Genotypic variation among different phenotypes within aphid clones

GUGS LUSHAI¹*, HUGH D. LOXDALE², CLIFF P. BROOKES²,
NICOLA VON MENDE², RICHARD HARRINGTON² AND JIM HARDIE¹

¹ *Aphid Biology Group, Department of Biology, Imperial College at Silwood Park, Ascot, Berkshire SL5 7PY, UK*

² *Entomology & Nematology Department, IACR-Rothamsted, Harpenden, Hertfordshire AL5 2JQ, UK*

SUMMARY

Most aphid species (Hemiptera: Aphididae) are parthenogenetic between periods of sexual reproduction. They are also highly polyphenic, with different adult morphs occurring in the life cycle, *viz.* winged, wingless, asexual and sexual. It is assumed that aphids born in a parthenogenetic clonal lineage are genetically identical regardless of the final adult form (with the exception of sexual forms). Using the randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) we have found that different asexual adult phenotypes (winged and wingless) of some clones of two cereal aphid species (the grain aphid, *Sitobion avenae* (F.) and the bird-cherry aphid, *Rhopalosiphum padi* (L.)) may be distinguished by the presence or absence of one or more RAPD-PCR bands. In three of nine clones examined, such differences were found, and Southern blotting and hybridization of the discriminating bands confirmed these to be of aphid origin, rather than due to endosymbiotic bacteria or contaminating fungi. The main 248 and 296 bp bands, in the two species, respectively, were sequenced and found to be A/T rich. The smaller band showed 57% homology with white striated muscle over a stretch of 90 bp. Genomic DNA treated with dimethyl sulphoxide to remove secondary structures still showed differences in RAPD-PCR profiles between winged and wingless morphs within the unusual clones. This discovery may be widespread and therefore it is important to understand the phenomenon in relation to clonal organisms.

1. INTRODUCTION

It is generally accepted that parthenogenetic organisms, such as aphids, remain genetically identical (Blackman 1979; Tomiuk & Wöhrmann 1982; Young 1983; Hughes 1989; Carvalho *et al.* 1991). However, previous studies on aphids using enzyme markers (e.g. cholinesterase; Beranek & Berry 1974) have shown heritable changes in banding profiles, which may be due to contamination of clones (Blackman 1979), and work on the esterases conferring resistance to pesticides in the peach-potato aphid, *Myzus persicae* (Sulzer), has shown variable patterns of intensity within highly resistant clones (Devonshire & Field 1991). These highly resistant clones, i.e. R3, the result of amplification of the esterase-4 (E4) gene, are 'plastic' in terms of genetic expression. The phenomenon is reversible in the absence of continuous chemical selection and is brought about by changes of DNA-methylation (Field *et al.* 1989; Field & Devonshire 1992; Blackman *et al.* 1995). Genetic variation within clones has been indicated in studies of the grain aphid *Sitobion avenae* (F.) by De Barro *et al.* (1994) using the radiolabelled oligonucleotide probe (GATA)₄ (changes in DNA fingerprints were observed in a clonal lineage

reared for 12 generations) and Lushai *et al.* (1997) using the randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR; Welsch & McClelland 1990; Williams *et al.* 1990) (germ line mutation noted in the 14th generation). In addition to intracolonial genetic variation, hitherto indistinguishable interclonal differences have been revealed by Simon *et al.* (1996) in holocyclic (with sexual phase) and anholocyclic (asexual) clones of the bird-cherry aphid, *Rhopalosiphum padi* (L.), using RAPD-PCR and mitochondrial DNA (mtDNA) markers.

In this paper, we report intracolonial, intermorph RAPD-PCR profile differences in two cereal aphid species.

