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SHORT COMMUNICATION

Presence of haemocyte-like cells in coccinellid reflex blood

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Abstract. Contrary to current assumptions, the reflex blood of two-spot ladybirds, *Adalia bipunctata*, and seven-spot ladybirds, *Coccinella septempunctata*, contains haemocyte-like cells. Furthermore, DNA can be extracted and amplified from coccinellid reflex blood, confirming the presence of haemocyte-like cells and demonstrating a nondestructive method of DNA extraction.

Key words. Coccinellids, DNA extraction and amplification, haemocytes, reflex blood.

Introduction

Many groups of insects, including coccinellid adults and larvae, exude haemolymph when disturbed or attacked, a process termed reflex bleeding (Kay *et al*., 1969; Nicolson, 1994; Dixon, 2000; Muller *et al*., 2001). Adult ladybirds release this yellowish fluid (reflex blood) from tibio-femoral articulations and larval coccinellids from dorsal glands (Dixon, 2000). Coccinellid reflex blood is strong smelling, bitter and, for some species, contains toxic alkaloids that contribute to its use as an antipredator defence mechanism (Majerus, 1994). Species of ladybird often produce a variety of alkaloids but there is generally one major alkaloid (Schroder *et al*., 1998); for example, adaline is the major alkaloid in *Adalia* species and coccinelline in most *Cocinella* species. The concentration of alkaloids is similar in the haemolymph and reflex blood, which suggests the fluid is exuded directly from the haemolymph (Dixon, 2000).

There is variability among species relating to the mechanism of secretion and presence of cells in the reflex blood. Nucleated haemocytes have been found in the defensive secretions of alder leaf beetle larvae, *Agelastica alni* (Bunnige & Hilker, 1999); however, it is generally stated that the composition of coccinellid reflex blood is similar to haemolymph but minus haemocytes (Dixon, 2000). The fluid released by coccinellids can be very high, comprising up to 20% of fresh body weight after a single attack (Grill & Moore, 1998). It has been assumed that a filtering mechanism exists to remove haemocytes from the fluid and so reduce the energetic costs of this process, but the physiology and biochemistry underlying reflex bleeding is poorly understood (Grill & Moore, 1998).

The aim of this study was two-fold. First, to look for the presence of cells in the reflex blood of the two-spot ladybird, *Adalia bipunctata* L., and the seven-spot ladybird, *Coccinella septempunctata* L., and second, to assess whether reflex blood could be used as a nondestructive method of sampling DNA from ladybirds. Modern molecular techniques such as the polymerase chain reaction (PCR) provide an effective tool for studying population structure and dynamics from a genetic perspective (Jarne & Lagoda, 1996; Sunnocks, 2000). Extraction of DNA from insects usually involves either partial dissection or complete destruction of an individual.

Materials and methods

Insect cultures

Field-collected (Cambridgeshire, U.K.) *A. bipunctata* and *C. septempunctata* adults were used in all experiments. The adult ladybirds were placed individually in 9-cm triple-vented Petri dishes at 20–25 °C and fed to satiation with a mixture of aphid species (mainly *Acyrthosiphon pisum* and *Sitobion avenae*).

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Assessment of cells in reflex blood

Reflex blood was collected by placing an individual adult ladybird (*C. septempunctata* or *A. bipunctata*) over a glass microscope slide and applying slight pressure until reflex blood was seen to exude from the ladybird. Gloves were worn throughout this procedure to minimize the risk of contamination. A drop of TE buffer [1 M Tris, pH 8; 0.5 M ethylenediaminetetraacetic acid (EDTA)] was added immediately and thoroughly mixed with the reflex blood. The preparation was then stained by application of a drop of acetic orcein and a coverslip was placed over the sample. The slide was then slowly heated, for 60 s, to approximately 60 °C over a spirit lamp. Finally, the preparation was gently squashed under a paper towel to flatten the cells. The reflex blood was viewed via oil immersion at × 1000 magnification using an Olympus BX40 microscope (Olympus, Japan).

