarose gels at 40-50 V for 6 hr along with the 1 kb ladder DNA size marker (Bethesda Research Laboratory), stained with ethidium bromide and photographed.

Three kits of 10-mer primers were obtained commercially from Operon Technologies (Kit C, Kit K, and Kit F). There are twenty different primer sequences in each kit. The following primers are specifically referred to in the results : CO7 : 5′-GTCCCGACGA-3′, C12 : 5′-TGTCATCCCC-3′, C14 : 5′-TGCGTGCTTG-3′, C15 : 5′-GACGGATCAG-3′, C16 : 5′-CACACTCCAG-3′, C18 : 5′-TGAGTGGGTG-3′, C19 : 5′GTTGCCAGCC-3′, K01 : 5′-CATTCGAGCC-3′, K02 : 5′-GTCTCCGCAA-3′, K15 : 5′-CTCCTGCCAA-3′, K19 : 5′-CACAGGCGGA-3′. Many of the other primers, especially from kits C and K were tested with two or more populations, but did not differentiate the populations sampled here.

RESULTS AND DISCUSSION

The amplification success of short random primers depends on the ability of the primers to bind to matching or near matching sequences in the genomic DNA. When two such sites are on opposite strands of the DNA with their 3′ ends facing each other and are approximately 100-2,000 bases apart, the PCR reaction is able to synthesize many copies of the region between the primer binding sites. These amplified regions are detected on gels that separate the fragments by size, producting a pattern of bands or different size DNA fragments. The amount of DNA in any specific amplification product (or band) can be a function of how precisely the primers blind to the DNA at either end of the fragment, how

often that particular fragment is repeated in the genome, how much secondary structure the DNA has in that region. These factors are in turn influenced by the annealing temperature, concentration of $MgCl_2$, the relative amounts of primer, template and nucleotides and other factors (Innis & Gelfand, 1990). An empirical method for selecting the $MgCl_2$ concentration used with all the primers is described elsewhere (Roehrdanz *et al.*, 1993). Because so many factors can produce artifacts that can be mistaken for polymorphism, both care and caution are necessary (Ellsworth *et al.*, 1993).

Using the 10 bp primers, almost any primer can produce polymorphisms that can be diagnostic for a given lady beetle species. Within species, many primers generate polymorphisms, but not all of these produce polymorphisms that are distinctive for different geographic populations. The procedure employed here to identify useful primers was to take one sample from each species (*C. septempunctata*, *H. variegata* and *P. quatuordecimpunctata*) and test the entire C - Kit and K - Kit (40 primers). The banding patterns produced were used to choose primers to be tested with additional samples and strains. Primers that produced a modest number of well defined bands received priority for further use. Those that produced many faint bands, or bands close together, or produced very few bands at all were relegated to the bottom of the list and used only if the selected ones did not provide the desired information. The rationale was that faint bands or many bands close together would be more difficult to score, while very few bands would be less likely to have a useful polymorphism. Since the diagnostic potential of polymorphisms produced by the primers not used is unknown, it may be just as efficient to proceed through the primer sets in numerical order.

Individuals from five different colonies of *C. septempunctata* were examined. Fig. 1A shows individuals of all five *C. septempunctata* strains and the single strains of *C. transversoguttata biinterrupta* using primer C-15. The *C. septempunctata* strains were originally collected in France (lanes 1-3), Delaware (4-6), Moldavia (10-12), Syria (13-15), and Crimea (16-18). Lanes 7-9 contain the *C. transversoguttata biinterrupta* samples. A total of 15 primers were used with at least two different strains. Table 2 shows how five of these primers can be used to distinguish among the five strains of *C. septempunctata*. The distinguishing bands, their approximate size, and whether they are present or absent are listed. Not every primer's pattern is given for each strain. For example, the C15 pattern for U (marked on fig. 1A) allowed it to be separated from the other four and no additional primers were needed for U. Primer C07 divided the remaining four into two pairs, F + D and M + S based on the presence or absence of bands 740-800 (table 2, no fig.). Strains F and D were separated by primers K15 (fig. 1B, band 1350) and C18 (band 600, not shown). For strains M and S, primers C16 (fig. 1C, band 310) and C18 (band 600, not shown) were diagnostic. Primer C18 could have been used to define the two groups F + M and D + S.

