Brief Communications

Microsatellite Diversity in Captive Bottlenose Dolphins (*Tursiops truncatus*)

A. P. Rooney, D. B. Merritt, and J. N. Derr

The utility of microsatellites for managing captive *Tursiops truncatus* was investigated. Specifically the level of genetic diversity among the loci examined and their usefulness for resolving paternity was assessed. Overall a relatively low level of genetic variation was found among captive dolphins. In addition, a high percentage of common alleles was found among dolphins belonging to different morphotypes (inshore versus offshore). The implications of these findings are discussed and suggestions are given for the use of genetic markers in captive propagation programs for *T. truncatus.*

There are three major goals in genetic management: (1) to prevent the deterioration of genetic variability through selective breeding programs which introduce new genetic variation (Frankel 1983), (2) to preserve as much of the genetic diversity of captive populations as it is reflected in their wild counterparts(Foose 1983), and (3) to verify pedigrees and avoid inbreeding. Traditional uses of genetic theory in captive propagation programs deal with the use of nonmolecular techniques. For example, one might estimate inbreeding coefficients from a pedigree constructed from direct observation. Then a plan could be designed to maximize genetic diversity based on the manipulation of effective population size combined with inbreeding coefficients. However, there are problems associated with such an approach. For example, the accuracy of pedigrees based on direct human observation is unknown, although such pedigrees are often assumed to be correct. In addition, when using effective population size theory in a breeding program to maximize genetic diversity, one assumes that a moderate amount of genetic diversity exists (or existed) in the founding population. Again, this might not always be the case. One way to overcome such problems is to use genetic markers.

Captive population managers can use genetic markers to verify or construct pedigrees or to assess the overall level of genetic diversity in captive populations. In addition, groups of animals with unique alleles or genotype combinations can be identified to develop a breeding plan. This is somewhat akin to the practice of marker-assisted selection in domestic farm animals, except that in zoo populations the goal is to use markers to select for genetic diversity and not for an economically advantageous phenotype. To characterize genetic diversity and to identify markers that are informative for paternity assessment in captive *Tursiops,* we isolated nine microsatellite markers that we developed from a *Tursiops* genomic library. The U.S. captive population of bottlenose dolphins is a mixture of animals representing two distinct morphotypes distinguishable by body and flipper size. In the wild, the larger morphotype tends to inhabit colder waters, whereas the smaller form tends to inhabit warmer waters (Mead and Potter 1990, 1995; Mitchell 1975). Consequently, because most coastal waters around the world are shallow and warm, the type usually inhabiting these waters has been commonly referred to as "inshore"; the larger morphotype, commonly seen in colder waters away from the coast, is known as "offshore." We looked at the level of allele sharing among morphotypes as a preliminary assessment of the possibility that these morphotypes are genetically distinct and not simply ecological adaptations.

Materials and Methods

Blood samples from bottlenose dolphins maintained by the United States Navy (73), Indianapolis Zoo (15), Mirage Dolphin Environment (4), Epcot Living Seas (1), and Marine World Africa USA (11) were collected in ACD buffer (Sambrook et al. 1989) and shipped to Texas A&M University for DNA analysis. DNA was extracted from white blood cells according to the protocol of Sambrook et al. (1989). Forty-two of these animals were wild caught, where three were *T. t. gilli* and the rest were inshore *T. t. truncatus* from the Gulf of Mexico. Two additional samples (skin) of the offshore *T. truncatus* morphotype from the northwestern Atlantic were received from the United States National Museum. DNA was isolated from these samples according to standard protocol (Sambrook et al. 1989).

For the construction of a genomic library, DNA was isolated from an animal maintained by the United States Navy. This DNA was then partially digested for 1 h with the restriction enzyme *Sau*3A1. Fragments between 300 and 700 bp were selected and cloned into a plasmid vector, pBluescript SK$^-$, which was previously digested to completion with *Bam*H1. Ligations were performed at room temperature with the Boehringer-Mannheim Rapid Ligation Kit™. A blue-white screen was used to select colonies for hybridization to a $(CA)_{15}$ oligonucleotide probe at 37°C. A total of 1000 colonies were screened. Nine of these were sequenced and found to contain microsatellites (Table 1). GenBank accession numbers are AF004901–AF004909.

The computer program MacVector 5.0 (International Biotechnologies, Inc.) was used to design PCR primers to amplify the nine microsatellite loci. At least two primer pairs were designed for each locus. Primers were labeled with γ-^{32}P-[dATP] according to standard protocol (Sambrook et al. 1989). PCR reactions were run with

Table 1. Microsatellite loci isolated from *T. truncatus*

Locus	Repeat size	Primer sequences (5′-3′)	Annealing temper-ature (°C)	Allele sizes[a]
TexVet1	$(CA)_4TA(CA)_2CC(CA)_2$	GAGTGAACATCAGTATCAAGAGGTGA TGTCAAGGTTTGAATTTGTGTGAG	50	165–166 (2)
TexVet2	$(CA)_8$	GCGTCACCATGATTTCTGTAGG CCAAGGTACTTTCAAGTTAGCCAC	51	188–192 (4)
TexVet3	$(CA)_{26}$	GCCCTCACGTTCATTCATGTTGTT CCGTGTTCACTCCAGCATTATTTCCACA	55	207–267 (16)
TexVet4	$(CA)_8$	CAGACTGTGGACCTCCCAGTTTCT GCGGTTTTCCCTTTTGTTTGTG	57.5	164 (1)
TexVet5	$(CA)_{24}$	GATTGTGCAAATGGAGACA TTGAGATGACTCCTGTGGG	51	236–260 (9)
TexVet6	$(CA)_9$	CGCACAGAAACGAAGACCCAAC AAAAGCTGAAGGCTAGACACTGGTC	52.5	192–193 (2)
TexVet7	$(CA)_{12}$	TGCACTGTAGGGTGTTCAGCAG CTTAATTGGGGGCGATTTCAC	54.5	155–163 (6)
TexVet8	$(CA)_5CG(CA)_5$	CCCTTCCTTTTAGCCAGAGGTG CGCTGTCACGTACACACCCATG	54.5	217–218 (2)
TexVet9	$(CA)_3CG(CA)_6$	TTTTCTTAGTACCAGAATCTTGTCAG TCCAGTTGCCTTTCAAGTCTAG	50.5	124 (1)

[a] Number of different alleles are shown in parentheses.

radiolabeled primers and PCR reagent concentrations were designed according to standard protocols (Hillis et al. 1996). PCR profiles contained an initial denaturation of 2 min at 96°C followed by 35 cycles of 30 s at 94°C, 30 s at locus-specific annealing temperature (as determined by MacVector; see Table 1), and 1 min at 72°C. Each set of 35 cycles was followed by a final extension of 10 min at 74°C. PCR products were denatured at 94°C for 5 min and run through 6% acrylamide, 7 M urea, 1× TBE denaturing gels in 1× TBE running buffer. Gels were run at 50°C and running times varied according to the PCR product size. Gels were transferred to 3 MM chromatography paper, dried, and exposed to autoradiograph film. Exposure time varied but was generally 14 h. The primer pairs which amplified the best were chosen to score all individuals and are reported in Table 1. Samples which failed to amplify were subjected to further amplifications in which the annealing temperature was lowered. If there was still no amplification, that individual was scored as "missing." For all subsequent analyses, individuals which were typed as "missing" were excluded. In addition, we calculated the number of alleles shared between morphotypes at all loci pooled together. For this analysis we compared the following populations (the number of individuals is indicated in parentheses): West Florida (10), Florida Keys (3), Texas (4), Louisiana (3), Alabama (3), Mississippi (16), mid-northwestern Atlantic offshore (2), Hawaii (1), California (2). Animals from California and Hawaii were *T. t.* gilli; those from the mid-northwestern Atlantic were *T. t. truncatus* "offshore"; all others are *T. t. truncatus* "inshore."

To assess genetic diversity among captive bottlenose dolphins, two statistics were used: heterozygosity was calculated at each polymorphic locus using the direct count method (Weir 1996); and polymorphism information content (PIC) (Botstein et al. 1980) values were estimated for each microsatellite locus. Although originally used for predicting marker usefulness in mapping studies, PIC also estimates the degree to which a marker is polymorphic (hence the name). We chose to estimate PIC values as a second measure of polymorphism because, as Falconer (1989) points out, simply estimating the proportion of polymorphic loci in a population gives undue weight to rare alleles. PIC values, however, take rare alleles into account and give an actual measurement of the degree of polymorphism at a given locus.

To test for deviations from Hardy–Weinberg proportions, two approaches were used: a chi-square test of heterozygosity and three exact tests. For the chi-square approach, Selander's *D* index (Selander 1970) was used to assess whether there was a deficiency or excess of heterozygosity for each locus and for all loci pooled. The significance of each locus-specific heterozygosity value was assessed with a chi-square test using one degree of freedom. The computer application GENEPOP (Raymond and Rousset 1995) was used to perform the following exact tests: the probability (Fisher's) test, the heterozygote deficiency test (Rousset and Raymond 1995), and the heterozygote excess test (Rousset and Raymond 1995). All tests were subject to a tablewide sequential Bonferonni correction (Rice 1989; Weir 1996). To investigate whether captive breeding had obscured results when all animals (wild caught and captive born) are pooled, a second analysis was performed where only wild-caught animals were analyzed (42 animals, or 40% of the total number of animals surveyed here). For those analyses, the individuals that were analyzed are described earlier in this section. The two *T. t. truncatus* "offshore" were not included in these analyses because they are museum specimens and not part of the captive population.

In order to identify markers that could be potentially useful for pedigree analysis and studbook registry, 14 cases of questionable paternity were investigated using the three most polymorphic loci: *TexVet3, TexVet5,* and *TexVet7.* The paternity test was conducted such that if neither allele from an offspring could be traced to a potential sire, that sire was excluded.

Results and Discussion

One major concern of captive bottlenose dolphin managers that can be addressed with genetic markers is pedigree accuracy. Often the sire of animals born in captivity is unknown because of management constraints. However, the resolution of paternity in captive settings is far easier than in the wild. This is due to the fact that generating a probability of exclusion is simply unnecessary. The probability of exclusion, as defined by Weir (1996), is the probability of excluding a random male from being the father and is based on allele frequencies. This assumes an infinite number of possible fathers. In a captive setting there is a finite number of possible sires, very often only two or three per institution. This makes paternity exclusion much easier provided that each potential sire has a unique genotype. An objective of this study was to use this logic in order to identify potentially useful markers for paternity analysis as well as future pedigree analysis, but not to construct a pedigree per se. We found that *TexVet7* was able to assign paternity in seven cases, *TexVet3* was able to assign paternity in four, and *TexVet5* was unable to assign paternity in any case. In no instance did any of the loci conflict in paternity assignment. Three cases were unable to be resolved by any marker. Therefore we suggest that *TexVet3* and *TexVet7* be used in future pa-

Table 2. PIC values and heterozygosity among all captives (wild caught and captive born) and tests of departure of observed locus heterozygosity from the expected value

Locus	PIC	H_o^t	H_e^t	D^t	$\chi^{2\,a}$	P^b	H_o^w	H_e^w	D^w	$\chi^{2\,a}$	P^b
TexVet1	0.031	0.011	0.032	−0.656	1.393219	.23786241	0.000	0.045	−1.000	1.980000	.15939047
TexVet2	0.181	0.213	0.193	0.104	0.209326	.64729619	0.262	0.238	0.101	0.106487	.74417964
TexVet3	0.849	0.879	0.849	0.035	0.115548	.73391412	0.767	0.878	−0.126	0.617453	.43199535
TexVet4	0.000	0.000	0.000	0.000	0.000000	1.00000000	0.000	0.000	0.000	0.000000	1.00000000
TexVet5	0.449	0.405	0.479	−0.155	1.246104	.26429806	0.425	0.550	−0.227	1.250000	.26355269
TexVet6	0.086	0.095	0.090	0.056	0.028056	.86697723	0.023	0.023	0.000	0.000000	1.00000000
TexVet7	0.636	0.573	0.680	−0.157	1.835207	.17551383	0.605	0.668	−0.094	0.261431	.60913874
TexVet8	0.268	0.227	0.316	−0.282	2.531712	.11157913	0.302	0.260	0.162	0.298523	.58480985
TexVet9	0.000	0.000	0.000	0.000	0.000000	1.00000000	0.000	0.000	0.000	0.000000	1.00000000
Average[c]	0.278	0.267	0.293				0.265	0.296			
Average[d]	0.357	0.343	0.377				0.341	0.380			

H_o = observed heterozygosity; H_e = expected heterozygosity; D = Selander's index; H_o^t, H_e^t = wild caught and captive born combined; H_o^w, H_e^w = wild caught only.

[a] Based on direct counts of heterozygotes.

[b] One degree of freedom used.

[c] All loci.

[d] Only polymorphic loci.

Table 3. Genetic variation within captive groups

	Observed heterozygosity[a]			
Locus	Indy[b]	MWA	USN	Mirage
TexVet1	0 (0)	0 (0)	0.011 (3)	0 (0)
TexVet2	0 (0)	0.091 (0)	0.254 (3)	0 (0)
TexVet3	0.778 (6)	0.875 (3)	0.918 (25)	1 (1)
TexVet4	0 (0)	0 (0)	0 (0)	0 (0)
TexVet5	0.417 (3)	0.546 (0)	0.387 (12)	0.333 (1)
TexVet6	0 (1)	0 (0)	0.069 (2)	1 (0)
TexVet7	0.714 (1)	0.364 (0)	0.616 (1)	0.25 (0)
TexVet8	0.444 (1)	0.182 (0)	0.397 (1)	0 (0)
TexVet9	0 (0)	0 (0)	0 (0)	0 (0)
Average[c]	0.261	0.228	0.295	0.287
Average[d]	0.336	0.294	0.379	0.369

Number of animals that failed to amplify in the PCR are shown in parentheses.

Indy = Indianapolis Zoo; MWA = Marine World Africa, USA; USN = United States Navy; Mirage = Mirage Dolphin Environment.

[a] Epcot Living Seas was not included because only one animal was genotyped from this group.

[b] Note that 15 animals from the Indianapolis Zoo were tested in only three cases: TexVet3, 5, and 7; only 10 animals were tested at the other loci.

[c] All loci.

[d] Only polymorphic loci.

ternity testing of captive bottlenose dolphins.

A second major concern of captive population managers that can be addressed with genetic markers is the level of genetic diversity in their population(s). In this study, PIC values ranged from 0 to 0.849, indicating a range of monomorphic to highly polymorphic (Table 2). The heterozygosity at individual loci ranged from 0 to 0.879 (Table 2). Among all captives (wild caught and captive born), average heterozygosity was 0.267 when all loci were considered and 0.343 when only polymorphic loci were considered (Table 2). The average heterozygosity among wild-caught captives only was 0.341 for polymorphic loci only and 0.265 when all loci are considered (Table 2). A high average heterozygosity among pooled microsatellite loci is often 0.6 or higher (Goldstein and Pollock 1997 and references therein). Compared to these studies, the average heterozygosity reported here for captive bottlenose dolphins was low. There are three possible explanations for this difference. First, microsatellite loci with large numbers of repeating units tend to display a higher level of polymorphism than loci with small to moderate numbers of repeating units (Weber 1990). Only two of our microsatellite loci had large numbers of repeating units, *TexVet3* and *TexVet5* (Table 1), while the others had low to moderate levels. Second, only approximately 25% of the total U.S. captive population, which numbers over 400 animals, was surveyed here. While this is a reasonable sample size, it is statistically possible that a higher amount of genetic variation may yet be uncovered when more animals are analyzed. Third, approximately 75% of the animals surveyed were either captured in Mississippi Sound or are descended from those animals. Thus coancestry may be lowering genetic diversity and heterozygosity may be higher if more animals are surveyed from other locations. However, sampling should reflect the percent composition of the total population with regard to geographic origin in order to provide a representative estimate of genetic diversity in a captive population.

When we looked at genetic diversity within groups (Table 3), we found that the levels were similar to the results of the combined group analysis (Table 2), although some groups displayed values that were slightly lower or higher. This is probably due to the fact that some institutions have smaller breeding groups than others (see Materials and Methods), or because some groups are composed of dolphins collected from either one or a few relatively close locations. The fact that intrainstitutional estimates of genetic variability are not different from the combined group analysis indicates that diversity is more or less at the same level in all groups.