DNA extraction and amplification

For DNA extraction and subsequent amplification, reflex blood was collected using a different procedure from that described above. An individual ladybird (either *C. septempunctata* or *A. bipunctata*) was placed in an Eppendorf tube and pressed gently against the side of the tube with an inverted end of a clean paintbrush. Droplets adhering to the side of the tube were washed to the bottom with 100 µL of TE buffer. DNA was then extracted using a Qiagen DNeasy Tissue Kit (Qiagen, U.K.). DNA was also extracted from whole ladybirds using a phenyl-chloroform method after snap freezing specimens in liquid nitrogen, grinding and incubating for 1 h at 65 °C in a 250-µL digestion buffer containing 100 mM Tris-HCl, pH 8; 80 mM EDTA; 160 mM sucrose; 1% SDS and 2.5 µL 20 mg/mL proteinase K. This latter DNA was used as a positive control in the PCR reactions.

The use of polymorphic microsatellite markers has been particularly effective in the study of population dynamics and mating systems in a wide range of species. PCR was therefore used to amplify microsatellite containing sequences recently identified within the two spot ladybird (*A. bipunctata*) genome (Haddrill *et al.*, 2002). A number of these same microsatellite sequences have been shown to be amplifiable within the seven spot ladybird genome (J. Bartolome, personal communication). All PCR reactions were carried out in 20 µL containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 10 ng each primer, 1 U of *Taq* DNA polymerase (ABgene, U.K.) and varying amounts of DNA. For whole ladybird extracts, 20 ng DNA was added to each PCR reaction. Levels of DNA extracted from the reflex blood were too low to be quantified; thus, each 20 µL PCR reaction contained 2 µL of the DNA extract. PCR reactions were carried out in a Perkin Elmer Gene Amplification PCR system 2400 DNA thermal cycler (Perkin Elmer, U.K.), using the following cycling conditions: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, annealing temperature for 1 min, 72 °C for 1 min; 72 °C for 10 min. Annealing temperatures were 60 °C for Ab35 and 58 °C for Ab38. Amplification products were visualized on a 2% agarose gel containing 20 µL of 20 mg/mL ethidium bromide. Fragments sizes were estimated by comparison to a ϕX174 RF DNA *Hae*III digest ladder (AB gene).

Results and discussion

Cells were clearly visible in the reflex blood of all six adult *A. bipunctata* and six *C. septempunctata* ladybirds from which samples were taken (Fig. 1a,b). The fact that cells were consistently seen at similar levels in all reflex blood samples examined strongly suggests that they are haemocytes which are released when these two species of ladybirds reflex bleed. This conclusion is strengthened by the morphological similarity with haemocytes from other species; for example, the haemocyctes identified in the defensive fluid of larvae of the alder beetle (Bunnige & Hilker, 1999). The possibility exists that the cells were not secreted with the haemolymph but are contaminants from other parts of the ladybird affected during the reflex bleed. However, the cells were present at a consistently high number in all samples examined and their uniform morphology supports the conclusion that the

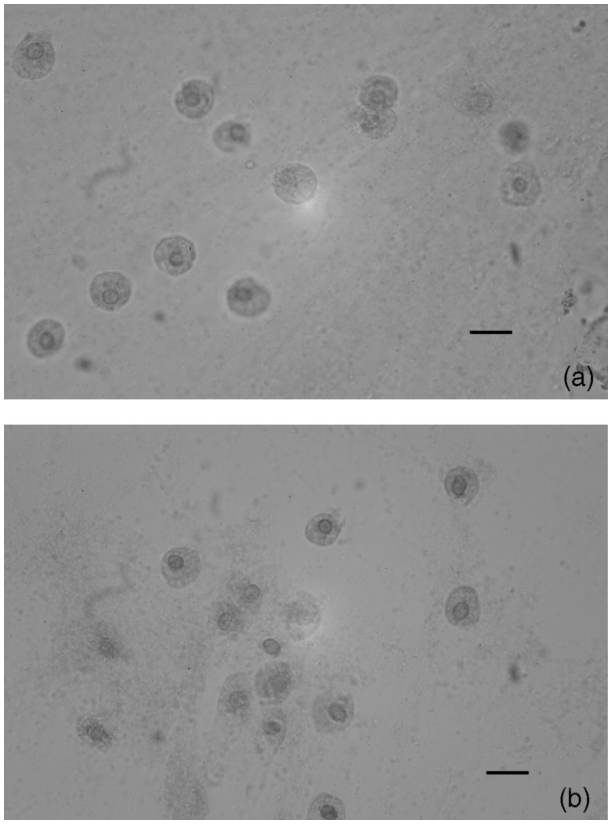

Fig. 1. Haemocyte-like cells stained within reflex blood samples taken from (a) *Adalia bipunctata* and (b) *Coccinella septempunctata*. Scale bar = 10 µm

cells are a true constituent of the haemolymph of these two ladybird species, and also that cells are regularly secreted at a reflex bleed.

