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Kevin A. Shufran
USDA-ARS

Z. B. Mayo
Univesity of Nebraska - Lincoln, zmayo1@unl.edu

Teresa Crease
University of Guelph, Guelph, Ontario N1G 2W1, Canada

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Intraclonal genetic variation: ecological and evolutionary aspects.
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Genetic changes within an aphid clone: homogenization of rDNA intergenic spacers after insecticide selection

KEVIN A. SHUFRAN[1,*], Z B MAYO[2] and TERESA J. CREASE[3]

[1]*USDA-ARS, Plant Science and Water Conservation Research Laboratory, 1301 N. Western Road., Stillwater, OK 74075, USA*
[2]*Department of Entomology, 202 Plant Industry Building, University of Nebraska, Lincoln, NE 68583, USA*
[3]*Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada*

A single asexual maternal lineage (i.e. clone) of the greenbug aphid, *Schizaphis graminum* (Rondani) was repeatedly selected with the insecticide disulfoton (*O*,*O*-diethyl *S*-[2-(ethylthio)ethyl] phosphorodioate). A parallel colony of the non-selected clone was also maintained. After approximately 200 generations (4 years) of continuous selection, both the selected and non-selected clones were assayed for changes in intergenic spacer (IGS) length variants of the rRNA cistron. No changes in sets of IGS variants were detected in the non-selected clone. However, the selected clone was found to have lost three variants present in the non-selected clone. This probably occurred by unequal cross-over between sister chromatids, whereby the cistron became homogenized by an increase of frequency of two smaller variants. This documents a large-scale genetic change occurring within the rRNA cistron in a parthenogenetically reproducing aphid. *Biological Journal of the Linnean Society*, 2003, **79**, 101–105.

ADDITIONAL KEYWORDS: Aphididae – chromosomal rearrangements – concerted evolution – greenbug – molecular drive – ribosomal DNA.

INTRODUCTION

Aphids are among the best known parthenogenetic insects, with asexual reproduction occurring apomictically, i.e. meiosis apparently does not occur and the egg undergoes a single mitotic maturation (Blackman, 1980; Suomalainen, Saura & Lokki, 1987). Therefore, all offspring descending from a single parthenogenetic aphid (i.e. clone) should (barring mutations) be genetically identical. Modern molecular genetic techniques generally have substantiated the clonal nature of aphids by showing the stability of various regions of nuclear DNA during parthenogenesis (Carvalho *et al*., 1991; Shufran, Black & Margolies, 1991; Black *et al*., 1992; De Barro *et al*., 1994; Fukatsu & Ishikawa, 1994). Recently, however, germ line and somatic mutations were detected with molecular markers in clones of *Sitobion avenae* (F.), thus confirming the occurrence of mutations in apomictic lineages (De Barro *et al*., 1994; Lushai *et al*., 1997, 1998).

The study herein documents another case of molecular evolution occurring within an asexually reproducing lineage of aphids. This was discovered while examining length diversity of the intergenic spacer (IGS) region of the rRNA cistron in a clone of the greenbug, *Schizaphis graminum* (Rondani), undergoing insecticide selection. Prior to this, IGS spacer length diversity was extensively studied in the greenbug. Previously, it was found that IGS variants were stable within greenbug clones, even after as many as 100 consecutive parthenogenetic generations (Fig. 1; Shufran *et al.*, 1991). For this reason, IGS profiles were useful as 'DNA fingerprint-like' markers in population studies. It was thought that IGS length diversity was generated by recombination during meiosis in the sexual cycle of the greenbug, and maintained via apomixis. New IGS size variants and combinations were apparent after laboratory crosses of *S. graminum* (Shufran, Peters & Webster, 1997). Here

*Corresponding author. E-mail: kashufran@pswcrl.ars.usda.gov

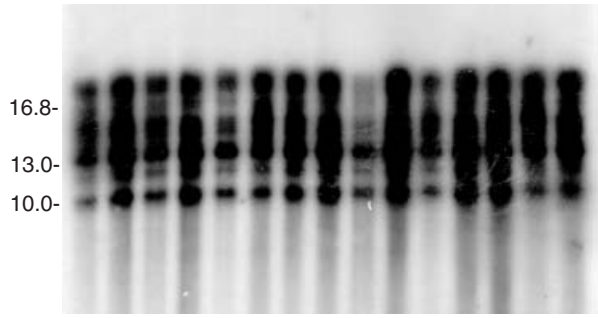

Figure 1. Southern blot showing rDNA IGS sizes are consistent within a single clone of *Schizaphis graminum* (from Shufran *et al.*, 1991).