Our analysis showed that the examined captive population sample is in Hardy–Weinberg equilibrium. As indicated by Selander's D index, a deficiency in heterozygosity was found at four polymorphic loci and an excess was found at three (Table 2). However, the deviation of the observed from the expected was not statistically significant for any locus when evaluated under a chi-square distribution (Table 2). When only wild-caught animals were examined, four loci displayed a deficiency in heterozygosity, while three showed an excess (Table 2), though none of these results were statistically significant. These findings are corroborated by the exact tests since they also failed to detect a significant departure from Hardy–Weinberg proportions, neither for the analysis of all animals pooled nor for the analysis of wild-caught animals only. Because we tried at least two different primer pairs for each locus and varied the annealing temperature, the probability that a null allele went undetected is unlikely.

Considering the above results, the question remains: Are the *Tursiops* morphotypes examined here distinct taxa? This is a question that is difficult to answer with genetic data alone. However, we found preliminary evidence that they are not genetically differentiated to a large degree by the observation of a substantial number of shared alleles among morphotypes when all loci are pooled: 19 of 22 alleles between *T. t. gilli* and inshore Gulf of Mexico *T. t. truncatus* and 16 of 17 in offshore mid-northwestern Atlantic and inshore Gulf of Mexico *T. t. truncatus*. If the two morphotypes are indeed distinct taxa, then this represents a large amount of shared ancestral polymorphism, indicating that the morphotypes have only recently begun to genetically differentiate (Lyons-Weiler and Milinkovitch 1997; Pamilo and Nei 1988; Tajima 1983; Takahata 1989; Takahata and Nei 1985; Wu 1991). However, there are three other explanations: (1) alleles that are electrophoretically indistinguishable are in fact different (i.e., homoplasy); (2) sample sizes for *T. t. gilli* and offshore mid-northwestern Atlan-

tic *T. t. truncatus* were too small and a more extensive characterization will reveal higher levels of different alleles; (3) there was an historical hybridization event among taxa. Regardless, this is an interesting observation and warrants further study of these morphotypes in the wild.

From the Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843–4467 (Rooney and Derr), and the Marine Mammal Department, Indianapolis Zoo, Indianapolis, Indiana (Merritt). We thank the following institutions for donating samples: Marine World Africa USA, the Indianapolis Zoo, the United States Navy, the United States Museum of Natural History (Smithsonian Institution), Epcot Living Seas, and the Mirage Dolphin Environment. We thank the following individuals for helpful comments and criticisms: R. C. Barber, A. R. Hoelzel, R. L. Honeycutt, E. Louis, J. G. Mead, M. Nedbal, P. Palsbøll, C. W. Potter, S. H. Ridgway, R. J. Tarpley, T. J. Ward, R. K. Wayne, B. W. Würsig, and two anonymous reviewers. We would also like to thank C. Curry, L. Gage, C. Lomax, C. W. Potter, T. Samansky, and J. Wignall for assistance in providing samples, and M. Holder, C. E. Kolenda, E. Louis, and D. Melendy for assistance in the lab. We are particularly grateful to SH Ridgway for his enthusiasm in helping to facilitate this study. This research was supported by a grant from the National Science Foundation to J.N.D. (NSF9622126) and by a grant from the Texas A&M University College of Veterinary Medicine to A.P.R. Address correspondence to James N. Derr at the address above or e-mail: jderr@cvm.tamu.edu.

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Received August 13, 1997
Accepted April 30, 1998

Corresponding Editor: Robert Wayne

Evidence of Female Heterogametic Genetic Sex Determination in White Sturgeon

A. L. Van Eenennaam, J. P. Van Eenennaam, J. F. Medrano, and S. I. Doroshov

To investigate the basic mechanism of sex determination in the aquaculturally important white sturgeon (*Acipenser transmontanus* Richardson), ploidy manipulation techniques were used to produce gynogenetic and polyploid individuals and the sex ratios present in these genetically distinct groups of fish were examined. Both sexes were observed in 23–24-month-old gynogenetic progeny groups derived from the ova of four different females supporting the hypothesis of a female heterogametic (ZZ{male}:ZW{female}) sex determination system. The percentage of males in diploid (48%), gynogenetic (18%), and triploid (14%) progeny groups derived from one batch of eggs reared under similar environmental conditions most closely approximated the percentages expected when the sex-determining element on the W chromosome segregates independently of the centromere during meiosis in females. This basic finding has implications for both the practical production of all-female populations and for conservation efforts designed to prevent the extinction of the many endangered sturgeon species.

The extant sturgeon species (Pisces: Chondrostei) are a phylogenetically ancient group of fish that evolved approximately 250 million years ago and today they are primarily known as the producers of valuable black caviar. The decline of the caviar fisheries in the Caspian Sea (Birstein 1993), coupled with strong international demand for caviar has created a potential market for farmed caviar. Confined production of white sturgeon (*Acipenser transmontanus* Richardson) began in the United States in 1979 (Conte et al. 1988). In addition to its value as a commercial food fish, this species has the potential to produce high-quality domestic caviar for national and international markets. The availability of monosex populations of caviar-producing females would significantly enhance the economic feasibility of domestic caviar production systems (Logan et al. 1995).

The mechanisms of sex determination present in the sexually monomorphic chondrostean species (sturgeon and paddlefish) are unknown. Cytogenetic studies have not revealed the presence of heteromorphic sex chromosomes in either sex of any sturgeon species (Fontana and Colombo 1974; Holcík 1986; Van Eenennaam 1997). In the absence of cytologically demonstrable sex chromosomes in most fish, genetic approaches have been used to show that sex determination in many of the gonochoristic species can be explained by a chromosomal mechanism. One genetic approach involves the use of artificial chromosome manipulation techniques to produce gynogenetic (all-maternal inheritance) individuals (Thorgaard 1983). Gynogenesis can be induced in fish by treating sperm with a radiation or chemical treatment before fertilization and then using a temperature or pressure treatment to induce the retention of the second polar body in the egg (Thorgaard et al. 1992). In species with female homogamety (XY{male}:XX{female}), gynogenesis will produce all-female progeny. In species with female heterogamety (ZZ{male}:ZW{female}), gynogenesis may produce ZZ males, WW "super"-females, and/or ZW females depending upon the rate of recombination between the sex-determining element and the centromere during meiotic

prophase (Nace et al. 1970; Thorgaard 1983). The objective of this study was to examine the sex ratios present in gynogenetic and polyploid white sturgeon to elucidate the mechanism of sex determination in this species.

Materials and Methods

In a previous article (Van Eenennaam et al. 1996) we reported the use of chromosome-set manipulation techniques to artificially produce experimental groups of gynogenetic, triploid, and normal diploid fish from batches of eggs collected from four different females (dams 1–4). Only a small number of gynogens from dams 1–3 survived, and triploid and diploid control groups from these dams were not maintained because of tank space constraints. The ploidy of all experimental fish was determined by the use of a Coulter counter to analyze erythrocyte nuclei size, and the maternal origin of putative gynogens was verified by the absence of sire-specific RAPD markers (Van Eenennaam et al. 1996). To allow time for gonadal differentiation the experimental fish populations were reared for approximately 2 years in identical flow through tanks under conditions of seasonal photoperiod and water temperatures ranging from 18°C to 21°C.

All groups of fish were necropsied at 23–24 months of age at an average weight of 2.4 kg (range 0.8–4.6 kg) and the gonads were removed. Sex was identified by the observation of either yellowish grainy ovigerous folds on the lateral sides of the ovaries in the females, or a smooth white cord of germinal tissue on the dorsolateral side of the testes in the males (Doroshov et al. 1997). Both gonads from each fish were examined for evidence of hermaphroditism. The sex identified by gonadal morphology was verified for all fish by the histological analyses of gonadal sections which had been fixed in 10% phosphate-buffered formalin, dehydrated in a series of alcohols and xylenes, embedded in paraffin, sectioned at a thickness of 5–6 μm, and stained by hematoxylin and eosin. Slides were examined using an Olympus BHS light microscope. Chi-square analysis was used to determine if the sex ratios found in the dam 4 diploid and triploid groups were significantly different from those expected for different sex determination systems.

Results and Discussion

Table 1 summarizes the sex ratios found in groups of offspring originating from batches of eggs collected from four different females (dams 1–4). The gonads of all fish were sexually differentiated and no ovotestes were found. Both sexes were observed in the gynogenetic progeny groups of all four dams with the percentage of males ranging from 18 to 50%. One hundred and twenty-three confirmed gynogens, 123 triploids, and 85 control diploids originating from the ova of dam 4 were sexed. The sex ratio of the control diploids did not differ significantly from 1{male}:1{female}. The percentage of males found in the triploid group (14%) was not significantly different from that of the dam 4 gynogenetic group (18%) and the sex ratios found in both groups was significantly different from 1{male}:1{female} ($P < .005$)

Table 1. Sex ratios of 23- to 24-month-old gynogenetic, triploid, and diploid white sturgeon (*Acipenser transmontanus* Richardson)

Dam	Group	*N*	Male (%)	Female (%)
1	Gynogen	12	50	50
2	Gynogen	15	33	67
3	Gynogen	23	26	74
4	Gynogen	123	18	82
	Triploid	123	14	86
	Diploid	85	48	52

Our finding of a 1{male}:1{female} sex ratio in the diploid progeny and a significantly different ratio in the triploid and gynogenetic progeny derived from the same batch of eggs reared under similar environmental conditions supports the contention of a genetic sex determination system. The fact that males were present in gynogenetic progeny groups originating from batches of eggs from four different dams suggests that a ZZ:ZW female heterogametic sex determination system is operating in white sturgeon.

The frequency of ZW heterozygotes in gynogenetic offspring derived from species with female heterogamety is expected to vary according to the frequency of second division segregation. No heterozygotes are expected if a crossover never takes place between the sex-determining element and the centromere; 100% heterozygotes are expected if an obligate single crossover takes place; and about 2/3 heterozygotes are expected if the sex-determining element is assorting independently of the centromere (Nace et al. 1970). In the absence of interference the expected proportion of females in gynogenetic groups derived from female heterogametic species where the sex-determining element is segregating independently of the centromere is (2/3 ZW + 1/6 WW) = 5/6 female if the WW "super"-female is viable. The sex ratio of dam 4 gynogens was not significantly different ($P > .5$) from 1/6 male: 5/6 female suggesting that in white sturgeon the sex-determining element may segregate independently of the centromere.

The fact that the sex ratio of the triploid group did not differ significantly from that of the gynogenetic group further supports the contention of a female heterogametic genetic sex determination system with triploid genotypes being present in the ratio of 1/6 ZZZ:2/3 ZZW{female}:1/6 ZWW{female}. The data do not allow us to distinguish between a dominant "W" sex determination switch mechanism and an autosomal balance mechanism where the ratio $Z/A < 0.67$ generates females, or to determine whether the WW "super"-female is viable since we could not differentiate between WW viability (1/6{male}:5/6{female}) and WW inviabilty (1/5{male}:4/5{female}) with the limited number of gynogenetic and triploid progeny that we had available to calculate the sex ratios in this experiment. The viability of the WW "super"-female is of commercial importance because such females would be the ideal brood stock for monosex culture at caviar production facilities as they would be expected to produce all female progeny when crossed to normal ZZ males. As sex chromosome–specific markers have not been identified for this species (Van Eenennaam 1997), lengthy progeny testing represents the only method available to determine if the WW "super"-females are viable and to differentiate them from the more numerous heterozygous ZW females. Further studies analyzing the sex ratios found in gynogenetic and triploid groups derived from a larger number of different females need to be undertaken to confirm the results of this preliminary research, especially given the recent finding of all-female gynogens in a related chondrostean species, paddlefish (Mims et al. 1997).

If female heterogamety is found in other sturgeon species then this information should be considered in conservation plans for the many endangered sturgeon populations (Birstein 1993). Cryopreservation of sperm from endangered fish species, followed by thawing and dispermic fertilization or induced mitotic diploid androgenesis in genetically inactivated "donor" ova from a closely related species has been postulated as one method for ensuring the survival of fish species or

strains on the verge of extinction (Ihssen et al. 1990; Grunina and Neifakh 1991; Thorgaard et al. 1992). Such a methodology in species with female heterogamety would preserve only the ZZ males of endangered species, making population recovery impossible. Knowledge of the basic sex determination mechanism operating in threatened sturgeon and other fish species is of importance when considering the use of sperm cryopreservation for species conservation.

From the Department of Animal Science, University of California, Davis, CA 95616–8521. This research was partly funded by the University of California Genetic Resource Conservation Program, a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce (grant no. NA36RG0537), project R/A-99 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies. The U.S. government is authorized to reproduce and distribute for governmental purposes. A. L. Van Eenennaam was supported by a Sea Grant traineeship. Address correspondence to A. L. Van Eenennaam at the address above.

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Received September 22, 1997
Accepted April 30, 1998

Corresponding Editor: Bernie May

The "Spotted" Locus Maps to Bovine Chromosome 6 in a Hereford-Cross Population

M. D. Grosz and M. D. MacNeil

The spotted locus is responsible for several phenotypically distinguishable piebald patterns in cattle, including Hereford, or white face (S^H), lineback (S^P), and recessive spotting (s), in addition to nonspotted (S^+). In a backcross mapping population, the S locus has been mapped by genetic linkage to bovine chromosome 6, between microsatellite markers BM4528 and EL03. This region corresponds comparatively to a region on mouse chromosome 5 which houses several coat color mutations, among which homology is possible with Hardy–Zuckerman 4 feline sarcoma viral oncogene homologue (*Kit*), patch (*Ph*), and rump white (*Rw*). Mutations at these loci resemble mutations at the bovine S locus in both phenotype and mode of inheritance. Data are presented which show genetic linkage between the bovine S locus and microsatellite markers on chromosome 6. Candidate genes for the bovine S locus are discussed.

Spotting patterns in cattle have interested breeders for hundreds of years. Historically, piebald patterns have been favored in selection (Epstein 1971). Also, as breeds were developed, certain breed consistencies were favored as a method of rapid and convenient identification (Olson 1992). In addition to the aesthetic values attributable to coat color variation, there are other, more economical reasons for interest in coat color phenotypes. In particular, breeds that possess lighter coat color and darker skin pigmentation tend to perform better in tropical climates and increased levels of solar radiation (such as Zebu breeds; Olson 1992).

An additional interest in coat color variation has arisen recently in the form of price discrimination at the sale barn for specific coat color patterns, in some cases regardless of the actual pedigree of the animal. Animals can sell at discounts or premiums to cohorts of similar ancestry simply because of coat color variations. Although such discounts or premiums may be temporary, one cannot dispute the effect of such economic selection on the breeding decisions made by the cattle rancher.

There are several genes which are known to affect coat color and/or spotting pattern. Included in this group (as reviewed by Olson 1992) are the *E* locus (red/black), *A* locus (removal of some red and black pigmentation), *C* locus (removal of red pigment with no effect on black pigment), *D* locus (dilution series), *Br* (brindle), and *Bp* (patterned blackish). Different allelic combinations at these loci may appear to lessen or heighten the effect of a particular gene.

White-spotting mutations are present in a variety of cattle breeds (e.g., Hereford, Pinzgauer, Holstein, Simmental, etc.). Several genes have been identified as playing a role in the amount and pattern of white-spotting (as reviewed by Olson 1992), and include the *S* locus (spotting), *Cs* (colorsided), *R* (roan, may be allelic with *Cs*), *Bl* (blaze), *Bc* (brockling), and *Bt* (belted). At least four alleles at the *S* locus have been identified. The S^H allele is present in Hereford and is described as white face, belly, feet, and tail, often with a white stripe over the shoulder when homozygous. This allele shows incomplete dominance over the wild type, S^+, in that S^H/S^+ heterozygotes will have white areas generally around the face only. The S^P allele is present in Pinzgauer, Longhorn, and Florida Cracker breeds and is expressed as variable amounts of white appearing along the dorsal and ventral areas extending forward from the tail or rump. The *s* allele is recessive and is expressed as irregular areas of pigment and white, with the feet, tail, and belly usually white (as seen in the Holstein breed). The *s/s* phenotype is also classically known as "piebald" (Olson 1992).

As part of a larger study to identify quantitative trait loci (QTL) affecting traits of economic interest, a Hereford-sired bull was bred to composite (CGC; ½ Red Angus, 1/4 Tarentaise, 1/4 Charolais) and Line 1 Hereford dams to produce 77 backcross offspring. The objectives of this research were to identify the chromosomal

location of the gene(s) causing dominant spotting in Hereford cattle, and to assess the viability of candidate loci based on the available bovine-mouse comparative map. Results of linkage analysis show that the *S* locus resides on bovine chromosome 6, and also suggests that the gene causing white spotting in cattle may be homologous to one of several coat color loci on mouse chromosome 5, which produce similar phenotypic observations, and lie in a region of conserved synteny between bovine and murine genomes.