The *H. variegata* strains did not separate in such convenient couplets as the *C. septempunctata* strains. The *H. variegata* strains from Kirghiz (formerly USSR), Canada, France, Chile, Moldavia (formerly USSR), and Morocco are abbreviated K, C, F, Ch, U, and M respectively. Key banding patterns are listed in table 3. The K, CH, and U samples are quite readily distinguishable with the primers K02 and C16. Strain C was variable with respect to the 225 bp band produced by K02 (fig. 2A). Individuals that contained the band were distinguished from K by the presence of several additional bands in the 500-1,000 bp range. Strain C individuals that did not have the 225 bp K02 band clustered with F and M. F, M, and C were then distinguished using primers K01 (fig. 2A). Individuals that contained the band were distinguished from K by the presence of several additional bands in the 500-1,000 bp range. Strain C individuals that did not have the 225 bp K02 band clustered with F and M. F, M, and C were then distinguished using primers K01 (fig. 2A) and K19 (not shown).

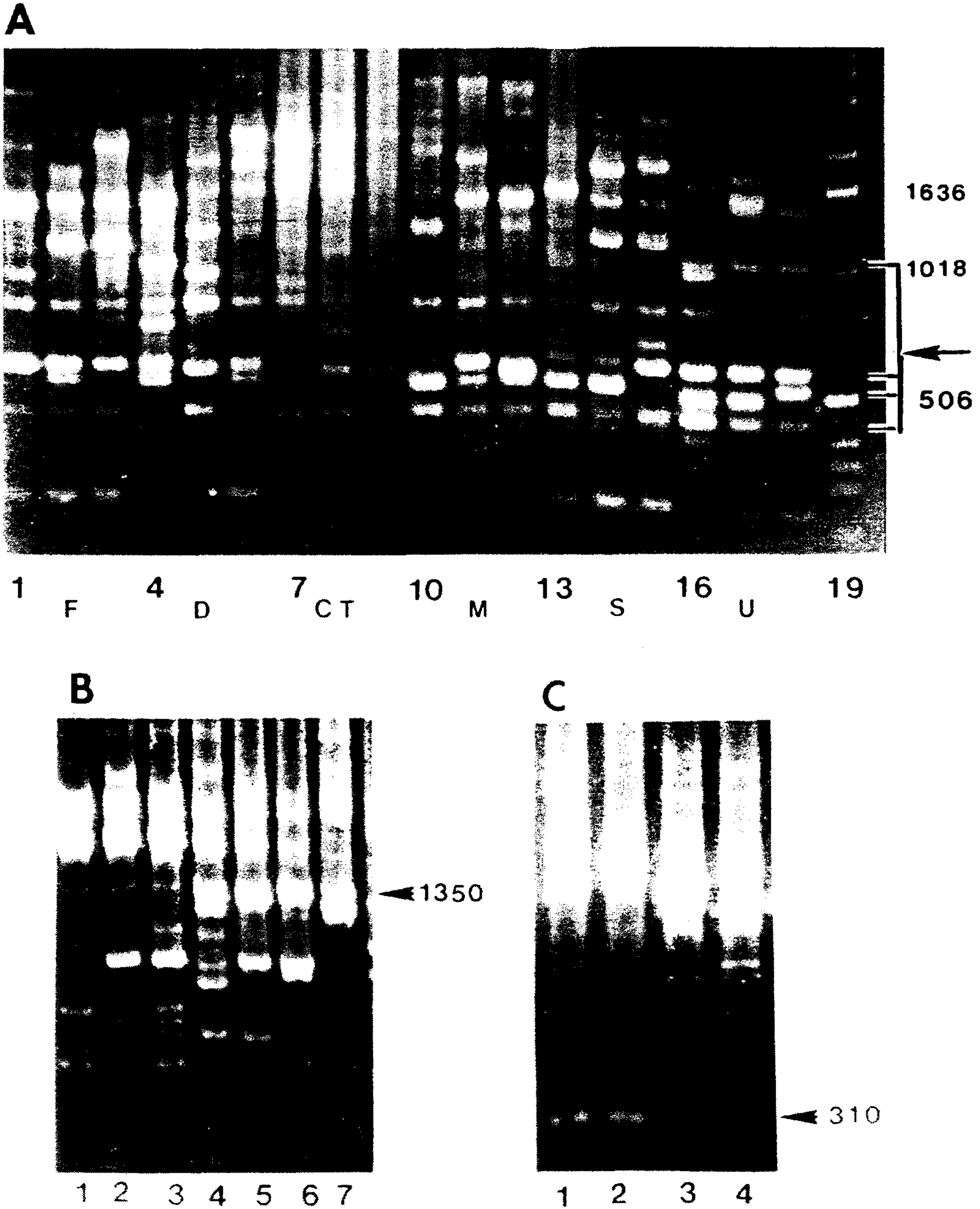

Fig. 1. [A] Amplified fragments of individuals from colonies of *Coccinella septempunctata* (CS) and *Coccinella transversoguttata biinterrupta* (CT) using primer C-15. (1-3) CS F, (4-6) CS D, (7-9) CT, (10-12) CS M, (13-15) CS S, (16-18) CS U, (19) 1 kb ladder from Bethesda Research Laboratories. [B] Comparison of CS F and CS D using K15 primer. (1-3) CS F, (4-7) CS D. Size estimates from 1 kb ladder. [C] CS M and CS S with primer C16. (1-2) CS M, (3-4) CS S.Size estimates from 1 kb ladder.

