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Generation of clonal diversity by sexual reproduction in the greenbug, *Schizaphis graminum*

K. A. Shufran,[1] D. C. Peters[2] and J. A. Webster[1]

[1] *USDA-ARS, Plant Science Research Laboratory, Stillwater, Oklahoma, and*
[2] *Department of Entomology, Oklahoma State University, Stillwater, Oklahoma, USA*

Abstract

In the United States, the greenbug, *Schizaphis graminum* (Rondani), reproduces primarily by apomictic parthenogenesis. Although a periodic sexual cycle exists, the extent to which it occurs naturally and its influence on the genetic variability of greenbug populations is unclear. Length variation in the intergenic spacer (IGS) of the rRNA cistron in the greenbug indicates that populations are made up of many genetically distinct clones. Previous laboratory studies have shown the stability of the IGS within parthenogenetic clones. By inducing the sexual reproductive cycle of the greenbug, we conducted both intra- and inter-clone matings and studied the inheritance of the IGS in the offspring. In both mating schemes, rearrangements in the IGS were apparent. IGS diversity found among the offspring could be attributed to unequal cross-over and probably other molecular drive events during meiosis. Periodic sexual reproduction is a primary mechanism for the generation and maintenance of genetic variability in greenbug populations, and explains the level of clonal diversity found in previous studies.

Keywords: aphid, holocycle, intergenic spacer, ribosomal DNA, molecular drive.

Introduction

Aphids (Hemiptera: Sternorrhyncha: Aphididae) reproduce primarily by apomictic parthenogenesis, a unique form of reproduction whereby adult females give birth to genetically identical daughters (Suomalainen *et al.*, 1987). In this form of reproduction, no genetic recombination occurs (Blackman, 1985; Suomalainen *et al.*, 1987) and members of single parthenogenetic lineages (mother, daughters, granddaughters, etc.) are commonly referred to as clones. Despite claims of 'endomeiosis' (Cognetti, 1961), there is good molecular genetic evidence for the apomictic form of reproduction in aphids. No variation in rDNA (Birch *et al.*, 1994; Shufran *et al.*, 1991), minisatellite DNA (Carvalho *et al.*, 1991; De Barro *et al.*, 1994), arbitrarily primed (AP-PCR) DNA fingerprints (Fukatsu & Ishikawa, 1994), or allozymes (Suomalainen *et al.*, 1980) has been detected within aphid clones. These same studies also revealed a great deal of variability among field-collected aphids, indicating the presence of many genetically distinct clones in naturally occurring populations. However, it does not adequately explain how clonal diversity was generated.

The clonal diversity of the greenbug, *Schizaphis graminum* (Rondani), has been extensively studied (Black, 1993; Shufran *et al.*, 1991, 1992; Shufran & Wilde, 1994). DNA fingerprint-like patterns were obtained using a probe that tested for length variants in the intergenic spacer (IGS) of the rRNA cistron. The rRNA cistron is a multigene family with genes occurring in tandem arrays and separated by an IGS. Although the coding areas are conserved, the IGS varies in length and sequence within and among individuals of the same species (Gerbi, 1985; Beckingham, 1982). There are approximately 145 copies of the rRNA cistron in the greenbug (Black, 1993). Previous studies showed that greenbug populations on wheat, *Triticum aestivum* L., and sorghum, *Sorghum bicolor* (L.) Moench, were made up of many genetically distinct clones, but did not explore how variation in the IGS was generated (Black, 1993; Shufran *et al.*, 1991, 1992; Shufran & Wilde, 1994).

Although parthenogenesis is its primary mode of reproduction, like many other aphids the greenbug also possess a sexual form of reproduction called the holocycle. In response to decreasing day length and cool temperatures during autumn, parthenogenetic females may produce males and sexual females (oviparae) which mate and lay eggs. Eggs overwinter and hatch during spring into apterous parthenogenetic stem mothers (fundatrices). Greenbug populations remain parthenogenetic throughout the summer until

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suitable conditions are experienced in the autumn again to initiate sexual reproduction. The majority of field reports of overwintering eggs have come from Kentucky bluegrass, *Poa pratensis* L., in areas north of approximately 36–38° N latitude (Puterka & Peters, 1990; Wadley, 1931; Webster & Phillips, 1912). Greenbug populations south of this region do not receive the proper environmental stimuli to trigger sexual reproduction, and thus remain parthenogenetic. However, the greenbug is not a serious pest in northern states, so the relationship of these sexually reproducing populations with southern populations that are problematic on wheat and sorghum is unknown.