Table 1 Linkage analysis of S locus

	BM143	BMS1242	BM4528	*S*	EL03	BMS2460
BM143	239	1 cM, 43.99	17 cM, 13.8	24 cM, 4.17	21 cM, 6.26	27 cM, 6.43
BMS1242	172	206	17 cM, 13.5	24 cM, 4.4	23 cM, 6.31	29 cM, 5.17
BM4528	149	150	172	9 cM, 11.66	10 cM, 13.46	12 cM, 18.9
S	67	72	70	75	6 cM, 13.59	13 cM, 18.69
EL03	100	105	94	69	130	7 cM, 17.6
BMS2460	187	173	161	73	109	230

Number of coinformative meioses are given below the diagonal, linkage distances (cM) and associated LOD scores are given above the diagonal and total genotyped meioses on the diagonal. All microsatellite loci have been previously mapped to chromosome 6.

Materials and Methods

An F_1 bull derived from a cross of Miles City Line 1 Hereford bull (Line 1) and Composite Gene Combination dam (CGC; ½ Red Angus, 1/4 Tarentaise, 1/4 Charolais) was mated to CGC and Line 1 dams to produce backcross calves. The phenotype of Line 1 Herefords is the classic Hereford pattern, with white face, belly, feet, and tail, and white stripe over the shoulder. Genotypically this pattern can be defined as S^H/S^H. CGC cattle represent all coat colors present in Red Angus, Charolais, and Tarentaise breeds, including red, dilution, and brindle. None of the CGC dams (nor any members of the CGC herd) have ever presented a spotted phenotype. Therefore the CGC dams are considered genotypically S^+/S^+, and the F_1 bull is S^H/S^+. Calves produced as a result of breeding the F_1 bull to Line 1 Hereford dams were not phenotyped for the *S* locus because of the difficulty in reliably distinguishing between S^H/S^H and S^H/S^+ phenotypes. However, all calves (77 CGC backcross calves and 73 Line 1 Hereford backcross calves) were used for linkage analysis concerning microsatellite markers.

The F_1 bull was mated to CGC dams by artificial insemination to produce 77 offspring. These offspring were genotypically either S^+/S^+ (completely nonspotted) or S^H/S^+ (white spotted on or around the face). Calves were scored for the presence (S^H/S^+) or absence (S^+/S^+) of white spotting at approximately 1 month of age. Three calves were solid white, making it impossible to determine the extent of their spotting. Solid white calves were not scored for the *S* locus. Of the 74 scored calves, 38 (51.35%) where scored as white faced (S^H/S^+) and 36 (48.65%) were scored as non-white faced (S^+/S^+), which is consistent with a 1:1 ratio as expected for the *S* locus.

Microsatellite markers were prescreened by genotyping the F_1 sire by the method of Bishop et al. (1994). Markers that were informative were then used to genotype calves, sires, dams, paternal grandsires, and paternal grandams. Eighty-four of 159 markers screened (53%) were informative in the sire and were used to genotype the entire population. With 74 informative meioses, a LOD score supporting linkage would be achieved with 21 recombination events (28.4 cM, LOD = 3.11). At least 1 marker was present on each chromosome, with the exception of 25, X, and Y.

All genotypic data was entered into a relational database housed at USDA-ARS, Meat Animal Research Center, Clay Center, Nebraska (Keele et al. 1994), and linkage analysis was performed using CRI-MAP (Green et al. 1990). Two-point analyses were performed to determine maximum likelihood estimates. If the maximum log-likelihood estimate was 3.0 greater than the log-likelihood estimate of a recombination frequency of 0.5 (unlinked), then the data was interpreted as evidence for linkage. All two-point linkages between microsatellite markers and *S* with a LOD score (*Z*) greater than 3.0 were identified. Markers which showed a two-point LOD score of greater than 3.0 were analyzed to determine the best order of markers using the CRI-MAP BUILD and ALL functions.

Results and Discussion

Two-point linkage analysis of the dataset revealed linkage between *S* and five markers on chromosome 6, with LOD scores ranging from 4.17 to 18.69 (Table 1). This was considered strong evidence for the assignment of *S* to bovine chromosome 6. To ensure that no other linkages existed, stringency on the test for linkage was reduced to a log-likelihood estimate greater than 1.5 times the LOD at a recombination frequency of 0.5. Under these relaxed conditions, no linkage was observed between *S* and the markers not located on chromosome 6.

To determine the order of linked markers along chromosome 6, the ALL function of CRI-MAP was used. Based on our mapping population, the order of microsatellite markers from the centromeric end of chromosome 6 was observed to be BM143-1 cM-BMS1242-16 cM-BM4528-8 cM-EL03-6 cM-BMS2460-34 cM-BL1038. By using the ALL function of CRI-MAP, the most likely order (after insertion of S) is BL1038-BMS2460-*S*-EL03-BM4528-BM143 (Figure 1).

The current status of comparative maps allows for selection of candidate genes from regions known to share homology between two species. In order for two genes to be considered homologous, several criteria must be met, including conservation of function and conservation of sequence. The most effective method of identifying homologous genes across species is by first identifying regions of conserved synteny, followed by analysis at the sequence and functional levels.

The majority of genes mapped to bovine chromosome 6 also map to mouse chromosome 5. There is additional conservation of synteny between the centromeric end of BTA6 and MMU3. A comparison of loci on BTA6 and homologous mouse loci is shown in Table 2. According to the map published by Barendse et al. (1997), the distance from BM4528 to SPP1 is 30 cM. Assuming the highest likelihood order, which places *S* between BM4528 and EL03, our data show BM4528 and *S* to be separated by 6 cM, suggesting that *S* lies on the region of bovine chromosome 6 which is homologous to murine chromosome 5.

A search of the Mouse Genome Database web site (The Jackson Laboratory, http://www.informatics.jax.org/) for coat color–associated loci located on MMU5 yields five loci, including Kit oncogene (*Kit*), patch (*Ph*), rump white (*Rw*), light ear (*le*), and buff (*bf*). All five loci are positioned between 42 and 64 cM from the centromere. Light ear and buff are clearly phenotypically dissimilar to bovine spotting, which leaves *Kit, Ph,* and *Rw* (all

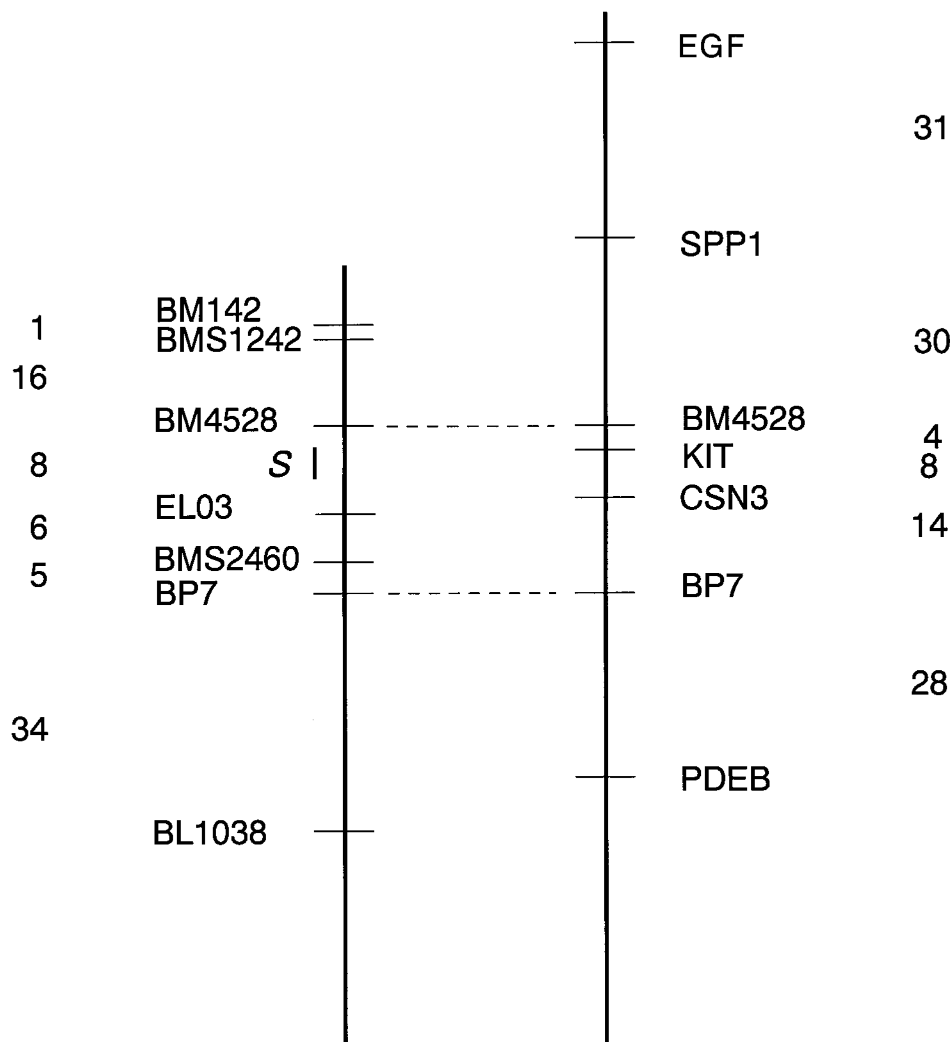

Figure 1. Linkage maps of bovine chromosome 6. Map on right represents markers used in this study (referenced in Kappes et al. 1997). Map on left is taken from Barendse et al. (1997). Linkage distances are in centiMorgans.

mapped to MMU5, 42 cM) as candidates for the bovine *S* locus.

The *Ph* mutation arose spontaneously in a C57BL strain. Homozygotes die in utero with malformations of the skull which interfere with proper development of the nose and palate. Heterozygotes survive and display variable levels of white spotting around the belly. The *Ph* locus is suggested to exert control over hematopoiesis (Loutit and Cattanach 1983). Several published reports have suggested that patch is a mutation involving the deletion of *Pdgfra* (Orr-Urtreger et al. 1992; Stephenson et al. 1991) and therefore would be more appropriately classified as an allele at the *Pdgfra* locus. However, because such a deletion may involve additional, adjacent genes (*Kit*; Duttlinger et al. 1995), *Ph* is not yet accepted as a *Pdgfra* allele.

Rump white was introduced by breeding (C3H/He × 101/H)F_1 males after exposure to low-intensity neutron irradiation. *Rw* behaves in a semidominant manner, with heterozygotes showing white spotting on the hind legs, tail, and posterior abdomen. Homozygotes die in utero at midgestation. Nagle et al. (1994) has suggested that rump white may well owe its effects on pigmentation to disruption of *Kit* and/or *Ph* regulatory sequences.

Kit is a member of the family of growth factor receptors that possess tyrosine kinase activity when bound to a specific ligand. The *Kit* gene is known to be involved with at least three populations of migratory stem cells: neural crest-derived melanocytes, hematopoietic stem cells, and primordial germ cells (Fleischman 1993). In the mouse, *Kit* and its alleles cause reduced and/or localized pigmentation, macrocytic anemia, and sterility (Geissler et al. 1988; Nocka et al. 1990).

Mutations at the *Kit* locus are semidominant and occur frequently (Schlager and Dickie 1967), with over 30 currently documented in the Mouse Locus Catalog literature. Examination of the listed phenotypes yields several general similarities. Homozygotes or compounds of two different mutations are generally white, sterile, and have severe macrocytic anemia, often causing death in utero. Animals containing one mutant allele show white spotting, are fertile, and may be slightly anemic. Mutations which affect the level of *Kit* kinase activity also tend to affect the severity of phenotypic expression. Mutations that abolish activity are homozygous lethal, while mutations with residual kinase activity are homozygous viable (Bernstein 1991).

In addition to white spotting, almost all *Kit* mutations also involve anemia. The condition is detectable at about 12 days of gestation and persists throughout life. Red cell count and hemoglobin are considerably reduced and the red cells are macrocytic (Bannerman et al. 1973; Russell 1970). This is an interesting finding, taking into account the discovery by Steffen et al. (1991) of a genetically based syndrome of anemia (along with progressive alopecia and dyskeratosis) transmitted genetically in a herd of polled Herefords.

This coincidence is compounded when it is considered that the frequency of bovine ocular squamous cell carcinoma ("cancer eye") is significantly higher in Herefords than other breeds (Anderson 1970; Blackwell 1956). Although the primary tumor type identified with *Kit* oncogene in mice is a papilloma of the forestomach and duodenum (Kitamura et al. 1980), the precise tumorigenic pathway is still poorly understood. Two hypotheses are presented: (1) during the formation of the Hereford breed, white-faced animals were combined and bred to produce true-breeding, white-faced (S^H/S^H) animals. However, these animals are only phenotypically similar. Underlying the mutation which causes spotting, there may be other mutations (in the same gene) which predispose animals to combinations of anemia, dyskeratosis, and tumors (as is seen in *Kit* mutants). (2) The presence of gene sequences which cause the phenotypic expression of S^H may also alter the expression and/or stability of nearby genes, which would predispose the animal to combinations of anemia, dyskeratosis, and tumors (as seen in *Rw* mutants).

This article has presented data which localizes the gene responsible for the Hereford white-face spotting pattern (*S* locus) to bovine chromosome 6 within a genomic region displaying conserved syn-

Table 2. Bovine chromosome 6 loci and map location in mouse and human

Gene symbol	Gene name	Mouse location (chr, cM)	Human location
EGF	Epidermal growth factor	3, 65.2	4q25
IF	I factor (compliment)	3, 66.6	4q24-q25
ADH3	Alcohol dehydrogenase 3	3, 71.2	4q21-q23
SPP1	Secreted phosphoprotein 1	5, 56	4q11-q21
GABRA2	Gamma-aminobutyric acid receptor, subunit alpha 2	5, 40	4p13-p12
KIT	Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue	5, 42	4q12
CSN3	κ-casein	5, 45	4p16.3-q21[b]
ALB	Serum albumin variant	5, 50	4q11-q13
GC	Group specific component	5, 50	4q12-q13
PDEB	Phosphodiesterase, cGMP, rod receptor, beta polypeptide	5, 57	4p16.3
QDPR	Quininoid dihydropteridine reductase	5, 30	4p15.3
PGM2[a]	Phosphoglucomutase 2	5, 38	4p14-q12
IDUA	Alpha-L-iduronidase	5, 57	4p16.3
PEP7	Peptidase 7	5, 39	4p11-q12
CNCG	Cyclic nucleotide gated channel, cGMP gated	5, 41	4p14-q13
PDGFRA	Platelet derived growth factor receptor, alpha polypeptide	5, 42	4q11-q12
GNRHR	Gonadotropin releasing hormone receptor	5, 44	4q21.2
SOD3	Superoxide dismutase 3, extracellular	Unknown	4p16.3-q21

Data obtained from Mouse Genome Database web site, The Jackson Laboratory, http://www.informatics.jax.org/, Barendse et al. (1997), Fries et al. (1993), Gallagher et al. (1992), Kappes et al. (1997), Threadgill and Womack (1990), and Zhang et al. (1992).

[a] PGM2 maps to MMU 4, 45 cM; however, PGM1 maps to MMU5, 38 cM, and most likely represents the appropriate homologous locus.

[b] 4p16.3-q21 represents the map location of CSN2, which along with CSN3 and other genes comprises the casein cluster.

teny with murine chromosome 5. Coincident with this finding are the observations of anemia and increased incidence of bovine ocular squamous cell carcinoma in the Hereford breed. Whether these observations are related through mechanisms involving the *Kit* oncogene or other genes remains to be proven. However, the prospect of a link between the bovine *S* locus and ocular squamous cell carcinoma is intriguing and warrants further study.

From the USDA-ARS, Fort Keogh Livestock and Range Research Laboratory, Rt. 1, Box 2021, Miles City, MT 59301. The authors wish to gratefully acknowledge Steven Kappes and John Keele of USDA-ARS, Meat Animal Research Center, Clay Center, Nebraska, for technical and material assistance. This research was conducted under a cooperative agreement between USDA-ARS and the Montana Agricultural Experiment Station and is published as contribution no. J-5152 from the Montana Agricultural Experiment Station. USDA-ARS, Northern Plains Area is an equal opportunity/affirmative action employer, and all agency services are available without discrimination.