2. STUDY SPECIES

The grain aphid, *S. avenae*, is a major pest of cereals in Europe (Vickerman & Wratten 1979). It is considered to be monoecious on Gramineae (grasses and cereals) and largely anholocyclic in southern Britain (Loxdale *et al.* 1985; Hand 1989), although in the north of Britain, a larger proportion of holocyclic clones occurs (Newton & Dixon 1988). Anholocyclic lineages have only two morphs (wingless and winged), whereas holocyclic lineages have up to seven morphs (three wingless and four winged). However, there are other aphid life cycles, i.e. androcyclic and inter-

* Present address and correspondence: School of Biological Sciences, Biomedical Sciences Building, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK.

mediate, full details of which are reviewed by Kawada (1987). Yield loss to cereals is caused by direct physical damage, and to a lesser extent in eastern England by the transmission of mild forms of barley yellow dwarf virus (BYDV) (Plumb 1977; Vickerman & Wratten 1979). In contrast, the bird-cherry aphid, *R. padi*, which is assumed to be predominantly holocyclic and host-alternating between bird cherry, *Prunus padus* L. (overwintering woody host), and Gramineae (secondary host) in Britain, causes significant yield loss in south and south-western England mainly as a result of transmission of more severe strains of BYDV (Plumb 1977).

3. METHODS

(a) *Insect cultures*

Aphids were maintained on barley seedlings (*cv.* Puffin (*S. avenae*); Igrí (*R. padi*)) under constant conditions (18 °C, 16 h light: 8 h dark). Six clones of *S. avenae* established in 1991–92 from field populations across eastern (Morley, MOR4, and Moulton, MOU5) and northern Britain (Stirling, S2 and S2.S19; Edinburgh, E3; and East Aquhorthies, EA2; Helden 1993) were screened for intracolonial differences between asexual summer wingless and winged females (apterous and alate virginoparae). These represented a range of life cycle types, including holocyclic (S2, S2.S19), anholocyclic (E3) and androcyclic (MOR4, MOU5, EA2). Two holocyclic clones of *R. padi* were collected in 1990 from central (Leeds) and northern Britain (Roslin) (Lushai *et al.* 1996) and examined for similar differences. Morphs studied in *R. padi* included apterous and alate virginoparae, asexual autumn winged females (gynoparae) and the sexual forms (oviparae and males). The gynoparae and sexual forms were induced by long night lengths (10 h light: 14 h dark) as described by Lushai (1994). In both *S. avenae* and *R. padi* the asexual alate individuals were induced by crowding (Lees 1967). All clones were reared either in Blackman boxes (Blackman 1971; *S. avenae*) or as described by Austin *et al.* (1991; *R. padi*).

(b) *DNA preparations and PCR reactions*

DNA was prepared as crude samples from live or liquid N₂-frozen aphids (after Black *et al.* 1992) or was purified using three phenol/chloroform DNA extraction methods (Sambrook *et al.* 1989; Milligan 1992; De Barro *et al.* 1995). The samples were stored at *ca.* 4 °C prior to use. PCR reactions were as described by Black *et al.* (1992) and De Barro *et al.* (1995), with Taq-polymerase from Northumbria Biologicals or Appligene. A selection of 10-mer primers from the Operon-B, -E and -F kits (Operon Technologies, California), as well as two cited by Black *et al.* (1992) (BAM and ECO) were tested. All the PCR reactions were done using Hybaid Omnigene thermal cyclers. The products of PCR and DNA size markers (Promega) were separated electrophoretically on 1.2% agarose gels (Sigma) at 100 V using 0.5 or 1.0 × TBE buffer and visualized by ethidium bromide staining and UV fluorescence. In all amplifications a negative control, containing all the reaction components except template DNA, was run. DNA was quantified using a fluorometer, and serial dilutions of DNA established that 25–50 ng gave reproducible RAPD-PCR profiles. Each sample was replicated at least 10 times for each PCR experiment. Individuals that were up to six months apart, and represented many parthenogenetic generations comprising alternations between wingless and winged morphs, were selected for DNA testing. DNA samples from head versus whole-insect

extractions were also tested by PCR to eliminate the possible involvement of endosymbiotic bacteria such as those detected by Simon *et al.* (1996). Dimethyl sulphoxide (DMSO) treatment was used in a PCR reaction to improve the stringency of the PCR reaction.