The greenbug sexual cycle may be artificially induced in the laboratory by manipulating temperature and photoperiod (Puterka & Peters, 1990). It is now known from controlled laboratory crosses that biotypic variation (i.e. ability to damage resistant lines or cultivars) is a result of sexual reproduction and the inheritance of 'virulence' genes (Puterka & Peters, 1989, 1995). Parthenogenetic morphs collected in Oklahoma are capable of producing males and oviparae in the laboratory (Ullah & Peters, 1996), but it is unknown if and to what extent sexual reproduction occurs naturally, or what effect this may have on genetic diversity. Previously, it was thought that biotypic and genetic diversity of greenbug populations were the result of mutations within clones (Starks & Schuster, 1976) or separate introductions (Blackman, 1980) and is still subject to debate.

The current study seeks to test whether genetic diversity found in greenbug populations may have arisen by sexual reproduction, as well as quantifying its effect. The IGS is stable, i.e. shows no variation, within parthenogenetic clones (Shufran *et al.*, 1991), yet field-collected populations exhibit much length variation in the IGS (Shufran *et al.*, 1991, 1992; Shufran & Wilde, 1994). Genetic recombination may occur during the sexual cycle of aphids, but only in the female gametes (Blackman, 1985). By artificially inducing sexual reproduction, we conducted within-clone and between-clone matings and studied the inheritance of the IGS in the rRNA cistron. Rearrangements in the IGS were apparent in the offspring, by the observation of new IGS size variants. This provides a mechanism for generating genetic and clonal diversity in field populations.

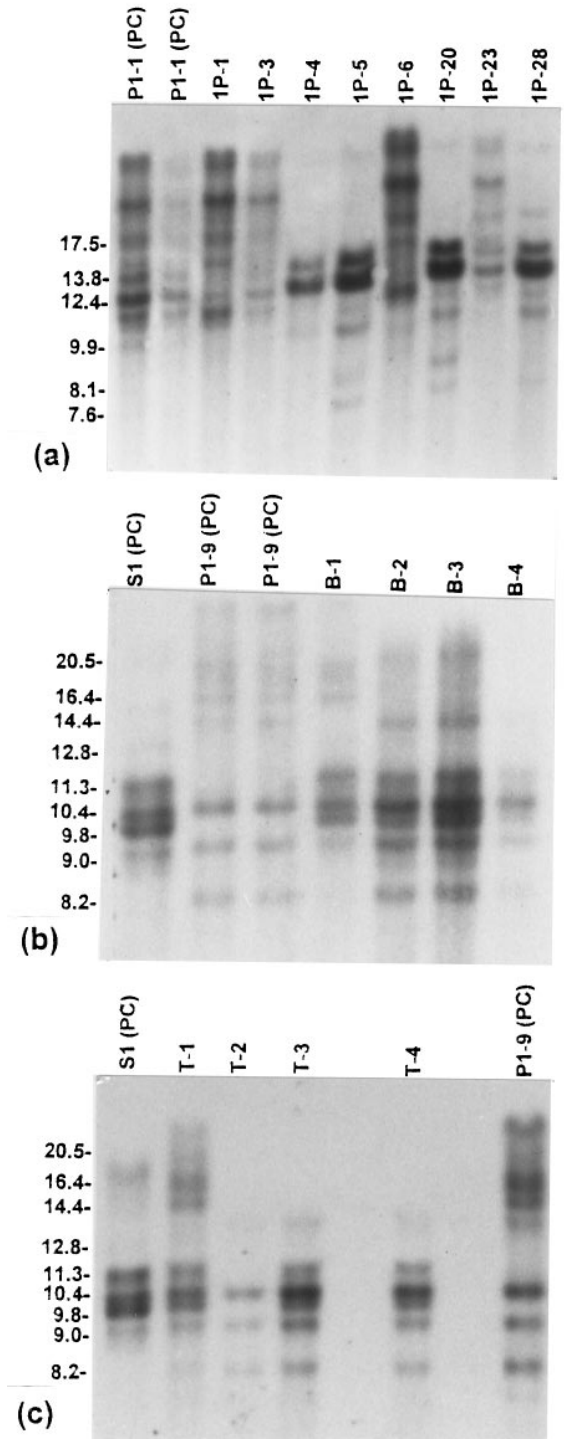

Figure 1. Length variations in the rRNA cistron intergenic spacer of the greenbug, *Schizaphis graminum*. Parental clones (PC) and daughters are shown. (a) Self mating of clone P1-1. (b) Mating of clones S1 × P1-9. (c) Reciprocal mating of clones P1-9 × S1. For space considerations, the mw of some fragments is not given.