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Received September 5, 1997
Accepted May 7, 1998

Corresponding Editor: James Womack

Hybridization Between Sika Deer (*Cervus nippon*) and Axis Deer (*Axis axis*)

G. W. Asher, D. S. Gallagher, M. L. Tate, and C. Tedford

We report an incidence of hybridization from natural mating between sika deer (*Cervus nippon*) and axis deer (*Axis axis*). A female exhibiting physical characteristics intermediate between the two species was born on a Tennessee deer farm sometime in 1995. Gel electrophoresis of three blood proteins (TF, HBB, and SOD) from the putative hybrid, the putative sika deer sire and three axis deer hinds from the

herd (not necessarily including the dam) initially verified that hybridization had occurred. Q-banded karyotypes further identified the offspring as a hybrid ($2n = 67$) between sika deer ($2n = 68$) and axis deer ($2n = 66$). Fertility of the hybrid remains to be assessed, although it is now of reproductive age.

The cervids comprise a complex taxonomic group of about 57 species and nearly 200 subspecies (Whitehead 1993) that is characterized by wide biogeographical, physical, physiological, and cytogenetic diversity within and between taxa. Numerous incidences of interspecies hybridization have been documented over the last century (Gray 1972; Whitehead 1993). With the recent growth of deer farming around the world, there has been an apparent increase in the incidence of purposeful or inadvertent hybridization. Examples of the former include successful hybridization between red deer (*C. elaphus*; $2n = 68$) and Père David's deer (*Elaphurus davidianus*; $2n = 68$), and between red deer and sambar deer (*C. unicolor*; $2n = 56$) by use of artificial insemination (Asher et al. 1988; Muir et al. 1997). Occasionally hybrids arise inadvertently due to enforced social interaction between taxa that would normally be geographically and behaviorally isolated. Recent examples of this include putative hybrids between red deer and rusa deer (*C. timorensis*; $2n = 60$) occurring on some Australian deer farms (Fennessy PF, personal communication).

We report here a case of inadvertent hybridization between sika deer and axis deer. This particular intergeneric cross does not appear to have been described previously.

Background History

For management reasons, a group of 25 axis deer hinds, 18 sika deer hinds, and a yearling sika deer stag on a deer farm in Tennessee were run together as a single group between August and December 1994. The subspecies status of the deer is unknown, although the sika stag is probably Formosian sika (*C. n. taiouanus*). In July 1996, the farmer noted that one of the female progeny (then a yearling) from the axis hinds was very "sika-like" in appearance and considered that it was perhaps a hybrid offspring.

At the time of reporting (June 1997), the putative hybrid is approximately 12–16 months of age (Figures 1 and 2). It exhibits certain physical and behavioral characteristics that distinguish it from its axis deer herdmates. In particular, white spotting and the dorsal stripe on the pelage are notably less distinct. It lacks the white throat patch characteristic of all axis deer but has a more distinct white rump patch and black tail similar to that of sika deer (Figure 1). The facial features are distinctly "sika-like," with a shorter skull and concave rostrum. Its tail is 5–6 cm shorter than that of an axis deer. The animal also exhibits a different posture when running, electing to hold it's head high rather than adopting the lowered-head stance of axis deer, and it also holds its tail erect in sika fashion (Figure 2).

Materials and Methods

In March 1997 blood samples and a skin biopsy were obtained from the putative hybrid (Y95-0108), its sika deer sire (63I 4537), and three axis deer hinds (63P 1308, 63I 4528, Y95 0027; not necessarily including the dam). For sampling, the deer were restrained individually in a cradle device.

Samples of blood were withdrawn from the external jugular vein into sterile vacutainers containing sodium heparin, held at approximately 5°C and couriered to the Invermay Agricultural Centre, New Zealand, within 48 h. Upon arrival, the samples were centrifuged at 1000 g for 15 min and the plasma fraction stored at –18°C for later electrophoretic evaluation. Using the methods of Emerson and Tate (1993), plasma was subjected to both polyacrylamide and starch-gel electrophoresis to assess allelic variation in transferrin (TF), hemoglobin (HBB), and superoxide dismutase (SOD). Previous studies have determined that these three protein markers distinguish sika deer and axis deer (Emerson and Tate 1993).

Skin biopsies were taken from the ear using a biopsy punch. The tissue was transferred immediately to sterile tubes containing DMEM medium (Gibco-BRL) supplemented with 10% fetal bovine serum and couriered overnight at ambient temperature to Texas A&M University. Fibroblast lines were established from the skin tissues of all five animals. Chromosomes were harvested following incorporation of 5-bromo-2′-deoxyuridine into early replicating bands, and then QFH banded according to published protocols (Gallagher and Womack 1992), the only exception being that after fluorescence staining with Hoechst 33258, chromosomes were mounted in an antifade rather than 2× SSC. Chromosome counts and analyses were then made on 15 QFH-banded metaphases for each individual and black-and-white photographs were taken of representative cells. To better resolve faintly fluorescent satellites, QFH-banded chromosomes were destained then counterstained with Giemsa stain, and representative cells rephotographed. Marker chromosomes were described according to standardized nomenclature of the domestic cow (Reading Conference 1976, 1980; ISCNDA 1989, 1990; Popescu et al. 1996).

Results and Discussion

Electrophoretic analysis provided initial confirmation that interspecies hybridization had indeed occurred (Table 1). At each marker the hybrid animal had one "sika" and one "axis" allele that fits exactly the expected phenotype of an F_1 hybrid. The results do not identify the putative dam (if present), as the axis deer all shared the same phenotype.

Chromosome counts taken on 15 cells per individual indicated diploid numbers of 68, 66, and 67 for the sika deer stag, axis deer hinds, and sika-axis hybrid hind, respectively. The chromosome complement of all individuals contained a pair of metacentric chromosomes (Figure 3A–C). This metacentric pair is of the Robertsonian type formed by centric fusion of acrocentric autosomes homologous to domestic cattle chromosomes 17 and 19 (BTA17 and 19) [see Gallagher et al. (1994) for the homologous mule and white-tailed deer chromosomes]. In addition to the metacentric pair, the axis hinds had a submetacentric autosomal pair (Figure 3B,D), and the hybrid was heterozygous, possessing only one of these submetacentric autosomes (Figure 3C,D). This submetacentric chromosome is also of the Robertsonian type with the q arm homologous to BTA10 and the p arm similar in size and banding to BTA27. The remaining chromosomes in the complement of all individuals were acrocentric, thus the differences in diploid number between the axis deer hinds and sika deer stag and the intermediate chromosome number of the hybrid was attributable to the presence and/or absence of the submetacentric Robertsonian chromosome. These karyotypic data are consistent with previous cytogenetic reports for axis and sika deer (Fontana and Rubini 1990).

In addition to variation in diploid number attributable to Robertsonian fusion, there were other noteworthy cytogenetic

Figure 1. Female sika × axis deer F_1 hybrid at 2 years of age (Mule Face Deer Farm, Tellico Plains, Tennessee).

differences between the sika deer stag and axis deer hinds that could be readily followed in the putative hybrid. The sika deer possessed an acrocentric autosomal pair homologous and identical in banding to BTA18 (Figure 3A,D), whereas the homologous pair in the axis deer appeared derivative, being slightly larger with a more prominent Q-positive region adjacent to the telomere (Figure 3B,D). The hybrid was heterozygous, possessing both chromosome band types (Figure 3C,D).

Along with this apparent species-specific difference in banding for axis and sika deer chromosomes homologous to BTA18, we also noted qualitative differences in the prominence of satellites associated with autosomes. We counted two to three very small and light-staining satellites on autosomes of the sika deer stag (Figure 3A,D); one of these satellites was on a chromosome homologous to BTA3, another one homologous to BTA4, and we occasionally visualized a satellite on a small acrocentric autosome. In contrast, the axis deer hinds revealed two light-staining but much larger satellites on a chromosome pair homologous to BTA4 (Figure 3B,D): axis hinds Y95-0027 and 63P1308 also possessed a third prominent satellite on an autosome homologous to BTA3. In the hybrid there were two to three light-staining satellites, with one of these of the axis-deer type. This prominent satellite in the chromosome complement of the hybrid was present on an autosome that appeared to be homologous to BTA4 (Figure 3C,D).

Figure 2. The same hybrid animal (right) with two adult female axis deer (left). Note differences in running posture and elevation of the hybrids' tail.

Table 1. Plasma protein phenotypes[a] for the axis deer hinds (n = 3), the sika deer sire, and the putative hybrid

	Locus		
Identifier	TF	HBB	SOD
63 I4528 (axis)	FF	FF	BB
63 P1308 (axis)	FF	FF	BB
95-0027 (axis)	FF	FF	BB
63 P1207 (sika)	BB	DD	CC
95-0108 (hybrid)	BF	DF	BC

[a] After Emerson and Tate 1993.

Fertility of the hybrid hind has yet to be assessed. At the time of reporting (June 1997) the animal is approaching the age of puberty for both parental species and will have the opportunity to mate with axis deer stags. A number of cervid hybrids with disparate karyotypes to their parents are of unknown fertility, including red deer × rusa deer (Fennessy PF, personal communication) and red deer × sambar deer (Muir et al. 1997). However, present indications for these two examples are that female fertility has been, at the least, compromised (i.e., no full-term pregnancies observed).

A further consideration is the potential seasonality entrainment of the sika × axis deer hybrid. The two parental species represent taxa of temperate (sika) and tropical (axis) origin, with distinctly different levels of reproductive seasonality. While sika deer are responsive to photoperiod cues, being "short-day" breeders with strongly delineated breeding seasons (Goss and Rosen 1973), axis deer appear to be largely aseasonal within a temperate environment (Louden and Curlewis 1988). Should the hybrid prove fertile, its potential breeding season would be of interest.

Neither morphological nor molecular evidence ally axis and sika deer. These species are typically separated by the deepest branch within the phylogeny of the Cervinae (Cronin 1991; Emerson and Tate 1993; Groves and Grubb 1987). Em-

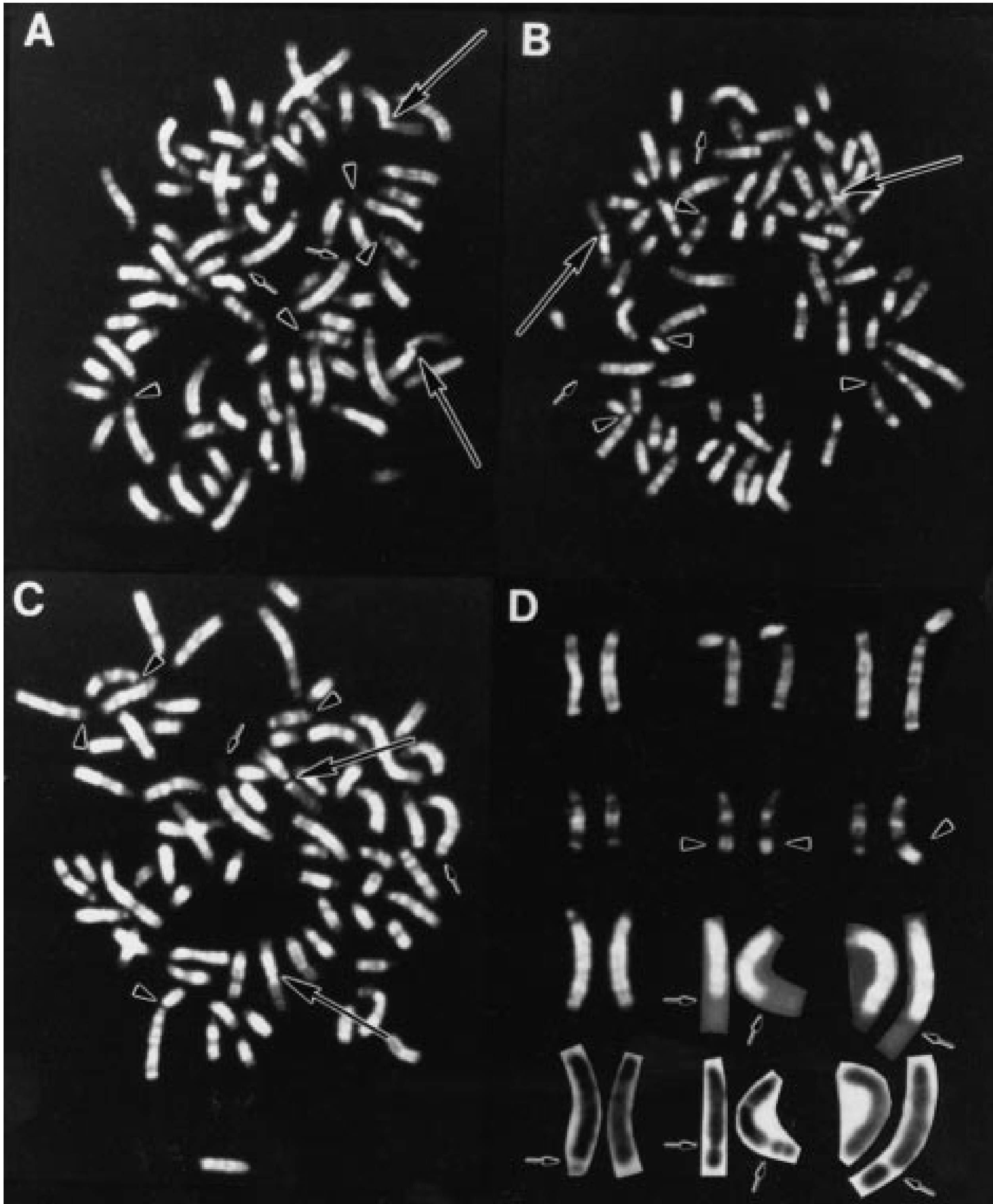

Figure 3. A composite showing a complete metaphase of the **(A)** sika deer stag, **(B)** axis deer hind 63I4528, **(C)** putative hybrid hind, and **(D)** select marker chromosomes taken from metaphases A–C. The large arrows point to the centromeres of the metacentric Robertsonian chromosome pair common to all individuals examined. The small arrows and arrow heads in (A–C) indicate marker chromosome displayed in (D). In (D), chromosome of individual deer are arranged in columns with the sika deer stag to the left, axis deer hind in the middle, and hybrid to the right. In (D), from top to bottom, row 1 corresponds to marker chromosomes of the variant submetacentric Robertsonian chromosome, row 2 of marker chromosomes homologous to BTA18, and rows 3 and 4 to marker chromosomes associated with qualitative differences in satellites. Note in (D) that the chromosomes in the last row are Giemsa stained to more clearly reveal the satellites, arrow heads point to the prominent Q-positive region near the telomere of axis deer chromosomes homologous to BTA18, and the small arrows point to secondary constrictions that are clearly visible on chromosomes homologous to BTA4.

erson and Tate (1993) calculated a genetic distance (Nei 1972) of 0.95 between axis and sika from an analysis of protein variants while Cronin (1991) found a mitochondrial DNA base substitution rate per nucleotide of 0.084 between axis and North American elk (*C. elaphus*), a close relative of sika. These distances exceed those of any previously reported interspecific cross within genus *Cervus* or between *Cervus* and *Elaphurus,* suggesting that the hybrid described here is phylogenetically the widest cross yet observed within the subfamily Cervinae.

From AgResearch, Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand (Asher and Tate), Department of Animal Science, Texas A&M University, College Station, Texas (Gallagher), and Mule Face Deer Farm, Tellico Plains, Tennessee (Tedford).

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Received September 22, 1997
Accepted June 30, 1998

Corresponding Editor: Oliver A. Ryder

Development and Characterization of Genetic Mapping Resources for the Turkey (*Meleagris gallopavo*)

H.-B. Huang, Y.-Q. Song, M. Hsei, R. Zahorchak, J. Chiu, C. Teuscher, and E. J. Smith

The development and partial characterization of turkey genomic libraries enriched for TG, GAT, and CCT simple sequence repeats (SSR) are described. The primary library, established using conventional methods, was enriched for each of the three SSR by single-primer polymerase chain reaction (PCR). The three enriched libraries were screened by standard hybridization and washing protocols under moderate to high stringency conditions. The utility of a fraction of the markers was evaluated based on the polymorphism of PCR-amplified products in a backcross reference DNA panel. The panel consisted of genomic DNA samples from three backcrossed families developed from a cross of a wild male turkey to three inbred Orlopp line C females. A total of 181 sequences from positive clones have been characterized and deposited in GenBank. About 60% of the 60 primer pairs designed from SSR-containing sequences detected polymorphism in the reference DNA panel. The turkey genomic DNA reference panel, the enriched libraries, and the markers described here provide an opportunity to begin to characterize the turkey genome and to develop a useful public genetic map for this economically important species.