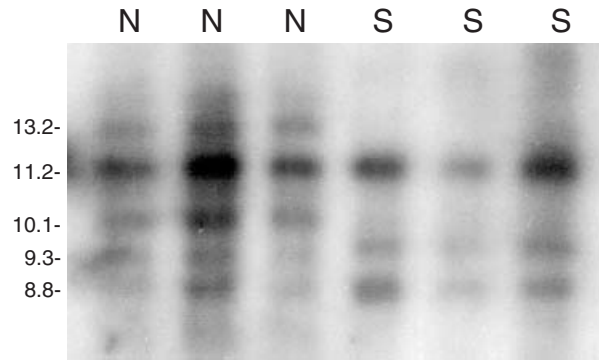

Figure 2. Southern blot showing rDNA IGS loss of a 13.2 kb, 10.1 kb, and 9.3 kb spacer in a single clone of *Schizaphis graminum*, after repeated selection with insecticides (N:-non-selected; S:-selected).

it was concluded that the large degree of IGS diversity seen in field populations was a result of recombination during meiosis in natural populations. However, the loss of IGS variants in a single parthenogenetic clone of *S. graminum* has been discovered; hence mechanisms other than conventional recombination contribute to the IGS diversity of field populations.

EXPERIMENTAL PROCEDURES

The greenbug clones used in this study were those which were selected for and maintained by Shotkoski, Mayo & Peters (1990). A clone was initiated from a single parthenogenetically reproducing individual and reared on sorghum (*Sorghum bicolor* (L.) Moench) to produce suitable numbers for selection experiments. Two colonies were maintained: one untreated with insecticides and another treated with the systemic insecticide disulfoton (*O*,*O*-diethyl *S*-[2-(ethylthio)ethyl] phosphorodioate) at a rate equivalent to 1.49 kg (AI) ha^{-1} (Shotkoski *et al.*, 1990). Greenbugs were under continual insecticide selection for a period of 8 months (approximately 32 generations of apomictic reproduction). During this time, the selected clone was determined to have gained between a 100- and 300-fold increase in resistance to disulfoton (Shotkoski *et al.*, 1990). The two colonies were then kept in the greenhouse for an additional 3 years under the same rearing and selection regime. For the entire duration of the experiments, the two colonies were kept in separate environmental chambers. Rearing conditions were 26°C with a photoperiod of 16:8 (L:D). The procedures for maintaining the identity of each clone were very stringent, i.e. the possibility of either colony being contaminated and taken over by an alien clone was remote (Shotkoski *et al.*, 1990). Furthermore, there were no other greenbug colonies being maintained at the facility that could have been a potential contamination source.

In 1993, we attempted to verify the clonal nature of each colony using the IGS of the rRNA cistron as a fingerprinting marker. The two clones (selected and non-selected) were still being maintained according to Shotkoski *et al.* (1990). Over a 4-year period, we estimated that both greenbug clones had undergone approximately 200 generations of continuous parthenogenetic reproduction. After this period of continuous parthenogenesis and selection, six adult, apterous individuals were chosen randomly from each of the selected and non-selected colonies and assayed for length diversity in the rDNA IGS according to protocols detailed in Shufran *et al.* (1991).

RESULTS

Genetic fingerprinting of two lineages of a single greenbug clone (one under insecticide selection and the other an untreated control) revealed multiple IGS size variants within individual greenbugs, and different sets of variants between the selected and non-selected clone (Fig. 2). A total of five IGS size variants were present in the non-selected clone, ranging from 8.8 to 13.2 kb. All individuals chosen from the non-selected clone had identical sets of IGS spacer sizes, i.e. zero variation within the clone. However, in the selected clone only three variants were detected. Two were identical in size to those in the non-selected clone, and a third was 0.3 kb larger (9.6 kb). After about 200 generations (4 years) of apomictic parthenogenesis and continuous insecticide selection, the selected clone lost the 13.2 kb, 10.1 kb and 9.3 kb variants (Fig. 2). Again, there was zero variation in IGS variants among individuals within the non-selected clone. This result is in contrast with those of Shufran *et al.* (1991) and Black (1993), in which IGS variants were found to be stable within clones, even after 100 generations of parthenogenesis (Fig. 1). However, in

these particular experiments, clones were maintained on sorghum plants without chemical selection pressures.

DISCUSSION

Large scale, nuclear genetic rearrangements have been documented in apomictic populations of the freshwater crustacean, *Daphnia pulex* Leydig (Crease & Lynch, 1991). Inter- and intra-chromosomal exchanges between rDNA arrays in the absence of meiosis lead to changes in the frequency of IGS variants. Over time, there was also an overall net loss of IGS repeats within obligately parthenogenetic clones. However, no new size variants were detected within clonal lineages (Crease & Lynch, 1991). Besides *D. pulex*, unequal cross-over and gene conversion within rDNA arrays has been documented during mitosis in the fruit fly, *Drosophila* (Tartof, 1974; Gillings *et al*., 1987), broad bean (Rogers & Bendich, 1987), and yeast (Szostak & Wu, 1980; Jackson & Fink, 1981; Klein & Petes, 1981; Keil & Roeder, 1984). Homogenization of rDNA arrays in parthenogenetic organisms may provide a mechanism to purge the gene family of deleterious mutations, thus avoiding the consequences of Muller's ratchet (1964) and offering new evolutionary opportunities within clones (Crease & Lynch, 1991).