Results

A total of six intra- and five inter-clone matings were evaluated. Clonal diversity was created by sexual reproduction in the greenbug, producing offspring with sets of IGS sizes unlike the parental clones. Within clone matings of greenbugs sometimes produced rearrangements and new IGS sizes in the off-

spring (Fig. 1a), but at other times did not (Table 1). The self-mating of clone S1 resulted in thirteen daughters which all carried the same sets of IGS sizes (Table 1a). Other intra-clone matings resulted in daughters which lacked parental spacers, and/or had new size variants (Fig. 1a and Table 1b–f). Some of the rearrangements could be explained by unequal cross-over. For instance, an unequal exchange of 500 bp within two copies of the 9.0 kb spacer resulted in two new variants (8.5 kb and 9.5 kb) in the daughter clone 7S4 (Table 1b).

Table 1. Within-clone matings of greenbugs. Patterns of inheritance of the intergenic spacer (IGS) between parental clones (PC) and offspring. + indicates presence and − indicates absence of a particular IGS. Sizes shown in kb.

(a)

Clone	14.4	12.8	11.3	10.4	9.7	9.0
S1 (PC)	+	+	+	+	+	+
13 offspring	+	+	+	+	+	+

(b)

Clone	16.4	14.9	11.5	10.2	9.5	9.0	8.5
S7 (PC)	+	−	+	+	−	+	−
8 offspring	+	−	+	+	−	+	−
7S4	+	+	+	+	+	−	+

(c)

Clone	20.5	13.3	12.4	11.3	9.7	8.8
P1-3 (PC)	+	+	+	−	+	+
3P1, 3P3, 3P4	+	+	+	+	+	−
3P6, 3P7	+	−	−	−	−	−
3P8, 3P13	−	+	−	+	+	−

(d)

Clone	20.2	18.6	16.5	15.8	14.8	12.6	11.8	11.2	9.6	8.9	8.0
P1-9 (PC)	+	+	+	−	−	+	−	+	+	+	+
9P1	−	−	+	−	−	+	−	+	+	+	+
9P3	+	+	+	−	−	+	−	+	+	+	+
9P5	+	+	−	+	−	+	+	+	+	+	−
9P8	+	+	+	−	+	+	−	+	+	+	+

(e)

Clone	17.5	14.8	13.8	12.4	11.4	9.9	8.1	7.6
P1-1 (PC)	+	+	+	+	+	+	−	−
1P1	+	+	−	+	+	−	−	−
1P3	+	−	+	+	+	−	−	−
1P4	−	−	+	+	−	+	+	+
1P5	−	−	+	+	+	+	+	+
1P6	+	+	−	+	+	+	−	−
1P20	−	−	+	+	+	+	+	+
1P23	+	+	−	+	+	−	−	−
1P28	+	−	+	+	+	+	−	+
1P42	−	−	+	+	+	+	+	+
1P38, 1P39, 1P50	+	+	+	+	+	+	−	−

(f)

Clone	20.5	18.2	17.2	15.0	13.9	13.4	12.5	12.1	10.8	10.1	9.5	8.7	7.3	7.2
S5 (P.C.)	−	−	−	+	−	+	−	−	−	−	+	−	−	−
5S1, 5S10	+	−	+	+	−	+	−	+	+	+	+	−	+	+
5S2	+	+	−	+	−	+	−	+	−	−	−	−	−	−
5S4, 5S9	+	−	+	+	−	+	−	+	−	−	+	−	−	−
5S6	−	+	−	+	+	−	−	−	+	+	+	+	−	−
5S14, 5S12	−	−	−	−	−	−	−	+	+	+	+	−	+	+