In recent years genetic maps have been reported for many species of animals including chickens (Cheng et al. 1995), cattle (Bishop et al. 1994), goats (Vaiman et al. 1996) and mouse (Dietrich et al. 1992). The construction of genetic maps has been made easier by the availability of highly polymorphic DNA markers that can be easily typed using the polymerase chain reaction (PCR). Among PCR-based markers, simple sequence repeats (SSR) or microsatellites have been the most widely used due to their ubiquity and high level of polymorphism.

Microsatellites or SSRs are a class of PCR-based DNA markers that contain tandem repeats of 1–6 bases in length. They are numerous, widely distributed in eukaryotic genomes, and are highly polymorphic (Hamada et al. 1982; Weber 1990). An additional advantage of microsatellite markers is a codominant mode of inheritance. These advantages have made microsatellites the markers of choice in the construction of genome maps for many organisms, including chickens (Cheng et al. 1995).

The turkey is among the agriculturally important animal species for which there is no public genetic map. Recently, however, different random genome analysis methods like randomly amplified polymorphic DNA (RAPD) and single primer amplification of simple sequence repeats (SPARS) have been used to evaluate diversity and relatedness (Smith et al. 1996b) and to develop DNA markers (Smith et al. 1996a) for the turkey. In this article we report the development of a backcross reference DNA panel and three SSR-enriched genomic DNA libraries. These resources provide the framework essential for developing a turkey genome map. In addition, a turkey genome map makes possible comparative mapping with other species including closely related ones like the chicken.

Materials and Methods

The backcross reference DNA panel was established at Brigham Young University from matings of two wild male turkeys each to three partially inbred Orlopp line C females [crosses were developed by Orlopp Turkey Breeding, Inc. (Orosi, California), in association with the parent company Moroni Feed, Inc. (Moron, Utah). The wild turkey males were obtained by Moreno Feed, Inc., from an exotic bird fancier in Pennsylvania. In the current work, a total of 72 birds from a single male (three F_1 females) backcross family were used to test the utility of the markers. Genomic DNA was isolated from blood samples using the method of Bartlett et al. (1996).

The primary and enriched libraries were constructed as described by Polido and Duyk (1994) in the *pJCP-1* cloning vector using genomic DNA isolated from a wild turkey male (the homogametic sex) blood.

The enriched libraries were screened by conventional colony hybridization screening using radiolabeled oligonucleotide probes as described by Smith et al. (1996a). The double-stranded plasmid DNA templates from positive clones were prepared using the QIAGEN plasmid DNA isolation kit (QIAGEN). Positive clones were sequenced at the Iowa State University Nucleic Acids Facility by the dideoxy chain termination method using an ABI 373 sequencer (PE Applied Biosystems). The resulting sequences allowed for the verification of repeat-containing clones and for the design of PCR primers flanking the repeat sequences.

Primers designed for PCR typing were 16–26 bp long and were selected (using a combination of DNAsis, Hitachi, and OLIGO™, National Biosciences) so as to amplify DNA fragments in the range of 100–250 bp. PCR was performed in a 25 μl final volume containing 60 ng genomic DNA, 1 μM of each primer (Research Genetics, Inc.), 200 μM of each dNTP, 2.5 U Amplitaq polymerase, 2.5 μl 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), and 2 mM $MgCl_2$. The amplification was performed in a PTC-100-60 Thermal Cycler (MJ Research). Cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing temperature of 50°C–60°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Ten microliters of the PCR-amplified products were analyzed on 8% nondenaturing PAGE gels and length polymorphism was detected by silver staining (Goldman and Merrill 1982).

Results and Discussion

The BYU reference panel consists of a total of 450 backcross progeny from three families (blood and tissue samples can be obtained from the Animal Genetics Laboratory at Tuskegee University). The backcross progeny in each family were obtained from mating each of three F1 females to the wild male parent. A separate panel, of about 190 birds produced from matings between F_1 males and the Orlopp parental females is also available and would be useful for Z chromosome mapping. The choice of wild and commercial birds as parents was based on the rationale of a higher likelihood of detecting polymorphism. This rationale is consistent with that used by Crittenden et al. (1993) in the development of the East Lansing chicken reference genomic DNA panel. In the present work, 72 backcross progeny from one male mated to three F_1 females were used to assess the percent polymorphism for the markers studied.

From the three enriched libraries, 11,705 clones were characterized including 6270, 3515, and 1920 from CA, GAT, and CCT libraries, respectively. The percentage enrichment based on analysis of these clones from the CA, GAT, and CCT libraries was 11, 7, and 2, respectively. The differences in the percent enrichment of the libraries are probably a result of the relative frequency of the CA, GAT, and CCT repeats in the turkey genome. This is consistent with a previous report based on single-primer amplification of repeats that suggested a higher frequency of TG than GAT and CCT in the turkey genome (Smith et al. 1996b).

A total of 181 positive clones, 20 each from CCT- and GAT- and 141 from TG-enriched libraries were sequenced and characterized. The sequences have been submitted to GenBank and have been assigned accession numbers U79305 to U79409, AF019665–AF019735, and AF021811–AF021815. The average number of repeats in SSR-containing clones ranged from 5 to 32 with an average of 14 for TG and 5 and 7 for CCT and GAT clones, respectively. The average size of the cloned inserts, based on agarose gel analysis after restriction enzyme digestion, was 300 bp.

Consistent with reports of SSR characteristics in other species (Ostrander et al. 1992), the repeats varied from nondescript, with no obvious simple repeat, to compound. Analysis of the sequence data from 26 randomly selected clones identified from the TG-enriched library, for example, revealed that 60, 16, 4, and 20% of the repeats were perfect, imperfect, compound, and nondescript, respectively. The

Table 1. Characterization of simple sequence repeat polymorphisms in the turkey

Locus[a]	Motif	Accession number	Forward primer	Reverse primer	bp[b]	EL[c]
TUM1	[AGT]7	U79346	GTTGATCTGAAAGGGATGGA	ATGGTATAGACTATGGAATA	105	+
TUM2	[CA][d]	U79358	TGCATGCACTCTGACAGCGC	CCGCGAGTGCGTGCGTGCAC	150	+
TUM5	[CA]18	U70357	TGCTCGTGCAACGTGATGGC	ATAGCTGTGTGCGGTCA	171	+
TUM6	[CA]10	U79372	AAATCAGTGTCATTGTGCAA	TTCTGCTACCTGACCATGTA	135	+
TUM8	[CT]24[d]	U79374	GAGCGCTACCTGCCACCA	TAGAGGGCGGATCAGGGGTC	136	0
TUM9	[ATC]12	U79376	CAACAGCACTCAGCCTATCT	CGAATTGGCCCCTCTAG	119	0
TUM10	[CA]6	U79376	CACTTTACCTTTATCAGAAG	CCCAACAGATGACCTTTCAA	218	−
TUM11	[CA]18	U79361	TTCCAACTTCTTTGTAGGTT	CTGATACTGAAGGATTATTT	120	+
TUM12	[CA]6	U79356	ATAGGAACAGCAAACACGCA	TGGTTCGCCAAGAATGAAAA	180	−
TUM13	[CAT]12	U79315	AGCCTTGTTACAACAGCA	CTCACTATAGGGCGAATT	182	−
TUM14	[CAT]5	U79317	CGAGCTCTTCAAGTCTAC	CTGTAGCAAACCCAAGTT	137	+
TUM15	[CA]6	U79308	ATGCTTGTAACATGTCAT	GTTAGAGAATCAGGTAAG	145	+
TUM16	[CA]16	U79309	CCCTTGTTGGTAACACTT	TCTCACCATCCTCACCTT	155	+
TUM17	[CA]16	U79312	GGTTTGAGCAGAGTGAAT	GATTACTGGTTAGCTGCA	175	+
TUM18	[CA]16	U79313	CCTATAGCTTCAGTAGAG	AGTAGGGGTAGTTCTTGT	145	+
TUM19	[CA]12	U79328	CCTAATGCAAGGAACTGA	CCCACCAGTGTGTTTAAA	154	+
TUM20	[CT]24 [CA]	U79310	TCAGTCCTGGCAGTTTAC	GTTCCTGTGTAGGACAAT	153	+
TUM21	[TG]18	U79329	CCTGTTGCTGTGTCTCAC	TACGACTCACTTTAGGGC	142	−
TUM22	[TG]12	U79397	CCAGTTTTATTGCAACTC	AGTGTCACGATTCCCCTT	175	+
TUM23	[TG]14	U79332	CGGCATCTCCAGCTCCAT	CCACGGAGAGTCCTGGAT	146	+
TUM24	[CA][d]	U79379	TGCACACGCACACCTCT	AGATGCATGCTCGAGCG	153	0
TUM25	[CA]15	U79387	GAGTGGCAGTGACATCA	GAGCAGCTGAATGTAAA	100	−
TUM26	[CA]16	U79389	TTGTTGGTAACACTTGT	CCATTTCCAGTGTCTCA	164	0
TUM27	[TG]18[d]	U79394	AGGACTGATCTTTTTCT	TGATTCAATTCTATTTT	150	−
TUM28	[TG]12	U79391	AAATAACCCATGTTAGG	AAATAACCCATGTTAGG	127	−
TUM31	[CA]11	U79401	CTTCCCATCCCTGCAGAC	CCAAATTTACAATGGTT	120	−
TUM32	[CA]9[d]	U79403	CACACGCTGCCTCGGGTT	AAGGGAAACGGAAACATG	183	+
TUM33	[CA]11	U79399	ACGAACCCACAGTGATCA	CCGCCAGTGTGCTCTAAA	180	−
TUM34	[AGG]12	U79323	CAGGTAGGCGGCTTCGTC	CCAACTCCAGCAAAGTGA	137	+
TUM35	[AGG]6[d]	U79322	TCGTCCGTCCGTCCGAT	CCCAGTCAGGACGTTGT	199	+
TUM36	[AGG]9	U79321	ACACCTGCGAGCCGCGCA	CCAGGCACAGCTGCACAA	160	+
TUM37	[AGG]4	U79318	GCATTCAGTAACCTTTC	GTACGATCCATTGTCTGA	140	−
TUM45	[AGG]10	U79341	GCTGTGCTGTCGCGCA	GACCCCATCCGCAGCA	150	+
TUM46	[CA]6	U79336	GCCAGGCAGGGACAGAC	TTCTCCATGGTCAGTGA	150	−
TUM47	[TG]13	U79327	TCTGGCATGGTGCTCGG	GTTCATCCCCGCACACA	115	+
TUM48	[TG]9	U79305	GAAAGAAATCAAGAAAA	TTAATAGCTTTTGTGTG	120	−
TUM49	[CA]11	U79378	CCTTTCCCATCCCTGCA	GAGCCACTTGACATTTG	188	0
TUM50	[TG]12	U79306	CTGATGTCTTAAAGGCT	ACAAAACGAACTGATCA	122	+

[a] The TUM designation stands for Tuskegee University Microsatellite and is in accordance with the standardized nomenclature procedure proposed by Crittenden et al. (1996). Additional primer and sequence information from our on-going turkey genome work can be found at http://agriculture.tusk.edu/caens/genome/genome.html.

[b] The amplified fragment size represents the polymorphic product in turkeys based on gel estimate.

[c] The +, −, and 0 signs indicate polymorphic, non-polymorphic, and no product, respectively, in the East Lansing (EL) chicken reference DNA panel using the same PCR conditions as that for amplification with the BYU turkey DNA panel as templates. No comparison has yet been made of fragment sizes in the two species for each locus.

[d] The sequence contains two or more repeat motifs, with interruptions between any two.

number of perfect repeats from the TG library, however, was lower than that reported for dogs (Ostrander et al. 1993) but similar to that in humans (Weber and May 1989) and chickens (Cheng et al. 1995).

The 60 primer pairs tested were chosen based on analysis of sequence information for repeat lengths greater than 3 and 5 within GAT and CCT, and TG-positive clones, respectively. All the 60 primer pairs tested amplified products detectable by silver staining after resolution on a 6% nondenaturing polyacrylamide gel. Sixty-eight percent of the primer pairs also revealed polymorphism in the BYU turkey reference panel. Allele sizes and sequences of primer pairs that detected polymorphism in the turkey DNA panel are presented in Table 1. In addition, preliminary information on the ability of these primer pairs to detect polymorphism (based on segregation of amplified products) in the East Lansing chicken DNA panel is also presented. While 80% of the primer-pairs amplified products in the chicken, only 55% of the primer pairs that detected polymorphism in the turkey DNA panel also amplified polymorphic products in the East Lansing chicken reference DNA panel under similar PCR conditions (Table 1).

The turkey-specific primers that amplified products in chickens at stringent amplification conditions suggest that the turkey markers may also be useful in chickens. However, the products amplified from chicken need to be sequenced for verification of homology with the turkey sequences. An earlier study by Levin et al. (1995) showed that 95% of chicken primers result in PCR products in the turkey, though only 60% were polymorphic. Parallel efforts to develop microsatellite markers in both species should increase the resources available to develop useful genetic maps for these two economically important poultry species. These efforts will also facilitate a clearer understanding of the genomic relatedness of chickens and turkeys. Such understanding may help resolve conflicting reports of the usefulness of chicken microsatellite markers for mapping purposes in turkeys (Liu et al. 1996).

In summary, we have constructed turkey genomic libraries enriched for SSRs TG, GAT, and CCT consisting of fragments ranging in size from 300 to 500 bp. Screening the libraries with synthesized SSR probes has been successful. Although the present work demonstrates that SSRs may be frequent in the turkey genome, their relative abundance could not be determined due to the bias inherent in the PCR enrichment process. The higher frequency of TG repeats relative to GAT and CCT, however, is consistent with reports of studies in other species. The reference panel and the polymorphic markers provide, for the first time, the resources necessary for the development of a public turkey genetic map.

From the Animal Genetics Laboratory (Huang, Song, and Smith) and the Department of Biology (Chiu), Tuskegee University, Tuskegee, Alabama; Research Genetics, Inc., Huntsville, Alabama (Hsei and Zahorchak); and the Department of Veterinary Pathobiology, University of Illinois, Urbana-Champaign, Illinois (Teuscher). Many thanks are owed to S. J. Lamont, Iowa State University, Ames, Iowa; T. F. Savage, Oregon State University, Corvallis, Oregon; and G. E. Smith and C. K. Bonsi, Tuskegee University, Tuskegee, Alabama, for their critical review of the manuscript. We are also very grateful to two anonymous reviewers and Lyman Crittenden (the Associate Editor) for comments and suggestions that greatly improved on the initial version of this manuscript. This research was sponsored in part by the National Science Foundation Research Improvement in Minority Institutions program (grant no. 94–50385) and the U.S. Department of Agriculture Capacity Building Grants program (grant no. 93–38814–8674). Address correspondence to E. J. Smith, 109 Milbank Hall, Tuskegee University, Tuskegee, AL 36088.

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Received September 30, 1997
Accepted April 30, 1998

Corresponding Editor: Lyman Crittenden

An STS Linked to a Hessian Fly Locus Controlling Virulence to Resistance Gene *H13* in Wheat

L. Zantoko and R. H. Shukle

We have identified a sequence tagged site ($STSE9_{617}$) linked to both the locus controlling virulence in Hessian fly to resistance gene *H13* in wheat (*vrH13*) and the white-eye (*w*) locus. *w*, $STSE9_{617}$, and *vrH13* are sex linked and occur on a single linkage group. The loci were mapped through analysis of recombinant genotypes in backcross progenies. These data contribute toward the development of a genetic map, anchored to the white-eye marker, that includes genes controlling virulence in Hessian fly to resistance in wheat.

Historically, the Hessian fly [*Mayetiola destructor* (Say)] has been the most important insect pest of wheat (*Triticum aestivum* L.) in the United States (Foster et al. 1986; Ratcliffe et al. 1994). Females of these members of the gall-forming midges, family Cecidomyiidae, lay eggs on the leaves of wheat plants. Upon hatching, larvae enter the leaf sheath and feed near the crown or at nodes. Feeding by larvae re-

sults in stunting of the primary culm and development of a dark green color in the leaves (Byers and Gallun 1972). The primary method of control of the insect is through genetically resistant wheat. However, the use of new resistance genes in wheat has resulted in the selection of new virulence genes in the insect (Gallun 1977; Ratcliffe et al. 1994). Resistance resources in wheat and wheat relatives are expected to be finite, and analysis of the genetic structure of field populations of the insect as well as knowledge of how the insect overcomes resistance in wheat will be essential to continue effective and durable control of the Hessian fly.