(c) *Digestion and hybridization of PCR-product*

A hybridization assay was carried out to see if morph-differentiating bands were of aphid genomic origin. The genomic DNA of apterous and alate *S. avenae* and of apterous, gynoparaous, oviparaous and male *R. padi* (head and thorax only) were restricted using excess amounts of *Hind* III and *Hinf* I (Appligene) (Sambrook *et al.* 1989). Southern blots of the digested DNA (Southern 1975; protocols after Pharmacia Biosystems) were probed under stringent conditions (washed at 58 °C) with a morph-discriminating band from each of the two species. Bands had been previously purified using a Qiaex II agarose gel extraction kit (Qiagen) and radio-labelled using an oligolabelling kit (Pharmacia).

(d) *DNA sequencing of isolated PCR fragments of S. avenae and R. padi and database searches*

PCR fragments from phenol-chloroform extracted DNA samples of *S. avenae* (250 bp) and *R. padi* (at 350 bp compared with the 100 bp ladder (Gibco)) were excised from agarose gels and purified as above. The prepared insert was ligated into a pGEM-T vector (Promega) and transformed into JM109 high efficiency competent cells (Promega) with colour selection for recombinants. The inserts of positive plasmid clones were sequenced from double-stranded DNA with T3 and T7 primers (Promega), applying the sequenase kit (USB).

The screening program BLAST (Altschul *et al.* 1990) was used to search the EMBL, Genbank, Eukaryotic-promoter (EPD) and *Escherichia coli* (ECOLI) databases for sequence homology.

4. RESULTS

Of 25 primers screened, one showed intracolonial differences in RAPD-PCR profiles in *S. avenae* (5'-CCTGATCACC-3' OPF-03; Operon Tech.) and one in *R. padi* (ECO; 5'-ATGAATTCGC-3'; Black *et al.* 1992).

(a) *Morph differences*

(i) *S. avenae*

Using purified DNA samples, apterous and alate virginoparae (wingless and winged asexual females) could be discriminated by the presence of a *ca.* 250 bp band in the former morph in one clone (E3; anholocyclic) of six clones tested over the five trials (figure 1*a*).

(ii) *R. padi*

RAPD-PCR of crude DNA preparations (Black *et al.* 1992) revealed polymorphism across all morph types (central clone; see §3). The profiles of morphs were reproducible between replicates and over time (6 months) (figure 1*b*). The apterous and alate virginopara were distinguished by a 180 bp band; both these morphs could be differentiated from the gynopara

Figure 1. RAPD-PCR of aphids showing different intraclonal profiles. (a) Purified DNA of *S. avenae*: lane 1 (L1 aptera, L2 alata); (b) crude DNA of *R. padi* (inverse image): L1 aptera, L2 alata, L3 alata heads, L4 gynopara, L5 male, L6 ovipara, L7 water control; (c) purified DNA of *R. padi*: L1 molecular marker, L2 aptera, L3 male, L4 gynopara, L5 ovipara, L6 water control; (d) purified DNA of *R. padi* with DMSO: L1 aptera, L2 male, L3 gynopara, L4 ovipara, L5 water control, L6 molecular marker.

(winged presexual female) by an additional *ca.* 820 bp band. In the male (also winged), a 280 bp band, common to all the other morphs, was missing. Lastly, the ovipara (wingless sexual female) was distinguished from the gynopara by two further bands above 820 bp. In contrast, the RAPD-PCR profile of crude DNA preparations of another *R. padi* clone tested (northern) did not show intramorphic differences (data not presented). However, when pure DNA was used from this clone, band differences were found (figure 1c). The asexual summer form (wingless aptera) could be differentiated from the gynopara/sexual forms by an additional 350 bp band, although there was no difference between the gynopara and the sexual forms (figure 1c).

(b) Investigating secondary structure in the DNA template

As it was considered that the observed differences in RAPD-PCR banding profile between aphid morphs could be the result of influences of secondary structures in the DNA template, the procedure was repeated following DMSO treatment of the DNA. This simplified the RAPD-PCR profile of the pure DNA prep-

aration (northern clone). The aptera and male of *R. padi* gave identical RAPD-PCR profiles, as did the gynopara and ovipara. However, both pairs could be readily distinguished by a 600 and 400 bp band (figure 1d).