The loss of the 13.2 kb, 10.1 kb and 9.3 kb variants in the insecticide-selected greenbug clone may have been due to unequal cross-over at the level of the entire rDNA repeat. Variants tend to cluster within an array, most likely as a consequence of the fact that misalignment of repeats occurs between close neighbours. If so, then unequal sister chromatid exchange events during apomixis could potentially create a chromatid in which an entire block of one variant is eliminated, or substantially reduced in frequency (Fig. 3). During apomixis, only one of the two chromatids migrate into the egg, while the other goes into the polar body (Blackman, 1980). Therefore, even though there is one equational division, only one mitotic product carries on to become an egg (Fig. 4). Because of unequal sister chromatid exchange, the chromatid entering the egg could be very different from the original chromosome.

Whilst with the methods presently used the relative copy number of each variant or subtle changes in frequency could not be estimated, we observed a stronger signal (darker band) representing the 8.8 kb variant in the insecticide-selected clone, suggesting a greater number of copies. We also detected a new sized variant of 9.6 kb, which is probably an increase in frequency of a previously rare variant (Fig. 2). This may represent a homogenization of these smaller variants of the IGS in the selected clone. The selected lineage went through numerous bottlenecks whereby a genotype bearing a new mutation that conferred increased resistance to disulfoton would have rapidly increased in frequency. If this lineage also contained an IGS array altered by unequal cross-over, that array would 'hitch a ride' in the resistant clone. It is also possible that aphids reared under continuous exposure to insecticides may have been more fit when containing these smaller variants, in which case they would have been rapidly selected for. We stress that there is no precedent for rDNA conferring insecticide resistance and make no claims that rDNA is involved in insecticide resistance in greenbug. In fact, the mechanisms of insecticide resistance in greenbug have been attributed to elevated esterases (Zhu & He, 2000).

Changes in rDNA have effects on the morphology and life-history of *Drosophila* (Frankham, 1988). Lynch, Spitze & Crease (1989) found that *D. pulex* with specific isozyme/mtDNA genotypes and differing in life-history characters from other populations could

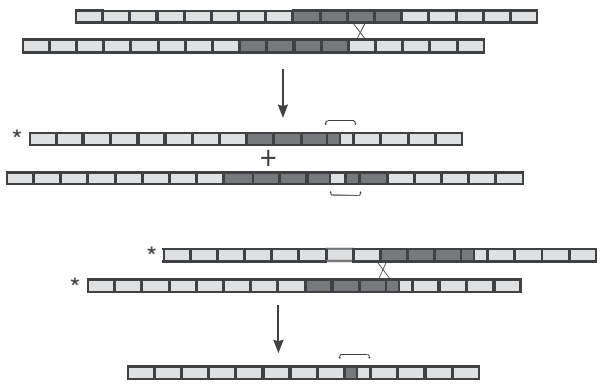

Figure 3. Results of two unequal cross-over events. The bottom chromatid has essentially lost the dark variant and four repeats. Further unequal cross-over could increase the number of light variants in this new array.

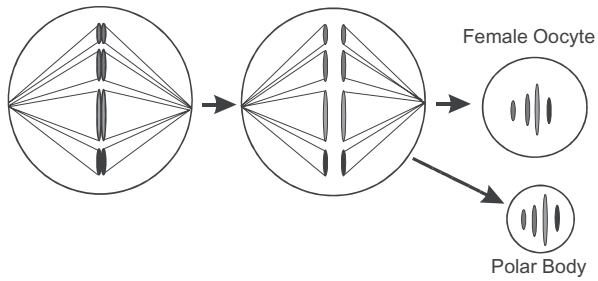

Figure 4. Behaviour of the aphid chromosomes during the mitotic division during development of the female oocytes. Adapted from Blackman (1980).

also be distinguished by their rDNA profiles. The IGS, while once thought to be non-transcribed, is now known to have important functions in the rRNA cistron. These include gene promoters, spacer promoters, transcriptional enhancers and the origin of replication. Therefore the IGS may be subject to greater selection than previously thought.

Ribosomal DNA is a multigene family coding for the RNA components of the ribosome. Members occur in tandem repeats and are usually found at one or a very few chromosomal locations. Each member contains three genes that code for the 18S, 5.8S and 28S subunits, with each member connected by an IGS (Beckingham, 1982; Gerbi, 1985). There are about 150 copies of each three-gene array in greenbug (Black, 1993). While we could not estimate the relative copy numbers of each IGS variant, it is safe to assume that there are multiple copies of each within different individuals. In the unlikely event these copies are equally distributed, there would have been an average of about 30 copies of each variant in the initial clone selected by Shotkoski *et al.* (1990). Thus, loss of multiple copies within the selected clone cannot be explained merely by a single mutation at a restriction site, or deletion of one fragment. Such changes occurring in multiple copies of a variant of specific size, and not others, would be very unlikely. Unequal cross-over is the most likely molecular drive event for the observed loss and homogenization of IGS variants in this case. Like *D. pulex* (Crease & Lynch, 1991), we observed a net loss in IGS variants in the greenbug. While the net loss occurred while the greenbug clone was under insecticide selection, we do not know what selection factors (if any) may have been acting upon *D. pulex* in nature.

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