Resistance in wheat to the Hessian fly is expressed as larval antibiosis and is controlled by dominant alleles at one or two resistance loci. Genetic studies have shown that virulence in the insect is controlled by recessive alleles at single virulence loci and operates in a gene-for-gene relationship with resistance in wheat (Gallun 1978; Hatchett and Gallun 1970; Zantoko and Shukle 1997). Knowledge of the gene products involved in such a virulence-resistance relationship will reveal how coevolution proceeds at the molecular level in gene-for-gene interactions.

Hessian fly chromosome cycles have several aberrant aspects. Somatic chromosome number in the insect is $n = 4$, two autosomes and two sex chromosomes (Stuart and Hatchett 1988a). The sex of individuals is determined through an XO system with males haploid for the sex chromosomes. In addition, there are 28–42 germline limited chromosomes that are eliminated from male gametes during early meiosis and from the soma of males and females during early embryonic development. During early spermatogenesis, paternally inherited somatic chromosomes are also eliminated, along with the germline limited chromosomes and no recombination occurs between paternal and maternal chromosomes (Gallun and Hatchett 1969; Stuart and Hatchett 1988a,b).

Females generally produce either all male or all female progenies. However, neither males nor females are heterogametic, and male progeny are established through elimination of paternally inherited sex chromosomes from the soma during early embryogenesis. The elimination or maintenance of sex chromosomes to establish male or female progenies is under the control of an autosomal, maternal-effect gene, chromosome maintenance (*Cm*) (Stuart and Hatchett 1991). This mechanism for determination of the sex of a progeny precludes inbreeding through sib matings for isolation and maintenance of alleles and hinders mapping in multiple families; however, the haplotype of sex-linked alleles can be determined by examining male genotypes. The haplotype of autosomal alleles can be determined by comparing the genotype of a male's mother with the genotype of his mate and their offspring through the crossing design described by Black et al. (1996).

Linkage between the sex-linked recessive marker white eye and the recessive allele causing virulence in Hessian fly to resistance genes *H13* and *H9* in wheat has been reported (Formusoh et al. 1996; Zantoko and Shukle 1997). The objective of the current work was to contribute toward development of a genetic map for sex-linked alleles in Hessian fly as part of a long-term goal of positional cloning of virulence/avirulence alleles and molecular characterization of the gene products. We identified an RAPD linked to white eye (*w*). The RAPD has been cloned and primers designed to convert it to a sequence tagged site (STS) (Olson et al. 1989). Recombination values and map distances between *w*, the STS, and *vrH13*, as well as the order of the loci, are reported.

Materials and Methods

Experimental Insect

Genotypes of the parental Hessian fly lines used in the current work were (1) white eyed (*w/w*), *H13* avirulent ($vrH13^{+}$/$vrH13^{+}$) and (2) dark eyed (w^{+}/w^{+}), *H13* virulent (*vrH13/vrH13*). Development of these lines and methods for rearing Hessian flies and conducting single-pair matings have been previously described (Shukle and Stuart 1993; Zantoko and Shukle 1997). In evaluating backcross progenies segregating for white-eyed and dark-eyed phenotypes, larvae were reared on the wheat cultivar Blueboy (no resistance genes). Backcross progenies segregating for virulence to resistance gene *H13* were evaluated by rearing larvae on the wheat cultivar Molly. In order to ensure accurate scoring of virulent/avirulent genotypes, one larva was reared per plant (Grover et al. 1989). Since larvae with an avirulent genotype died within a few days on resistant plants, only virulent genotypes were recovered in these analyses.

Isolation of DNA

DNA was isolated from adults by adaptations of the techniques described by Black et al. (1992)and Lis et al. (1983). The technique of Lis et al. was used for bulk isolation of DNA from progeny samples, while the technique of Black et al. was used for isolation of DNA from individuals.

Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

RAPD-PCR amplifications were done in a volume of 50 μl with Promega *Taq* polymerase buffer, 2 mM MgCl, 200 μM dNTPs, 500 pmol of primer, and 2 units *Taq* DNA polymerase (Promega, Madison, Wisconsin). Genomic DNA (about 5 ng) prepared from adult *M. destructor* was used as the template in amplifications. Amplification of RAPDs was with OPERON Technology (Almeda, California) primer sets A–E.

Amplifications were carried out in a programmable temperature cycler (MJ-Research Inc., Watertown, Massachusetts) with the following program: 80°C for 20 min, 94°C for 1 min, 36°C for 1 min, 72°C for 2 min for a total of 45 cycles, and 4°C for storage. The initial 80°C step was used to load the *Taq* polymerase to prevent artifacts due to nonspecific binding of primer to template DNA. A control lacking template DNA (DNA negative control) was included in all PCR amplification sets.

Bulk Segregant Analysis

Initial screening of 100 RAPD primers was performed with bulk DNA isolated from the two parental lines. Primers detecting apparent variation between the parental lines were then evaluated for detection of an RAPD linked to *w* by bulk segregant analysis (Michelmore et al. 1991). Crosses conducted to produce backcross progenies were reciprocal crosses between the white-eyed, *H13* avirulent and dark-eyed, *H13* virulent lines and backcrosses of F_1 females to males from the dark-eyed, *H13* virulent line. Resulting backcross male progenies were divided into white-eyed and dark-eyed bulks for analysis. Linkage disequilibrium for RAPDs in both coupling and repulsion phase with *w* could be evaluated in backcross males which are hemizygous for maternally inherited sex-linked alleles and reflect meiotic recombination in F_1 females (Black et al. 1996; Shukle and Stuart 1993; Zantoko and Shukle 1997).

Cloning and Sequencing of RAPDs

RAPDs linked to *w* were recovered from agarose gels using GeneClean (BIO 101, Vista, California) and cloned in the T vector pT7Blue (Novagen, Madison, Wisconsin). The identity of a cloned fragment was confirmed by DNA gel blot analysis to se-

quences amplified with the RAPD primer producing the variation and template DNA isolated from backcross males divided into white-eyed and dark-eyed bulks. DNA gel blots were prepared by standard procedures using a Hoefer Trans-Vac (Pharmacia Biotech, Piscataway, New Jersey). Cloned sequences hybridized to DNA gel blots were labeled with $[\alpha^{32}P]$-dCTP (sp. act. 3000 Ci/mmol; Amersham, Arlington Heights, Illinois) by oligonucleotide random priming reaction (Feinberg and Volgelstein 1983).

A cloned fragment confirmed by DNA gel blot analysis as having identity to an RAPD was sequenced on both strands over its full length using the Pharmacia ALF DNA sequencer system (Pharmacia Biotech, Piscataway, New Jersey). Sequence analysis was performed using the Genetics Computer Group program package (Devereux et al. 1984). Primers to convert the RAPD to an STS were then designed based on the DNA sequence obtained. The BLASTX program (Altschul et al. 1990) was used to search protein databases with the putative translation of the STS.

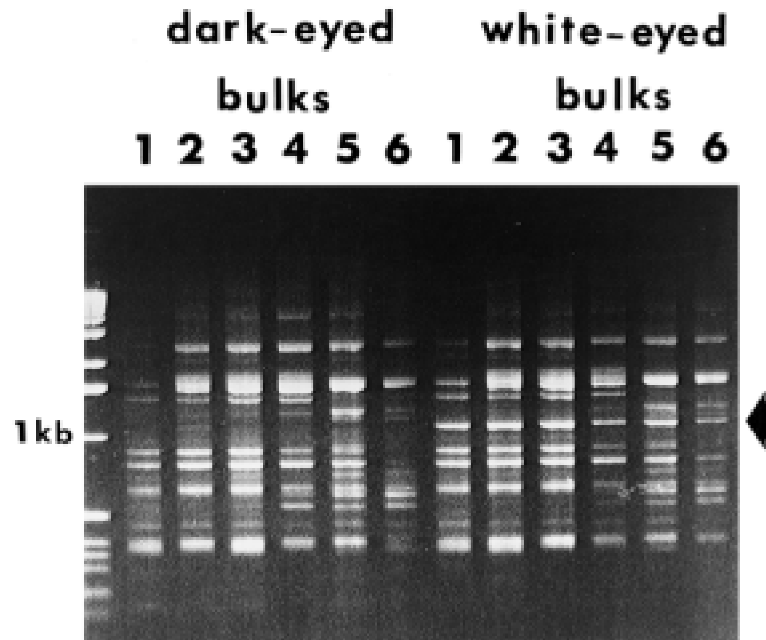

Figure 1. Linkage of an RAPD with white eye color in Hessian fly. Polymerase chain reaction (PCR) was with primer OPE9 and template DNA isolated from flies divided into dark-eyed and white-eyed bulk segregant classes for six backcross male progenies. Lanes 1–6, dark-eyed bulks, show amplification products lack the 1.1 kb marker band. Lanes 1–6, white-eyed bulks, show the 1.1 kb marker. Arrow indicates the 1.1 kb marker band. A 1 kb ladder was run on the left. The location of the 1 kb marker is indicated.

Analysis of Segregation Genotypes in Backcross Progenies

Recombination between *w* and a DNA sequence identified in bulk segregant analyses was determined by analysis of individuals in backcross male progenies segregating for white and dark eye color with PCR primers specific for the target DNA sequence. Backcross progenies for these analyses were constructed in the same manner as for bulk segregant analysis and larvae reared on the wheat cultivar Blueboy (no resistance genes). PCR amplification with primers specific for a polymorphic sequence was similar to that described for RAPD primers, except that the annealing temperature for binding of primers to template DNA was increased to 55°C and the primer concentration reduced to 50 pmol per primer. The integrity of template DNA isolated from backcross flies in a reaction where specific primers failed to amplify the DNA sequence was verified by PCR with the decameric RAPD primer producing the variation. Production of an expected pattern of amplified sequences with the RAPD primer was taken as evidence the template DNA was intact.

Recombination between an STS linked to *w* and *vrH13* was evaluated by analysis of individuals in backcross progenies segregating for virulence to *H13*. Backcross progenies for these analyses were constructed as previously described and larvae reared with one larva per plant to ensure accurate scoring of virulent and avirulent genotypes on the wheat cultivar Molly (resistance gene *H13* only). Linkage disequilibrium between an STS in either coupling or repulsion phase to the recessive virulence allele could be evaluated in backcross male progenies. Linkage in backcross females could be evaluated when an STS was in *cis* with avirulence.

Analysis of segregation genotypes in backcross male progenies for mapping loci was with the aid of MapMaker Macintosh version 2.0 (Copyright 1992 E. I. duPont de Nemours and Company). Conversion of recombination values between loci to map distances was with the Kosambi mapping function (Kosambi 1944).

Results and Discussion

Of the 23 RAPD primers found to detect variation between the parental white-eyed, *H13* avirulent and dark-eyed, *H13* virulent lines only one detected an RAPD that was linked to *w* in bulk segregant analyses of seven backcross male progenies. The seven backcross male progenies consisted of 284 and 267 white-eyed and dark-eyed flies, respectively. This primer, OPE9, amplified a 1.1 kb fragment that consistently appeared in white-eyed bulks but not in dark-eyed bulks of backcross males (Figure 1). The amplified 1.1 kb fragment, named $OPE9_{1100}$, was cloned and the identity of the cloned fragment confirmed by DNA gel blot analysis to sequences amplified with primer OPE9 and template DNA isolated from backcross males divided into white-eyed and dark-eyed bulks. The fragment $OPE9_{1100}$ was sequenced over its entire length and specific primers designed to amplify an STS of 617 bp near the middle of the fragment. The primers had the following sequences: forward primer *STSE9*–617F-5′-CGCGTCAACACTTTCGAATAG; reverse primer *STSE9*–617R-5′-ATTTAATTCGGGGACAATTTT. These primers amplified a 617 bp sequence that was linked to white eye color in backcross males and were used to evaluate recombination between $STSE9_{617}$ and *w* in backcross male progenies (Figure 2). BLASTX of the STS sequence to peptide sequence databases revealed only one match. This was for a putative translation sequence near the middle of the STS that showed 57% identities and 76% positives (identities plus conserved substitutions) to 21 amino acids from cucumber (*Cucumis sativus* L.) lectin.

Use of an STS increased the reliability of accurately scoring backcross genotypes for the presence of the target DNA sequence compared with the RAPD analysis. Parental genotypes in these analyses were (1) *w*, $STSE9_{617}$ positive and (2) w^+, $STSE9_{617}$ negative. Recombinant genotypes were (1) *w*, $STSE9_{617}$ negative and (2) w^+,

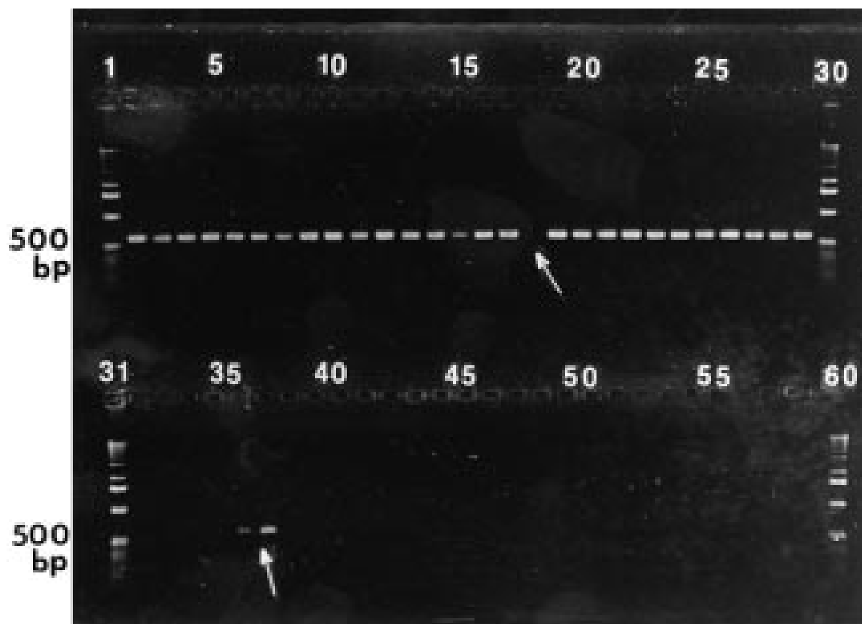

Figure 2. Segregation of $STSE9_{617}$ and white versus dark eye color in individual backcross males. PCRs with template DNA from individual white-eyed flies are shown in lanes 2–29. The arrow in lane 18 indicates the result with a recombinant white-eyed fly negative for $STSE9_{617}$. PCRs with template DNA from dark-eyed flies are shown in lanes 32–59. The arrow indicates the results with two recombinant dark-eyed flies positive for $STSE9_{617}$. A 1 kb ladder was run in lanes 1, 30, 31, and 60. The location of the 500 bp marker is indicated.

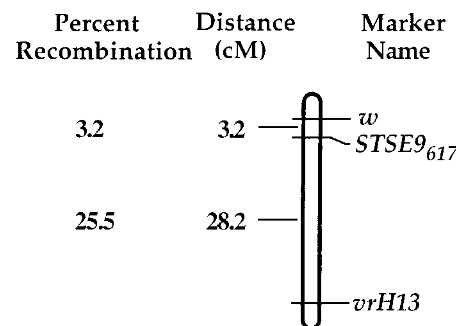

Figure 3. Map of the loci white eye (*w*), $STSE9_{617}$, and virulence to resistance gene *H13* (*vrH13*) based on analysis of segregation genotypes in backcross male progenies using MapMaker Macintosh v2.0. The Kosambi mapping function was chosen to convert recombination values to map distances because of interference in the data (no individuals with the recombinant genotype w^+, $STSE9_{617}$ positive, *vrH13* were recovered; see Table 2).

$STSE9_{617}$ positive. The results of these analyses (Table 1) revealed a recombination value between $STSE9_{617}$ and the white locus of 1.4 ± 0.4%.