An unequal exchange of 1.5 kb between the 16.4 kb spacer and the 10.2 kb band also appeared to have occurred. We saw the generation of a new variant (14.9 kb) due to the 1.5 kb loss, with the corresponding gain by the 10.2 kb band yielding another band of about 11.5 kb. Because the rRNA cistron is a multigene family, more than one copy of each IGS may be present. The most intense bands on the 'Lumigraphs' are the predominant spacers. Due to the diminished power of resolution of the larger sized fragments, it would not be possible to detect all unequal cross-over events, but changes in many of the lower weight bands can be explained in this way. Other IGS sizes which were lacking in the offspring or represented new variants cannot be readily explained by unequal exchange. Insertions or deletions within the IGS may, however, account for some of these. Variation in the banding patterns occurred in multiple individuals from within clone matings. Multiple individuals of the same clone run on the same and different gels yielded identical IGS banding patterns; therefore this is likely to reflect genetic variation in the IGS generated by sexual reproduction and is not simply due to incomplete digestion of the DNA samples.

Table 2. Between-clone matings of greenbugs. Patterns of inheritance of the intergenic spacer (IGS) between parental clones (PC) and offspring. + indicates presence and − indicates absence of a particular IGS. Sizes shown in kb.

(a)

Clone	20.5	18.2	16.4	14.4	12.8	11.3	10.4	9.8	9.0	8.2
S1 (male PC)	−	−	−	+	+	+	+	+	+	−
P1-9 (female PC)	+	+	+	+	−	−	+	−	+	+
B1	+	+	+	−	−	+	+	+	+	−
B2, B3	+	−	−	+	−	+	+	+	+	−
B4	−	−	−	+	−	+	+	+	+	+

(b)

Clone	20.5	18.2	16.4	14.4	12.8	11.3	10.4	9.8	9.0	8.2
P1-9 (male PC)	+	+	+	+	−	−	+	−	+	+
S1 (female PC)	−	−	−	+	+	+	+	+	+	−
T1	+	+	+	+	−	+	−	−	+	+
T2	−	−	−	+	−	+	−	+	−	+
T3, T4	−	−	−	+	−	+	+	+	+	+

(c)

Clone	16.4	14.4	12.8	11.3	10.4	9.7	9.0
S1 (male PC)	−	+	+	+	+	+	+
P1-1 (female PC)	+	−	+	+	+	+	−
A1, A4	+	−	+	+	+	+	+
A5, A3	+	−	−	+	+	+	+

(d)

Clone	18.4	17.4	15.2	13.5	11.8	10.5	9.5	8.4
S5 (male PC)	−	−	−	−	+	−	−	+
P1-9 (female PC)	+	+	+	+	−	+	+	+
D2	+	+	+	−	−	−	−	+
D3, D5	+	+	+	−	+	+	+	+

(e)

Clone	19.4	17.4	15.9	14.6	13.5	12.2	10.5	10.0	9.5	8.5
P2-7 (male PC)	+	+	+	−	−	−	+	−	+	+
S1 (female PC)	−	−	+	−	+	+	+	+	+	−
SQ2, SQ5	−	−	+	−	+	+	+	+	+	−
SQ4	+	+	+	−	−	−	−	−	−	−
SQ6	−	−	−	+	−	+	+	+	+	+
SQ7	+	+	+	−	+	+	+	+	+	−
SQ8	+	+	+	−	+	−	−	−	−	−
SQ10	−	−	−	−	−	−	+	−	+	+

Crosses between different clones also generated rearrangements of IGS sizes among offspring (Fig. 1b, c; Table 2a–e), but only one new size variant not present in the parental clones was detected in the offspring (clone SQ6 in Table 2e). No offspring were produced that contained all the IGS sizes present in both parental clones. There was always a rearrangement or absence of some bands in the offspring which were present in the parental clones. Absence of some bands can be explained by meiosis and segregation, as can the combination of IGS sizes which were unique to each parental clone. The one new size variant (14.6 kb) found in daughter clone SQ6 can be explained by unequal cross-over in the S1 female parent (Table 2e). This entailed a 1.3 kb loss from the 15.9 kb band, which may have been added to the 12.2 kb band. This would have yielded a duplicate IGS of about 15.9 kb.