TABLE 2
Key to Coccinella septempunctata *populations*

Strain	Primer	Key Bands in Base Pairs
U	C15	~ 420, 500-520, 560, 1050 whole pattern
F	C15	"U" pattern absent or incomplete
	CO7	740-760 present
	K15	~ 1350 faint or absent
	C18	600 absent
D	C15	"U" pattern absent or incomplete
	CO7	740-760 present
	K15	~ 1350 present
	C18	~ 600 present
M	C15	"U" pattern absent or incomplete
	CO7	780-800 medium present or 600-900 absent
	C16	~ 310 present
	C18	~ 600 present
S	C15	"U" pattern absent or incomplete
	CO7	780-800 medium present or 600-900 absent
	C16	~ 310 absent
	C18	~ 600 absent, extra 1700 and 1900

TABLE 3
Key to Hippodamia variegata *populations*

Strain	Primer	Key Bands in Base Pairs
K	KO2	~ 225 present, 500-1000 absent
	C16	~ 650 absent
C	KO2	~ 225+/−, several 500-1000 present, ~ 460 absent,
	KO1	~ 600 absent
	K19	~ 750 absent or faint
F	KO2	~ 225 absent, 460 absent
	KO1	~ 600 absent
	K19	~ 750 present (bright)
CH	KO2	~ 225 absent, 460 present
	C16	~ 650 absent
U	KO2	~ 225 absent, 460 present
	C16	~ 650 present
M	KO2	~ 225 absent, 460 absent
	KO1	~ 600 present

Three strains of *P. quatuorde cimpunctata* from Canada (C), France (F), and Moldavia (U) were studied (table 4). The Canadian strain was readily separated from the other two with several different primers such as the 480 bp band with C12 (not shown) and the 950 bp band with C19 (fig. 3). However, the F and U lines proved to be very similar and the distinctions reported represent slight differences in band sizes and spacing rather than clear cut presence or absence of well defined bands.