Analysis of eye color and $STSE9_{617}$ within backcross males selected for *H13* virulent genotypes on the wheat cultivar Molly revealed the order of the loci (Figure 3) and the recombination values (Table 2) between them. The parental genotype recovered in these analyses (only virulent genotypes survived) was w^+, $STSE9_{617}$ negative, *vrH13.* The recombinant genotypes were (1) *w,* $STSE9_{617}$ negative, *vrH13*; (2) *w,* $STSE9_{617}$ positive, *vrH13*; (3) w^+, $STSE9_{617}$ positive, *vrH13.* The recombinant genotypes *w,* $STSE9_{617}$ negative, *vrH13* and w^+, $STSE9_{617}$ positive, *vrH13* could have resulted from double crossover between *w* and *vrH13,* depending on the order of the loci (i.e., *w,* $STSE9_{617}$, *vrH13* or $STSE9_{617}$, *w,* *vrH13*). The frequencies of segregation genotypes within *H13* virulent backcross male progenies (Table 2) suggested the rare genotype w^+, $STSE9_{617}$ positive, *vrH13* would have resulted from a double crossover between *w* and $STSE9_{617}$ (region 1) and between $STSE9_{617}$ and *vrH13* (region 2). The genotype *w,* $STSE9_{617}$ negative, *vrH13* resulted then from a single crossover between *w* and $STSE9_{617}$. This suggested the order of the loci was *w,* $STSE9_{617}$, *vrH13.* This hypothesis was supported by analysis of the data from backcross male progenies with MapMaker Macintosh. Recombination in backcross male progenies between *w* and *vrH13* was 28.7 ± 4.6%. Recombination between $STSE9_{617}$ and *vrH13* was 25.5 ± 4.4% and recombination between *w* and $STSE9_{617}$ was 3.2 ± 1.7%.

Recombination between $STSE9_{617}$ and *vrH13* in backcross female progenies selected for *H13* virulent genotypes on cultivar Molly was also evaluated ($STSE9_{617}$ in *cis* with the dominant allele controlling avirulence to *H13* in the parental genotype *w,* $STSE9_{617}$ positive, $vrH13^+$). Results revealed a recombination value between $STSE9_{617}$ and *vrH13* of 28.9 ± 4.1% (Table 2). Within *H13* virulent backcross female progeny the possible double-crossover genotypes could not be distinguished from the parental genotype or single-crossover recombinant genotype since the white-eye allele was covered by the dominant wild-type dark allele in females (Table 2).

We previously reported recombination values between the white-eye locus and the locus controlling virulence to *H13* with the alleles *w* and *vrH13* in both *trans* and *cis* (Zantoko and Shukle 1997). These results estimated the recombination between *w* and *vrH13* with the alleles in repulsion phase at 21.5 ± 2.0% and with the

Table 1. Segregation of eye color and $STSE9_{617}$ within backcross male progenies

Mating[a] $\frac{W\ E9^+}{W^+\ E9^-} \times W^+\ E9^-$

N	Sex of progeny	Totals	Segregation genotypes and their frequencies				Phenotypic ratios	Observed frequencies	Exp. ratios[b]	χ^{2c}	*P*	Percent recombination
7	Male	**664**					W^+:W	344: 320	1:1	0.63	.3–.5	
			NTR $\underline{W^+\ E9^-}$	340	NTR $\underline{W\ E9^+}$	315						
			RT $\underline{W^+\ E9^+}$	4	RT $\underline{W\ E9^-}$	5						
		344					$W^+\ E9^-$:$W^+\ E9^+$	340: **4**	1:1	324.2	<.001	$0.6^d \pm 0.2$ $1.4^f \pm 0.4$
		320					$W\ E9^-$:$W\ E9^+$	**5**: 315	1:1	300.3	<.001	$0.8^e \pm 0.3$

W and W^+, white and dark-eyed alleles; $E9^-$ and $E9^+$, $STSE9_{617}$ negative and $STSE9_{617}$positive; NRT, nonrecombinant type; RT, recombinant type.

[a] F_1 females from reciprocal crosses between *W,* $STSE9_{617}$ positive, $vrH13^+$ and *W,* $STSE9_{617}$ negative, *vrH13* flies.

[b] Based on independent assortment of alleles controlling eye color and $STSE9_{617}$.

[c] Chi-square goodness-of-fit test. The null hypothesis is that percent recombination is 50%. There is one degree of freedom.

[d] Percent recombination between *W* and $STSE9_{617}$ in dark-eyed flies.

[e] Percent recombination between *W* and $STSE9_{617}$ in white-eyed flies.

[f] Percent recombination between *W* and $STSE9_{617}$ in both dark-eyed and white-eyed flies.

Total number of individuals scored and numbers of recombinant genotypes are shown in bold.

Table 2. Segregation of eye color and *STSE9*$_{617}$ within *H13* virulent backcross genotypes

Mating[a] $\frac{W\ E9^+\ vrH13^+}{W^+\ E9^-\ vrH13} \times \underline{W^+\ E9^-\ vrH13}$

N	Sex of progeny	Totals	Segregation genotypes and their frequencies				Phenotypic ratios	Observed frequencies	Exp. ratios	$\chi^{2\,b}$	*P*	Percent recombination
3	Male	**94**					W^+:W	67:**27**	1:1	17.02	< .001	28.7[d] ± 4.6
			NRT $W^+\ E9^-\ vrH13$	67	RT $W\ E9^+\ vrH13$	24						
			RT $W\ E9^-\ vrH13$	3	RT $W^+\ E9^+\ vrH13$	0						25.5[e] ± 4.4
		27					W, $E9^-$:W, $E9^+$	**3:24**	1:1	16.33	< .001	3.2[f] ± 1.7
4	Female	**121**					W^+:W	121:0	1:0[c]	0	1	
			NRT $\frac{W^+\ E9^-\ vrH13}{W^+E9^-\ vrH13}$, $\frac{W\ E9^-\ vrh13}{W^+\ E9^-\ vrH13}$	86	RT $\frac{W\ E9^+\ vrH13}{W^+\ E9^-\ vrH13}$, $\frac{W^+\ E9^+\ vrH13}{W^+\ E9^-\ vrH13}$	35						
		121					W^+, $E9^-$:W^+, $E9^+$	86:**35**	1:1	21.49	< .001	28.9[g] ± 4.1
7		**215**					$E9^-$:$E9^+$	156:**59**	1:1	43.76	< .001	27.4[h] ± 3.0

W and W^+, white and dark-eyed alleles; *vrH13* and $vrH13^+$, *H13* virulence and *H13* avirulence alleles; $E9^-$ and $E9^+$, *STSE9*$_{617}$ negative and *STSE9*$_{617}$ positive; NRT, nonrecombinant type; RT, recombinant type.

[a] F_1 females from reciprocal crosses between *W, STSE9*$_{617}$ positive, $vrH13^+$ and W^+, *STSE9*$_{617}$ negative, *vrH13* flies.

[b] Chi-square goodness-of-fit test. The null hypothesis is that percent recombination is 50%. There is one degree of freedom with this test.

[c] Based on dominance of the dark-eyed allele in backcross females.

[d] Percent recombination between *W* and *vrH13.*

[e] Percent recombination between *STSE9*$_{617}$ and *vrH13* in male progenies.

[f] Percent recombination between *W* and *STSE9*$_{617}$ in male progenies.

[g] Percent recombination between *STSE9*$_{617}$ and *vrH13* in female progenies.

[h] Percent recombination between *STSE*$_{617}$ and *vrH13* combined data from male and female progenies.

Total number of individuals scored and numbers of recombinant genotypes are shown in bold

alleles in coupling phase at 27.3 ± 3.0%. Considering both repulsion and coupling data, recombination between the loci was then estimated at 24.3 ± 2.0%. The current data estimate recombination between the white-eye locus and *STSE9*$_{617}$ at 1.4 ± 0.4% (segregation of *STSE9*$_{617}$ in male progenies, virulent and avirulent genotypes considered; Table 1) and recombination between *STSE9*$_{617}$ and *vrH13* at 27.4 ± 3.0% (segregation of *STSE9*$_{617}$ in male and female backcross progenies, only virulent genotype considered; Table 2). These combined data estimate the recombination between the white-eye locus and the locus controlling virulence to *H13* at 28.8 ± 3.4%, which is in good agreement with our previously reported value. The estimate of recombination between the white-eye locus and *STSE9*$_{617}$ when only *H13* virulent backcross male genotypes were taken into consideration (Table 2) of 3.2 ± 1.7% is not considered to as accurately reflect recombination between these loci as data obtained when both virulent and avirulent genotypes were considered (Table 1) because of the smaller sample size. The recombination value between the white-eye locus and the locus controlling virulence to *H13* within *H13* virulent male progenies (Table 2) of 28.7 ± 4.6% is also in good agreement with the combined estimate of 28.8 ± 3.4% for recombination between *w, STSE9*$_{617}$, and *vrH13* and our previously reported recombination value of 24.3 ± 2.0% (Zantoko and Shukle 1997).

There is economic interest in Hessian fly genetics because plant resistance is the primary method of control and fly genotypes continue to evolve that can live on resistant wheat. The lack of genetic markers and maps for this economically important insect has hindered the application of molecular genetics to understand the virulence-resistance relationship between the insect and its host plant. Recently it has been reported that the locus controlling virulence to resistance gene *H13,* the locus controlling virulence to resistance gene *H9,* and the locus controlling white eye are sex-linked loci and part of the same linkage group (Formusoh et al. 1996; Zantoko and Shukle 1997). This suggests the alleles controlling virulence/avirulence to resistance genes *H13* and *H9* could be isolated through genetic mapping and a positional cloning strategy. The present results provide an initial step toward development of such a genetic map. We are currently in the process of hybridizing *OPE9*$_{1100}$ and *STSE9*$_{617}$ to salivary polytene chromosomes via in situ hybridization. These results should allow us to map *w, STSE9*$_{617}$, *vrH13,* and *vrH9* to a specific X chromosome.

From the Department of Entomology (Zantoko) and the USDA-ARS (Shukle), Purdue University, West Lafayette, IN 47907. This is a joint contribution of the USDA Agricultural Research Service and Purdue University. Purdue University AES Journal paper 15513. Supported through USDA CRIS no. 3602-22000-010-00D. Mention of a commercial or proprietary product does not constitute an endorsement by the USDA. Address reprint requests to R. H. Shukle at the address above.

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Received December 17, 1997
Accepted June 30, 1998

Corresponding Editor: Gary E. Hart

Inheritance of Intersimple Sequence Repeat Markers in Citrus

D. Q. Fang and M. L. Roose

Inheritance of intersimple sequence repeat (ISSR) markers amplified by 14 primers was studied in 87 progeny trees from a cross between two *Citrus* × *Poncirus* hybrids. A total of 223 unambiguous polymorphic fragments were scored. ISSR markers in citrus were generally inherited in a dominant fashion. Of ISSR loci, 22.9% deviated significantly from Mendelian ratios ($P \leq .05$), a proportion similar to that observed for RFLPs and microsatellites in the same cross. At most aberrant loci, the allele from *Poncirus* was overrepresented. The simple inheritance of ISSR markers combined with the easiness of detecting a large number of polymorphic markers gives this system great potential in studies of citrus genetics and breeding.

Studies of inheritance of genetic markers provide important basic information about the genome. Moreover, genetic markers have become major tools in ecology, systematics, evolution, and many aspects of genetics. In citrus, isozymes, restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNA (RAPD) markers have been extensively studied and utilized in genetics and breeding (Cai et al. 1994; Durham et al. 1992; Jarrell et al. 1992; Roose 1988). More recently, microsatellite markers were integrated into the RFLP and isozyme linkage map in citrus (Kijas et al. 1997). These four marker systems are usually inherited in a Mendelian fashion in citrus, with isozymes, RFLPs, and microsatellites often behaving as codominant markers while most RAPDs segregate as dominant markers.

Recently we studied genetic diversity in citrus using a new marker system—intersimple sequence repeat (ISSR) markers (Fang et al. 1997; Fang and Roose 1997). ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence such as $(GA)_8$ anchored at the 3′ or 5′ end by two to four arbitrary, often degenerate, nucleotides (Zietkiewicz et al. 1994). The sequences of repeats and anchor nucleotides are arbitrarily selected. Coupled with the separation of amplification products on a polyacrylamide gel, ISSR amplification can reveal a much larger number of fragments per primer than RAPD. The diversity studies showed that citrus cultivars commonly differed for many ISSR fragments from each primer, suggesting that ISSR markers would be a powerful tool for linkage mapping in citrus. Using ISSR markers we identified mutationally derived citrus cultivars that were difficult to distinguish by any other genetic markers (Fang and Roose 1997). In order to facilitate using ISSR markers in citrus genetics, genomic map construction, and breeding, knowledge of inheritance of this marker system is important. In this article we describe the inheritance of ISSR markers in citrus.

Materials and Methods

The Cross

Sacaton citrumelo [*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.] was hybridized with pollen from Troyer citrange (*C. sinensis* Osbeck × *P. trifoliata*). Zygotic seedlings derived from hybridization were distinguished from apomictic ones using morphology and seven isozyme loci that were heterozygous in Sacaton. Eighty-seven progeny were chosen for the present study. Of them, 60 were used by Jarrell et al. (1992) and Kijas et al. (1997) to construct genetic maps. Cultivars selected to represent grandparental lines were *P. trifoliata* cv. Pomeroy, *C. paradisi* cv. Duncan, and *C. sinensis* cv. Olinda Valencia.

ISSR Analysis

Total DNA extraction, ISSR-PCR amplification, electrophoresis, and silver staining were performed according to Fang et al. (1997). Fourteen ISSR primers that produced clear banding patterns were chosen based on our previous results (Fang and Roose 1997).

Segregation Analysis

Polymorphic fragments were scored as present or absent. Scoring was conducted independently by two researchers. Any disagreements between the two datasets were rechecked and 16 fragments with ambiguous scorings were deleted from the final dataset. Loci were tested for single-locus Mendelian segregation using chi-square goodness-of-fit values with or without sequential Bonferroni correction (Holm 1979). Loci were named after the primer and the fragment size. For example, $(TCC)_5$RY-195 means the 195 bp fragment generated by primer $(TCC)_5$RY.

Results and Discussion

Each of the 14 ISSR primers generated a banding profile composed of many amplification products. About 820 fragments were detected. Of these, 239 fragments were polymorphic and 223 were scored identically by both recorders and analyzed further. The number of unambiguous polymorphic fragments ranged from 7 [primer $(CA)_8$RG] to 27 [primer VHVG$(TG)_7$], with an average of 16 polymorphic fragments

Table 1. Primers used and numbers of loci scored and of loci with skewed segregation

Primer[a]	Number of loci scored	Number of loci with skewed segregation ($P \leq .05$)
$(TCC)_5RY$	16	4
$HVH(TCC)_5$	8	4
$BDB(TCC)_5$	10	5
$VHVG(TG)_7$	27	4
$HVH(TG)_7T$	17	4
$(GA)_8YT$	25	5
$(GA)_8YC$	20	3
$(GA)_8YG$	17	4
$(AG)_8YC$	10	1
$(AG)_8YT$	19	4
$(CA)_8RG$	7	1
$HVH(CA)_7T$	17	5
$DBDA(CA)_7$	20	6
$BDB(CA)_7C$	10	1
Total	223	51

[a] R = purine, Y = pyrimidine, B = non-A, D = non-C, H = non-G, V = non-T.

per primer (Table 1). The ISSR fragments were scored as dominant markers, that is, fragment present in some progeny individuals but absent in others. In this analysis, the scored loci were of three crosstypes: 68 of them were identified as Aa × aa (fragment present in Sacaton but absent in Troyer), 94 were of the type Aa × Aa (fragment present in both Sacaton and Troyer), and the remaining 61 were of type aa × Aa (fragment present in Troyer but not in Sacaton). These numbers indicated that Sacaton and Troyer had approximately equal levels of heterozygosity at these loci. Alleles at the loci of Aa × aa and aa × Aa types were expected to segregate in a 1:1 ratio in the present cross, while those at the loci of Aa × Aa type should segregate in a 3:1 ratio. The chi-square goodness-of-fit test showed that the segregation of 172 loci fit the expected Mendelian ratios.

In order to identify possible redundant markers, we conducted pairwise linkage tests using the computer program JoinMap 2.0 (Stam and Van Ooijen 1995). Sixteen fragments with highly skewed segregation ($P \leq .001$) and a few individuals with marginal scoring were eliminated from this analysis. Out of the 21,321 pairwise linkage tests, 115 pairs of fragments had LOD scores greater than 4.0 and recombination estimates of 0.0. Of these 115 pairs, there were 29 pairs in which all progeny trees had identical phenotypes (fragment present or absent) at both loci. Six pairs were composed of two fragments amplified by a single primer, and the remaining 23 pairs were composed of two fragments generated by two different primers. One explanation for this cosegregating inheritance pattern is simply close linkage between two loci. Pairs of fragments amplified by the same primer [e.g., $(GA)_8YG$-720 and $(GA)_8YG$-795] or by two different primers [e.g., $(GA)_8YT$-1530 and $BDB(CA)_7C$-690] might belong to this type. Another explanation is that two different primers with the same core repeat might bind to sequences at the same location, thus the same or similar size ISSR products were amplified from the same genomic sequence by two primers. Such redundant "loci" would not provide any new information. Twelve fragment pairs detected in the present study were consistent with this model. Fragments $HVH(TCC)_5$-382 and $BDB(TCC)_5$-377 were one possible example. Cloning and sequencing these pairs of fragments would elucidate whether they are two cosegregating or redundant loci.