Discussion

Intra- and inter-clonal matings of the greenbug generated clonal diversity as determined by DNA fingerprint-like patterns based on the IGS. This is very strong evidence that unequal crossing-over and independent assortment at meiosis generates genetic diversity of greenbug populations in the USA. Not only were different combinations of IGS sizes present in the offspring, but new size variants not present in the parental clones were also created. We found that two or more greenbug clones arising from different eggs (but full sibs of the same inter- or intra-clone mating) could have the same sets of IGS sizes, i.e. have identical fingerprints (e.g. clones 1P5 and 1P20 in Fig. 1a, and clones B2 and B3 in Fig. 1b). This implies that previous studies of clonal diversity utilizing the IGS as a fingerprinting probe may actually have *underestimated* clonal diversity, if one accepts the definition of an aphid clone as a purely parthenogenetic lineage arising from a single fundatrix. Full sibs with identical IGS profiles would likely be homogenous at other loci; however, nothing is known about the incidence of intra-clone mating of greenbugs in field populations. Some within-clone matings did not produce any variants among the offspring (Table 1a), while others did (Fig. 1a and Table 1b–d). Unequal crossing-over apparently did not always occur during meiosis.

The level of clonal diversity found in previous studies of greenbug populations on wheat and sorghum in Kansas, Oklahoma and Texas (Shufran *et al.*, 1991, 1992; Shufran & Wilde, 1994) probably is the result of sexual reproduction. The populations tested were from a region which lies in and north of an area (approximately 36–38° N latitude) where the necessary conditions occur for the induction of the holocycle (Wadley, 1931; Webster & Phillips, 1912). Populations remain parthenogenetic to the south of this zone (Wadley, 1931; Webster & Phillips, 1912). Interestingly, the zone of sexual reproduction includes southwest Kansas where many greenbug biotypes were first discovered. Sporadic reports of greenbug eggs in winter wheat have come from this area, but the eggs did not hatch when brought into the laboratory (John D. Burd, USDA-ARS, Stillwater, Oklahoma, personal communication).

Our results suggest that even a minor proportion of the greenbug population reproducing sexually would be sufficient to generate and maintain the amount of clonal diversity present on wheat and sorghum. This certainly is plausible, as up to 73% and 65% of greenbugs collected from wheat in Oklahoma produced males and oviparae, respectively, when subjected to short day-lengths and cool temperatures in the laboratory (Ullah & Peters, 1996). Sexual reproduction may be occurring on hosts other than wheat, because about seventy different species of grasses have been identified as greenbug hosts (Michels, 1986). The same greenbug clones were found on weedy grasses and sorghum, indicating that populations move between these hosts (Shufran & Burd, unpublished data). The formation of new clonal variants gives cyclically parthenogenetic species an advantage in heterogenous environments (Dixon, 1987). With the formation of new genotypes each year from sexual reproduction, the greenbug may better exploit less uniform or predictable conditions associated with non-cultivated hosts.

The patterns of variation present in the offspring of intra- and inter-clonal matings can be explained by unequal crossing-over and independent assortment, which occurs during meiosis of the female gametes (Blackman, 1985). Unequal cross-over is known to generate diversity in the IGS of *Drosophila melanogaster* Meigen (Williams *et al.*, 1990). Loss or gain of some bands may be due to segregation of heterozygotes within clones. Other factors, e.g. gene conversion, transposition, and single cross-over events, were also probably responsible for generating IGS diversity in *D. melanogaster* (Williams *et al.*, 1990), and may occur in the aphid genome as well. Substantial length variation also exists in the IGS of the large raspberry aphid, *Amphorophora idaei* (Borner), and has also been attributed primarily to molecular drive events (Birch *et al.*, 1994; Fenton *et al.*, 1994). *A. idaei* has a regular sexual cycle which also is apparently responsible for clonal variation.

The rRNA gene family is thought to undergo 'concerted evolution' whereby genes evolve in concert instead of acquiring independent mutations. Forces

acting to maintain homogeneity in the coding regions, while creating variation in the IGS, are known collectively as 'molecular drive' and mostly involve unequal cross-over, gene conversion, and transposition (see Elder & Turner, 1995, for a recent review). Models of concerted evolution are largely based on sexual reproducing populations, but less is known about the evolution of multigene families in parthenogenetic species. However, new rDNA variants spread within arrays faster than the new variants spread within populations of the cyclical parthenogen, *Daphnia pulex* Leydig, which was contrary to models for strictly sexual species (Crease & Lynch, 1991). The greenbug is another cyclical parthenogen in which molecular drive apparently also acts to create diversity in the IGS.