(c) Aphid band verification

To verify that the morph-discriminating RAPD bands were truly of aphid genomic origin rather than due to microbial or fungal contamination, a Southern blot analysis was done with both species. With *R. padi* the analysis of purified genomic DNA showed homology to the diagnostic probe in the region of 23 kb as well as to several small bands (0.6–0.8 kb) in the *Hind* III digest and an additional band *ca.* 3 kb in the *Hinf* I digest (figure 2). There was weaker homology indicated by the *S. avenae* probe (results not shown).

(d) DNA sequencing

The two discriminating bands (*S. avenae*, 250 bp (see figure 1a), and *R. padi*, 350 bp (see figure 1c)) were sequenced to confirm that these were not of microbial or fungal origin, as well as to search for possible known

Figure 2. Southern analysis. (a) Autoradiograph of genomic DNA of *R. padi* probed with the diagnostic fragment: L1 *Hind* III digest, L2 *Hinf* I digest of DNA from apterae. Molecular sizes given in kb. (b) Complementary agarose gel showing complete digestion of the purified genomic DNA with the first lane representing the molecular marker.

sequence homologies. The *S. avenae* fragment (248 bp) was found to have an A/T content of 63%, the *R. padi* fragment (296 bp), 57% (table 1). Searches for looping structures revealed 7 and 15 stems in the *S. avenae* and *R. padi* sequences, respectively (setting: minimum stem = 6; minimum bonds/stem = 12 and maximum loop

size = 20). For both sequences, no high homology was found to any known sequence. Short stretches of DNA (15–20 bp) showing homology (80–90%) were found in a diverse array of species sequences and most of the homologies were based on A/T-rich regions. Of particular interest were matches of the *S. avenae* sequence with the promoter region of the myosin gene sequence for white striated muscle (accession# EPD:EP011080)) showing 57% homology over a stretch of 90 bp.

5. DISCUSSION

With *S. avenae*, previous work has shown a different (GATA)₄ DNA fingerprint after the twelfth generation in culture, which, disregarding clonal contamination, could have been due to a mutation in the repetitive sequence hybridized (De Barro *et al.* 1994). More recently a germ-line mutation has been reported after the fourteenth generation in a clonal lineage of *S. avenae* using RAPD-PCR (Lushai *et al.* 1997). The present results on *S. avenae* and *R. padi* using RAPD-PCR are clearly different, as some genetic variation observed is between sister phenotypes within a generation (apterous and alate virginoparae). What is strange is why adult aphids should differ when a lot of the DNA being sampled in apterae, alatae and gynoparae is from brooded embryos that have yet to undergo morph determination. This suggests that only fully differentiated tissues vary, and that there is a lot of tissue-specific endoreplication of DNA that differs between morphs (R. L. Blackman, personal communication).

Besides changes in DNA methylation related to the action of regulatory genes as occurs in the spontaneous loss of insecticide resistance status in *Myzus persicae* (Devonshire & Field 1991; Field & Devonshire 1992; Hick *et al.* 1996), another type of chromosomal methylation mechanism is responsible for genetic differences observed at different stages of development, i.e. those noted in the nuclear organizer region (NOR) of the B-chromosome in grasshoppers, *Eyprepocnemis plorans* (Charpentier) (Lopez-Leon *et al.* 1995). Because of the nature of RAPD-PCR amplification and as