The consistent presence of nucleated cells in reflex blood suggests that it would be possible to extract sufficient DNA from a reflex bleed for PCR analysis. Preliminary work with whole ladybird DNA demonstrated that locus Ab35 in the two-spot ladybird genome and locus Ab38 in the seven-spot ladybird genome showed the most reliable amplification. These two microsatellite loci were therefore used in this investigation. The results were mixed. Amplification of the Ab35 locus occurred in only 20% ($n = 10$) of *A. bipunctata* adult reflex blood DNA extracts (Fig. 2). Fifty percent ($n = 8$) of *C. septempunctata* adult reflex blood DNA extracts gave amplification of the Ab38 locus.

Our results show that it is possible to extract amplifiable DNA from the reflex blood of these two species. Indeed, they confirm the presence of nucleated cells in the reflex blood of *C. septempunctata* and *A. bipunctata* adults. The amplifiable DNA can be assumed to have been extracted from the cells observed microscopically in preparations of the reflex blood. However, failure to successfully amplify the Ab35 and Ab38 microsatellites in all extracted DNA samples might be the result of problems in the DNA extraction procedure or of the PCR. Non-destructive methods of extracting DNA are routinely used to recover DNA from animals (Taberlet & Luikart, 1999). Thus, the most probable explanation for failure with respect to amplification is inappropriate PCR amplification conditions; for example with the low levels of DNA that would have been recovered from the reflex blood, adjustments may need to be made to $MgCl_2$, primer and/or dNTP concentrations. Weak or absent PCR amplification also occurs if the *Taq* DNA polymerase is inhibited by contaminants in the DNA sample. It is possible that one of the alkaloids or other toxic substances present in the reflex blood is not fully removed by the extraction process and remains to interfere with the PCR.

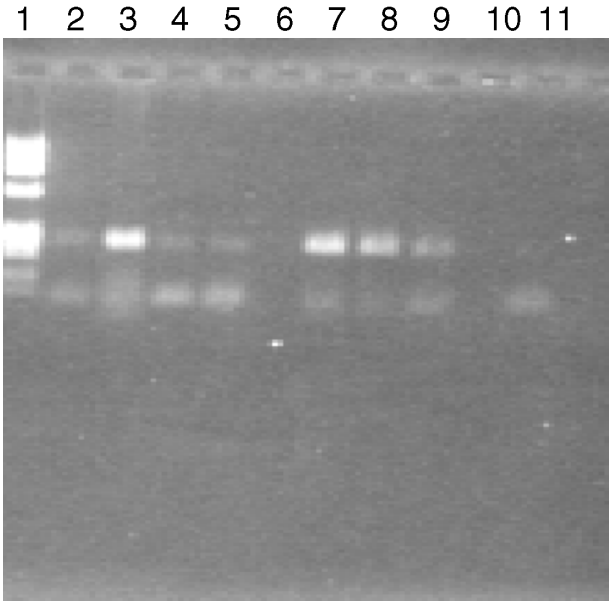

Fig. 2. Amplification of microsatellite Ab35 in DNA extracted from whole *Adalia bipunctata* (lanes 7–11) and reflex blood (lanes 2–5, with clear amplification in lane 3). Lane 1 = ladder.

In conclusion, both *A. bipunctata* and *C. septempunctata* contain nucleated cells in their reflex blood and these are likely to be haemocytes derived from the haemolymph. It has proved possible to extract amplifiable DNA from the reflex blood of both species, although amplification by PCR was sporadic. For reflex blood to provide a reliable non-destructive source of amplifiable DNA, further work is needed to optimize the extraction and PCR procedures.

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