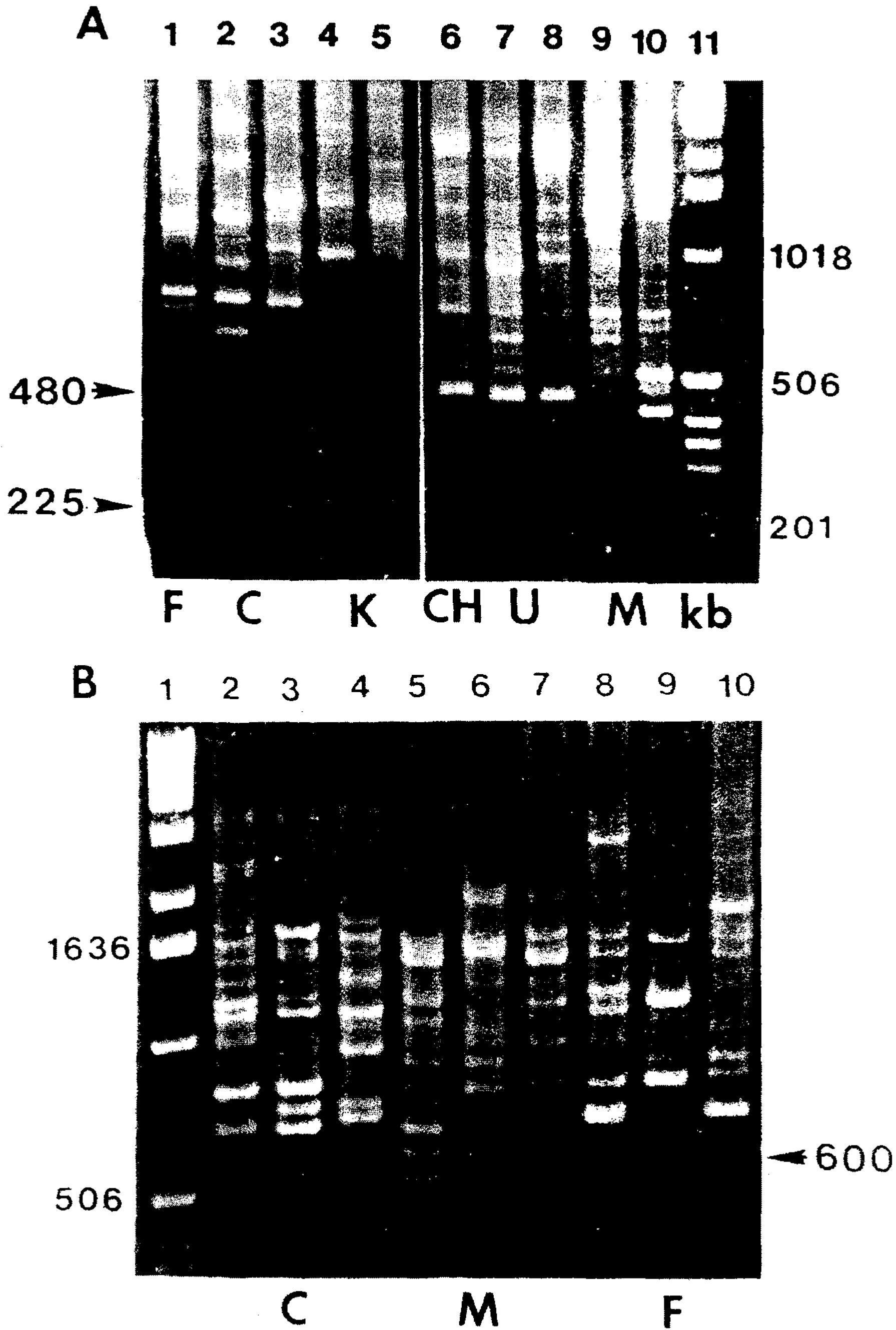

Fig. 2. [A] Amplified fragments of *Hippodamia variegata* (HV) DNA using primer K-02. (1) HV F, (2-3) HV C, (4-5) HV K, (6) HV CH, (7-8) HV U, (9-10) HV M, (11) 1 kb ladder. [B] HV strains C, M and F with primer K-01. (1) 1 kb ladder marker, (2-4) HV C, (5-7) HV M, (8-10) HV F.