Table 1. *Sequence of the PCR fragments from S. avenae and R. padi*

species/fragment (bp)	sequence
<i>S. avenae</i> (248)	5'- CCTGATCACCAAGGAGAGTACGTAGCTGAGCAAAATAT GGCCCATGACGATTATTATTACCAAAATCATCCAAAGGA AGGGGAAAATGAAAATCAAGTTGATCAACACTATCAAG AGCATCAAGGAAATTATGCCAATGAGAGCAAGACAATT ATTATTACGATAATAATTCTAATGTTAACACAGAAGGCA TCCTGAAGACCAATATGACGAAAATGCTCAACAGGAAA CATACACAGGTGATCAGG-3'
<i>R. padi</i> (296)	5'- ATGAATTCGCACTTCGAATATCAAGCCGTTTCAGCAACGC TTAAGCGGGCGGCAACCTTTTGTGTATATCAAGGGCGT AATATAGCCAAATATATTAAAAAATTAACACTTGTA TCGAGCCGGTATGTCGTTGACTCTGGAGATGCTGACTGC TGACCTGCTGACGACACGTGTTGTGTAAAAAAAATGC ACTCATGGGCTGTAATCGCAATAGGTACACTACTATTTCG TGAGTACGTATAGCCGGACGACCACTTGAGTCCGATCGA TAAATGCCAACCTGCGAATTCAT-3'

primers bind to A/T-rich regions, both these phenomena are unlikely to account for the present results (L. M. Field, personal communication).

The fact that the radioactive diagnostic probes annealed to the genomic DNA excludes the possibility that foreign DNA, i.e. symbiotic bacteria (Rouhbachsh *et al.* 1994) or fungi (Fenton *et al.* 1994) have contributed to intraclonal differences. Maternal transmission of these associated organisms from generation to generation, makes it unlikely that DNA contamination by them would have resulted in repeatable intraclonal variation. We attribute probe homology at several sites throughout the aphid genome to hybridization to repeat motifs. The RAPD-PCR primer could amplify across one or more 'middle repetitive DNA' sequences in such tandem repeats, which are regions renowned as sites where transposons operate (Charlesworth 1987). This may explain the morph changes noted, but does not explain replicable differences over time.

Results from the sequencing data fail to show any high homology with known sequences, although with *S. avenae*, the best fit was congruent with a striated muscle myosin sequence. This is of interest considering the wingless *versus* winged aphid phenotypes studied. However, the high A/T content of the sequences indicates non-coding regions or repetitive sequences as revealed by Southern blotting.

The present phenomenon cannot be analogous to that reported by Perrot-Minnot & Navajas (1995), who observed RAPD-PCR profile differences between the sexes of predatory mites, *Typhlodromus pyri* Scheuten. They attribute these RAPD-PCR differences to partial paternal genome loss (PGL) in early embryogenesis. This is quite different to the present case because no sexual recombination step has occurred in producing the morphs tested (Blackman 1987). However, if under-replication of chromosomes took place early in embryogenesis, the resulting somatic mutation would involve a number of cells that may be detectable by RAPD-PCR, but not transmitted in the germ-line. Zacharias (1993) has noted such genomic redundancy resulting in the under-representation of heterochromatic sections relative to euchromatin in endonucleated nuclei of the fruit fly, *Drosophila nasutoides* Okada. The diminution of chromosome 4 in somatic nuclei appeared to occur in many tissues. Therefore the phenomenon we describe may be similar.

The present phenomenon and that described by Simon *et al.* (1996), i.e. differences between holocyclic and anholocyclic clones, are probably of a different nature, yet both are possibly related to factors involved in the binding of RAPD-primers to the original template DNA, as the differences in the DMSO trial indicate. In the case of the phenomenon described by Simon *et al.* (1996), the difference between RAPD-PCR profiles noted between holocyclic and anholocyclic clones of *R. padi* appears to be well correlated with mtDNA and symbiotic-bacterial DNA PCR profiles. Therefore, it is probable that a degree of behavioural and ecological specialization may operate in these particular clones and that there may be restricted gene flow between them. In contrast, our

finding is exclusively intraclonal, and the differences presently observed within clonal lines of cereal aphids may be common to morph or developmental stages of other invertebrates. If the phenomenon is widespread, researchers studying the population genetics of clonal organisms using RAPD-PCR and other molecular markers (Loxdale *et al.* 1996) will have to screen for the occurrence of such intraclonal variability and, if detected, will have to limit studies to a single morph or life stage. Such an approach will hopefully further elucidate the genetics of the phenomenon.

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