Experimental procedures

Sexual crossing

Laboratory colonies of insecticide susceptible (S) and resistant (P1 and P2) greenbugs (Shufran *et al*., 1996) were obtained from Gerald Wilde (Kansas State University). These clones were originally collected from sorghum in Kansas and were used in a concurrent study of the inheritance of esterases associated with insecticide resistance. At Oklahoma State University, isofemale lines (clones) of greenbugs were re-established from single, parthenogenetic adults. All clones were maintained in cup cages consisting of 227 cm^3 Styrofoam cups with two or three 'Triumph 64' wheat seedlings grown in sandy soil and caged by 3.5 cm diameter $\times$ 15 cm tall clear plastic tubes. For ventilation, the tops of the cages were covered with fine-mesh cloth and sealed with hot glue. Greenbug clones were maintained on the caged plants at 18°C and 15:9 h (L:D) in environmental chambers.

Sexual reproduction was induced by adjusting temperature and photoperiod (Puterka & Peters, 1989). Inter-clone crosses were achieved by removing the first produced oviparae from clone colonies and placing ten per cup with three to five males collected from another clone. The remaining oviparae and males in the clone colonies were allowed to mate and acted as parents to produce the within-clone matings. All eggs were collected by cutting those sections of the top cloth or plastic edge containing concentrations of black (viable) eggs and were incubated for seven weeks (Puterka & Peters, 1989). Parthenogenetic clones from each surviving fundatrix were maintained on 'Triumph 64' wheat seedlings at 18°C and 15:9 h (L:D) until needed for DNA isolation.

Visualization of IGS

Genomic DNA was isolated from single adult, apterous greenbugs of the parental clones and parthenogenetic offspring of the fundatrices according to Shufran *et al.* (1991). This procedure yielded on average 1.52 ($\pm$ 0.13) μg DNA per aphid, as determined by reading OD_{260}. The ratio of OD_{260}/OD_{280} averaged 1.85 ($\pm$ 0.01). The entire amount of DNA from each aphid was digested overnight at 37°C, with 12 units *Xho*I, in a total volume of 20 μl, and in the presence of 0.25 μg RNase. Digested DNA was loaded on 0.6% agarose, 0.5 $\times$ TBE gels, and run at 35 V for 24 h, and transferred to nylon membranes using standard Southern transfer techniques (Sambrook *et al*., 1989). The probe pGBS1 is specific for the greenbug IGS (Black, 1993) and was digoxigenin labelled, hybridized to the nylon membranes, washed, and 'Lumigraphs' produced using the Genius kit (Boehringer-Mannheim, Indianapolis, Indiana) according to manufacturer's instructions.

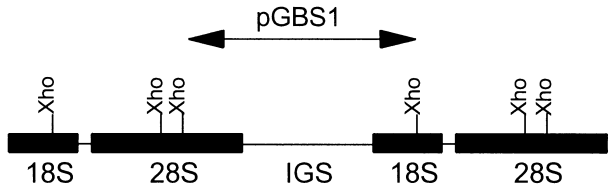

Figure 2. Simplified map of the greenbug rRNA cistron. *Xho*I digestion and hybridization with pGBS1 tests for total length variation in the intergenic spacer (IGS) (adapted from Black, 1993).

This procedure cuts out the entire IGS and allows for visualizing length diversity in this region of the rRNA cistron (Fig. 2) (also see Black, 1993). The fragment containing the IGS also contains about 1.5 kb and 2.0 kb of the flanking 18S and 28S coding regions, respectively. However, since the coding regions are highly conserved, size variation in this fragment is due to diversity in the IGS. Size determinations were made by measuring bands with a ruler and comparing distances to a high molecular weight DNA marker (Gibco-BRL, Gaithersburg, Maryland). In this and previous studies (Shufran *et al*., 1991, 1992), these procedures have not revealed any variation in the IGS among individuals of single parthenogenetic clones, or among unmated oviparae and males of the same clone (Shufran & Peters, unpublished data). Furthermore, multiple individuals of the same clones run on the same and different gels yielded identical IGS banding patterns.

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