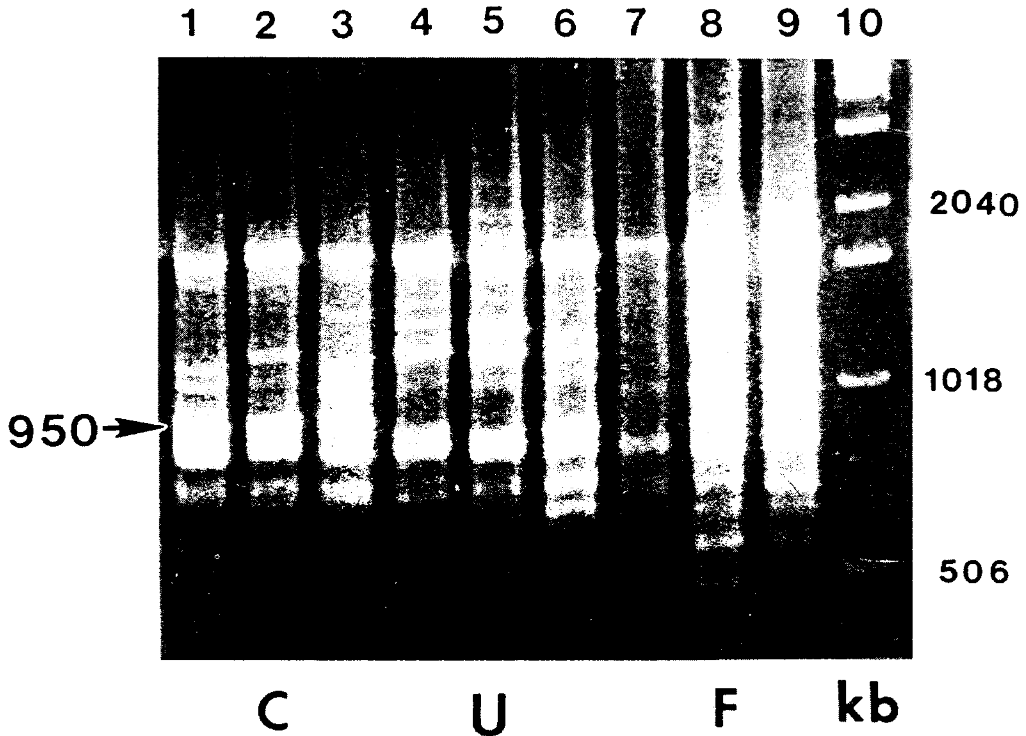

Fig. 3. Amplified DNA from *Propylea quatuordecimpunctata* (PQ) using primer C-19. (1-3) PQ C, (4-6) PQ U, (7-9) PQ F, (10) 1 kb ladder marker.

C. transversoguttata biinterrupta is represented by a single collection from Kirghiz in the former Soviet Union. It is morphologically similar to *C. septempunctata*. With most of the primers tested, its patterns were distinct from the *C. septempunctata* samples, however, it was much more similar to *C. septempunctata* than *C. septempunctata*, *H. variegata*, and *P. quatuordecimpunctata* were to each other. In a separate experiment, the 12S-16S mitochondrial rDNA region was amplified by standard PCR and cut with restriction endonucleases. The *C. transversoguttata biinterrupta* had RFLP patterns that indicated a small deletion in that region relative to the other *C. septempunctata* strains (Roehrdanz, 1992). A comparison of *C. septempunctata* and *C. transversoguttata biinterrupta* for primer C 15 is shown in fig. 1. The patterns for *C. septempunctata*, and *P. quatuordecimpunctata* for primer K01 are shown in fig. 4. It is evident that differences between the species can be quite distinctive.

Most of the products of RAPD-PCR are inherited as dominants and it is not possible to distinguish between homozygotes and heterozygotes (Welsh *et al.*, 1991b and Williams *et al.*, 1990). Therefore in designing experiments to follow the markers in a release project or a laboratory study of competition between two strains, it would seem preferable to have both strains positively marked, where each contains at least one marker that is absent from the other. This would permit recognition of at least some of the hybrids if interbreeding between strains occurs. Interstrain crosses should be done to find markers that are homozygous within a single strain. Release of marked exotics should, if possible, be

TABLE 4
Key to Propylea quatuordecimpunctata *populations*

Strain	Primer	Key Bands in Base Pairs
C	C12	~ 480 present
	C19	~ 950 presetn
F	C12	~ 480 absent
	C19	~ 950 absent, pair 1000 & 1300 present
	C14	widely spaced around 1000
U	C12	~ 480 absent
	C19	~ 950 absent, No pair 1000 & 1300
	C14	close double band ~ 1000

preceded by a survey of the extant population of that species in the region of the intended release. The release strain could be selected so as to maximize difference with the native population. Data is needed regarding the fate of banding patterns when two distinctively marked strains are reciprocally crossed and the progeny monitored for several generations.

One caveat regarding RAPD markers needs to be emphasized here. A RAPD marker is essentially a single allele in the genome. As such it is a member of a single linkage group and is subject to the normal processes of segregation and recombination. Over the course of generations of random mating with different populations in the field, more and more of the genome of the original marked strain will be scattered. In time only those genes most closely linked to the RAPD marker itself will remain with the marker. Unless there are no representatives of the same species in the field to interbreed with the released strain, what ultimately will be monitored is the establishment of alleles defined by RAPD marker. Theoretically a strain could be constructed that had multiple RAPD markers on each linkage group. However, that would be extremely impractical and would only serve to delay the genetic breakdown of the released strain in the field, not prevent it. Tracking a RAPD marker would therefore be most informative only in the first couple generations.

The behavior of RAPD markers as single alleles does suggest another potential use for them in biological control. If an important genetic character (e.g. insecticide resistance) is incorporated into a biological control species, it may be possible to find an identifiable RAPD marker tightly linked to the genetic character. This RAPD could be used to follow the progress of the desirable gene independent of the rest of the genetic make up of the carrier individuals.

The ease with which primers producing distinctive polymorphisms can be found using RAPD-PCR varies somewhat with species. Williams *et al.*, 1990 found an average of 1 polymorphism detected per primer for corn, 0.5 per primer in soybeans, and 2.5 per primer tested in Neurospora. Many more primers were tested to find diagnostic polymorphisms in samples of Hymenoptera that are parasitic on aphids (Roehrdanz *et al.*, 1993). Pairwise combinations of primers can also be used to generate additional patterns (Welsh & McClellend, 1991). Once the polymorphisms are identified, the procedure is much quicker than RFLP analysis and can be done with very small amounts of DNA (Ballinger-Crabtree *et al.*, 1992). This approach may be universal in the sense that fingerprints and useful polymorphisms are theoretically obtainable for virtually any species.

The availability of DNA markers for insect strains that are involved in biological control projects has significant potential. Some of these applications are discussed by Black *et al.*,

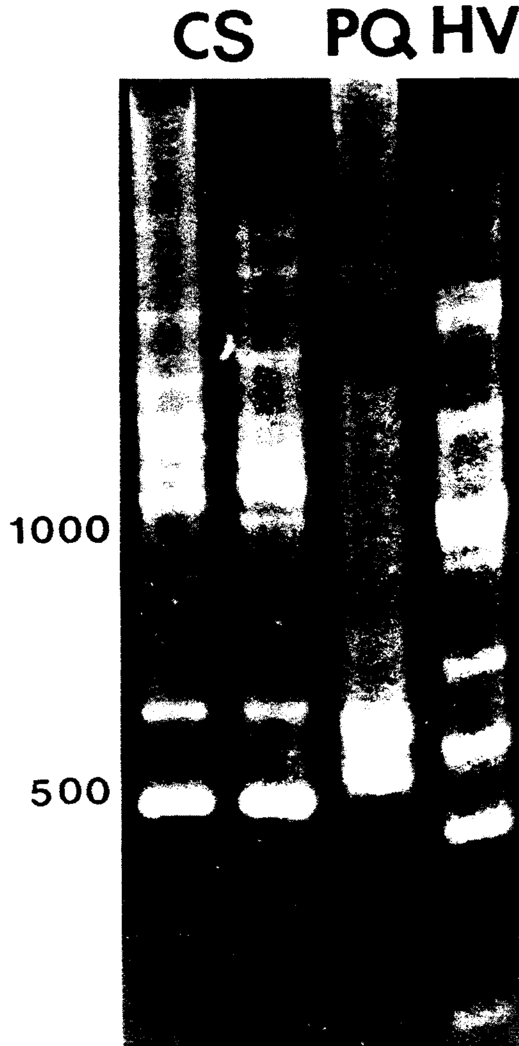

Fig. 4. Comparison of amplified DNA from different species with the same primer. (1-2) *Coccinella septempunctata* individuals with primer K-01, (3) *Propylea quatuordecimpunctata* with primer K-01, (4) *Hippodamia variegata* with primer K-01. Size markers from 1 kb ladder.

1992, Roehrdanz, 1992, and Roehrdanz *et al.*, 1993. Among the various uses would be tracking the survival of genes from a released strain in an area where a wild population of the same species already exists. The markers could also assist in the analysis of competition between two different strains to determine their relative survivability or effectiveness in the laboratory, in a cage, or in the field. Another important application would be in helping maintain the purity of commercial or research colonies. Previous work with the screwworm fly (Roehrdanz, 1989) and boll weevil (Roehrdanz & North, 1992) has demonstrated how

easy it is for laboratory colonies to become cross contaminated when there are no markers, visible or otherwise, to identify the individual strains of the same species.

The technique may also assist systematics determinations by providing a means of identifying closely related species that are morphologically similar. It may be especially helpful in identifying the species of immature insects which are often not as morphologically distinct as the adults. In the lady beetles both the larvae and adults are predators. Whether RAPD markers can provide important insights into the genealogical relationships between strains and species remains a matter of question. Ballinger-Crabtree *et al.*, 1992, Chapco *et al.*, 1992, and Landry *et al.*, 1993, describe methods of comparing the presence/absence of a large number of RAPD bands and inferring genetic relatedness.

In the lady beetles used here, there is extensive intra strain variation. Fig. 1A shows the banding patterns generated by a single primer with three individuals from each of five strains of *C. septempunctata.* The fraction of conserved bands ($F = 2 N_{xy}/N_x + N_y$) in each pair-wise combination was calculated for the 26 discrete bands identified on the gel photo. The average inter strain value was 0.56 (range 0.47-0.67) while the average intra strain value was only slightly higher at 0.66 (range 0.50-0.74). It was not really possible to distinguish the strains using this approach. Because the size of the founding population for each strain is not known, it is not possible to draw any inferences about the history of each strain. There is no correlation between the number of individuals that initiated the Niles colonies and the ease of finding unique markers or, in the case of *C. septempunctata,* the calculated value for F. Since the primary goal of this work is to quickly recognize strains that are being raised for biological control projects, the emphasis has been to recover single band markers that are easily screened and readily discriminate among the colonies. Questions regarding the genetic variability of extant populations would require additional study and might be more easily interpretable from PCR + sequencing or PCR + RFLP data than from RAPDs.

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RÉSUMÉ

Caractérisation du polymorphisme de l'ADN chez des prédateurs coccinellides par l'utilisation de la réaction de polymérisation en chaîne et de marqueurs *arbitraires* (RAPD-PCR).

On a caractérisé le polymorphisme de l'ADN chez quelques prédateurs Coccinellides qui ont été expérimentés comme agents de lutte biologique contre des pucerons et d'autres insectes en Amérique du Nord. La technique emploie une variante de la réaction de polymérisation en chaîne (PCR),

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appelée RAPD-PCR, qui utilise des amorces uniques choisies arbitrairement pour amplifier un groupe de séquences génomiques prises au hasard. L'utilisation de cette technique permet de faire la distinction parmi des colonies élevées au laboratoire et d'origines géographiques variées. Plusieurs colonies de chacune des 3 espèces ont été examinées (*Coccinella septempunctata, Hippodamia variegata et Propylea quatuordecimpunctata*). Il a été également possible de distinguer *C. septempunctata* d'une espèce voisine très proche, *C. transversoguttata biinterrupta*. La technique semble pouvoir constituer une source de marqueurs très utile pour maintenir des colonies et des gènes traceurs dans les projets de lutte biologique et pour identifier les espèces et les stades immatures des insectes.

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