For the remaining 86 fragment pairs which showed no recombination, progeny phenotypes were not identical within a pair. It seems likely that a few of these fragment pairs are codominant alleles, particularly among the 24 fragment pairs amplified with the same primer. However, our experiment did not allow rigorous tests for codominant inheritance, and we have a low level of confidence that any particular fragment-pair is codominant.

The segregation analysis revealed that 51 loci (22.9%, including 14 Aa × aa type, 21 Aa × Aa, and 16 aa × Aa type) deviated significantly at $P \leq .05$, and 19 of them were highly skewed ($P \leq .005$). Although some of the skewed segregation may be caused by sampling error and conducting a large number of tests, the sequential Bonferroni correction test showed that six and seven loci deviated significantly at the $P \leq .005$ and $P \leq .05$ levels, respectively. This analysis provides good evidence that segregation distortion affects many loci in this cross. Examination of the direction of segregation distortion (i.e., between *Citrus*- and *Poncirus*-derived alleles) showed that 30 distorted loci skewed toward *Poncirus,* 18 toward *Citrus,* and 3 were unknown because both *Poncirus trifoliata* and *C. paradisi* or/and *C. sinensis* had fragments. Among the 19 highly skewed loci, 13 were distorted in favor of the alleles from *Poncirus,* 3 toward the alleles from *Citrus,* and 3 unknown. A direct comparison could be made with the proportion of aberrant loci at RFLPs (9/37, 24.3%) and trinucleotide microsatellites (2/9, 22.2%) in the same cross (Jarrell et al. 1992; Kijas et al. 1997). This indicates that the proportion of skewed loci for the above three marker systems are in the same range, though ISSR markers are generally dominant while the other two are codominant. Of interest, for the skewed ISSR loci, the alleles from *Poncirus* were overrepresented while those from *Citrus* were underrepresented in most cases (30/48, 62.5%). For those highly skewed loci, the proportion in favor of *Poncirus* alleles were even higher (13/16, 81.2%). This is in agreement with the findings of Jarrell et al. (1992) and of Kijas et al. (1997) where the *Citrus* alleles were underrepresented in eight of nine aberrant RFLP loci and the two skewed microsatellites. Apparently the unequal allele segregation toward *Poncirus* appears independent of marker type and of the type of polymorphism being assayed in the cross used in these studies. The distorted ratio may result from linkage to genes subject to directional selection at either gametic or zygotic stages (Gadish and Zamir 1987; Pham and Bougerol 1993). Wide hybrids frequently show distorted segregation (Zamir and Tadmor 1986), but although the two parents of our cross are intergeneric hybrids, the proportion of loci with skewed segregation is about average for interspecific crosses (Jenczewski et al. 1997). The reduced transmission of *Citrus* alleles in crosses involving both *Citrus* and *Poncirus* should be considered when designing breeding crosses aimed at introgressing specific genes from *Poncirus.*

From the Department of Botany and Plant Sciences, University of California, Riverside, CA 92521. We gratefully acknowledge financial support from the California Citrus Research Board and the generous assistance from Dr. C. T. Federici. Address correspondence to M. L. Roose at the address above or e-mail: roose@citrus.ucr.edu.

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Received November 12, 1997
Accepted June 30, 1998

Corresponding Editor: William F. Tracy

Genetic Analysis of Two Terpenoid Variants in Cotton (*Gossypium hirsutum* L.)

R. J. Kohel and A. A. Bell

We conducted genetic analysis of two terpenoid aldehyde (TA) variants transferred to *G. hirsutum* L. A high level of methylation (ME) was transferred from *G. barbadense* L. by direct backcrossing to *G. hirsutum.* Raimondal accumulation (RA), unique to *G. raimondii* Ulbr., was transferred by producing a synthetic amphidiploid and backcrossing to *G. hirsutum.* Progeny tests of the two TA variants were conducted in the field to ensure that the phenotypic expression was repeatable under field conditions and that plants used in the genetic analysis were homozygous. The TA variants were crossed on to TM-1 and the F_1 TA profiles were determined. The F_1 with the ME line had a wild-type (WT) TA profile that indicated that ME was recessively inherited. The F_1 was self-pollinated to produce an F_2, and the F_1 was crossed with the ME line to produce a backcross (BC). Segregation in both populations confirmed that ME was conditioned by a single recessive allele, and we assigned it the symbol *meme.* The F_1 with the RA line expressed raimondal that indicated that RA was dominantly inherited. The F_1 was self-pollinated to produce an F_2, and the F_1 was crossed to TM-1 to produce a BC. Segregation for the RA variant was complex. In the F_2, segregation was 9 raimondal : 7 none, and in the BC segregation was 1 raimondal : 3 none. This segregation conformed to the segregation of alleles at two loci with epistasis in which a variant allele has to be present at both loci before raimondal is produced. We assigned the gene symbols $Ra_1Ra_1Ra_2Ra_2$ to the RA variant. Linkage tests were conducted with virescent-1 (v_1), cup leaf (*cu*), glandless-1 (gl_1), frego bract (*fg*), cluster-1 (cl_1), Red plant (R_1), Okra leaf (L_2^o), Tomentum (T_1), Petal spot (R_2), Yellow pollen (P_1), Yellow petals (Y_1), Brown lint (Lc_1), and Naked seed (N_1). Neither of the TA variants showed any linkage with the markers in our tests.

Gossypium is characterized by the production of lysigenous glands that contain TA and appear as small dark specks in foliar tissue and seeds. The glands are located below the epidermis among the mesophyll cells in cotyledons, leaves, and bracts; and they are scattered throughout the cortex of the stem and upper root bark (Bell 1984). In the seed of cultivated *G. hirsutum,* glands contain gossypol and methylated derivatives, and although glands in all tissues contain some gossypol, those in young leaves contain predominantly hemigossypolone (HGQ) while those in old leaves contain mostly heliocides H1, H2, H3, and H4 derived from HGQ (Bell et al. 1987; Elzen et al. 1985). These same TA predominate in lysigenous glands located in other organs and tissues, such as the bract, capsule, and stem surface.

In surveys of 24 *Gossypium* species (Bell et al. 1975, 1978), four qualitative variations were found in the TA accumulated in glands. Differences in methylation (3-methyl ethers) varied from undetectable levels in *G. hirsutum* to more than 70% of the total TA in *G. barbadense.* Second, all American wild diploid cottons (D genome), except *G. gossypioides* (Ulbr.) Standl., did not accumulate HGQ or heliocides in foliar glands. Third, the American wild cotton *G. raimondii* accumulates mostly raimondal in leaves. Finally, certain species do not accumulate heliocides H2, H3, B2, or B3 in glands because they lack the ability to synthesize myrcene.

We are reporting the genetic analysis of two TA variants transferred to *G. hirsutum.* The variants for high levels of methylation from *G. barbadense* and raimondal accumulation from *G. raimondii* were analyzed for their genetic segregation and linkage relations in *G. hirsutum.* These are the first two TA variants for which the genetic analyses were completed.

Table 1. Segregation and analysis of F_2 and BC families of ME

Family	Segregation (*n*) Mutant	Normal	χ^2	*P*
F_2			(1:3)	
1	24	76	0.05	.90–.80
2	22	63	0.04	.90–.80
3	19	76	1.27	.30–.20
Pooled	65	215	0.47	.50–.30
Heterogeneity (df = 2)			0.89	.70–.50
BC			(1:1)	
1	53	44	0.84	.50–.30
2	47	50	0.09	.80–.70
Pooled	100	94	0.18	.70–.50
Heterogeneity (df = 1)			0.75	.50–.30

Materials and Methods

Enhanced methylation was transferred from *G. barbadense* by direct backcrossing to *G. hirsutum,* and the raimondal trait was transferred from *G. raimondii* by crossing *G. arboreum* L. × *G. raimondii,* chromosome doubling to produce a synthetic amphidiploid, and backcrossing to *G. hirsutum* (conducted by A. A. Bell). In each case, the desired trait was identified in the segregating progeny and repeatedly backcrossed four times to Tamcot CAMD-E as female parent before the genetic analyses were begun. The transference process provided preliminary information as to the recessive versus dominance of the trait. Populations were not designed to accurately determine genetic control; however, preliminary observations were reported (Bell et al. 1987, 1994) which suggested recessive inheritance of ME and dominant inheritance of RA.

The approximate ratio of hemigossypolone methyl ether (MHGQ) to HGQ to measure methylation and the presence or absence of raimondal were both monitored with TLC fingerprint methods (Bell and Stipanovic 1977). In both analyses, the first fully expanded leaf below the terminal was analyzed from individual plants prior to full bloom (Bell and Stipanovic 1976).

We began the genetic analyses with the lines of *G. hirsutum* that expressed the variant TA transferred from *G. barbadense,* ME, and *G. raimondii,* RA. These lines were grown in 20 plant progeny rows in the cot-

Table 2. Segregation and analysis of F_2 and BC families of RA

Family	Segregation (*n*) Mutant	Normal	χ^2	*P*
F_2			(9:7)	
1	48	44	0.62	.50–.30
2	58	39	0.50	.50–.30
3	51	45	0.39	.70–.50
Pooled	157	128	0.16	.70–.50
Heterogeneity (df = 2)			1.35	.70–.50
BC			(1:3)	
1	23	73	0.06	.90–.80
2	28	77	0.16	.70–.50
3	14	67	2.57	.20–.10
Pooled	65	175	0.56	.50–.30
Heterogeneity (df = 2)			2.23	.50–.30

ton genetics field nursery and evaluated for their TA profiles. Individual plants were self-pollinated, and the progeny testing and evaluation process was repeated for 2 more years to ensure repeatability of phenotypic expression under field conditions and to ensure that the plants used for genetic analysis were homozygous. All subsequent analyses were conducted in the field nursery.

The genetic standard for *G. hirsutum*, TM-1, was used as the normal tester for the inheritance studies (Kohel et al. 1970). It is an inbred line maintained by individual plant pedigree, 42 generations, and it was included in the above evaluation process to verify that it expressed the WT *G. hirsutum* TA profile. For the analysis of genetic linkage relations, the TA variants were crossed with T582, multiple recessive marker tester line, and T586, multiple dominant marker line. T582 includes the recessive marker loci virescent-1 (v_1), L.G. XVII on chromosome 20; cup leaf (*cu*), location unknown; glandless-1 (gl_1), location unknown; frego bract (*fg*), L.G. VI on chromosome 3; and cluster-1 (cl_1), L.G. III on chromosome 16. T586 includes the dominant marker loci Red plant (R_1), L.G. III on chromosome 16; Okra leaf (L_2°), L.G. II on chromosome 15; Tomentum (T_1), L.G. IV on chromosome 6; Petal spot (R_2), L.G. I on chromosome 7; Yellow pollen (P_1), L.G. XI on chromosome 5; Yellow petals (Y_1), L.G. XII on an unknown A chromosome; Brown lint (Lc_1), L.G. I on chromosome 7; and Naked seed (N_1), L.G. V on chromosome 12 (Endrizzi et al. 1984).

Results and Conclusions

The TA variants were crossed with TM-1 and the F_1 TA profiles were determined. The F_1 with the ME line had a WT TA profile that indicated that ME was recessive. Therefore, the F_1 was self-pollinated and crossed to produce an F_2 and a BC to the ME line, respectively. The F_1 with the RA line expressed raimondal which indicated that RA was dominant. In this case, the F_1 was self-pollinated to produce an F_2 and crossed to TM-1 to produce the BC.

In the F_2 with TM-1 the ME variant segregated 24 plants with high methylation (>70%) and 76 plants with none. This segregation was not significantly different from that expected for a single recessive allele ($\chi^2_{(1:3)} = 0.05$). The BC to ME segregated 53 plants with high methylation to 44 plants with none ($\chi^2_{(1:1)} = 0.84$), which confirmed that ME was conditioned by a single recessive allele. Segregation of ME in the linkage populations, F_2 and BC, is reported with the TM-1 populations, and the pooled F_2 data ($\chi^2_{(1:3)} = 0.47$) and pooled BC data ($\chi^2_{(1:1)} = 0.18$) confirm the inheritance data with no significant heterogeneity (Table 1). We assigned the gene symbol *meme* to the ME variant.

Segregation for the RA variant was complex. In the F_2, 48 plants produced raimondal and 44 plants produced none ($\chi^2_{(9:7)} = 0.62$), and in the BC to TM-1 23 plants produced raimondal and 73 plants produced none ($\chi^2_{(1:3)} = 0.06$). This segregation conformed to the segregation of alleles at two loci with epistasis. The production of raimondal is dominant to WT, but a variant allele has to be present at both loci before raimondal is produced. Segregation of RA in the linkage populations, F_2 and BC, are reported with the TM-1 populations, and the pooled F_2 data ($\chi^2_{(9:7)} = 0.16$) and pooled BC data ($\chi^2_{(1:3)} = 0.56$) confirm the inheritance data with no significant heterogeneity (Table 2). We assigned the gene symbols $Ra_1Ra_1Ra_2Ra_2$ to the RA variant.

Linkage analysis of ME was limited to the F_2 with T582 because both lines contained recessive alleles for testing. Tests with T586 included both F_2 and BC progeny to the ME line. Linkage analysis of RA included F_2 and BC progeny with both testers. In the case of the recessive tester, T582 was the BC parent, and with the dominant tester, TM-1 was the BC parent. Since RA was transferred from a D genome species, we expected linkages only with markers of the D genome, chromosomes 14–26. Neither of the TA variants showed any linkage with the markers in our tests (Table 3). Tests with individual linkage group lines and aneuploid stocks will be pursued to determine chromosome location and/or linkage relations. These TA variants provide two additional mutants to the genetic collection. However, they will have limited use as genetic markers because of difficulty of identification without the resources of a chemical laboratory.

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Table 3. Linkage tests of ME and RA in F_2 and BC with T582 and T586

Marker locus	ME F_2 RC% ± SE[a]	ME F_2 χ^2	ME F_2 *P*	ME BC RC% ± SE[a]	ME BC χ^2	ME BC *P*	RA F_2 RC% ± SE[a]	RA F_2 χ^2	RA F_2 *P*	RA BC RC% ± SE[a]	RA BC χ^2	RA BC *P*
v	65.00 ± 4.26	1.60	.30–.20				69.00 ± 12.11	1.56	.30–.20	52.24 ± 6.10	0.004	.95–.90
cu	42.50 ± 5.63	0.85	.50–.30				63.70 ± 10.60	1.08	.30–.20	40.30 ± 5.99	0.40	.70–.50
gl_1	75.00 ± 3.52	4.35	.05–.02				54.50 ± 8.48	0.13	.80–.70	52.24 ± 6.10	0.04	.80–.70
fg	60.00 ± 4.58	0.34	.70–.50				70.00 ± 12.47	1.41	.30–.20	41.79 ± 6.03	1.12	.30–.20
cl_2	56.00 ± 4.83	0.52	.50–.30				44.00 ± 8.82	0.28	.70–.50	46.27 ± 6.09	0.84	.50–.30
R_1	51.00 ± 5.55	0.03	.90–.80	46.39 ± 5.06	0.51	.50–.30	35.50 ± 10.58	1.12	.30–.20	53.26 ± 5.20	1.17	.30–.20
L_1^0	42.50 ± 5.01	1.26	.30–.20	54.64 ± 5.05	0.84	.50–.30	56.00 ± 7.04	0.43	.70–.50	56.52 ± 5.17	1.45	.30–.20
T_1	54.00 ± 5.72	0.22	.70–.50	41.24 ± 5.00	2.98	.10–.05	51.00 ± 7.70	0.03	.90–.80	52.17 ± 5.21	0.52	.50–.30
R_2	49.00 ± 5.42	0.01	.95–.90	48.45 ± 5.07	0.09	.80–.70	51.00 ± 7.70	0.03	.90–.80	42.39 ± 5.15	1.75	.20–.10
P_1	38.50 ± 4.78	2.89	.10–.50	46.39 ± 5.06	0.51	.50–.30				53.26 ± 5.20	1.17	.30–.20
Y_1	59.00 ± 6.01	1.79	.20–.10	53.61 ± 5.06	0.51	.50–.30	48.00 ± 8.14	0.02	.95–.90	46.74 ± 5.20	0.01	.95–.90
Lc_1	45.50 ± 5.21	0.29	.70–.50	52.55 ± 5.07	0.26	.70–.50	69.50 ± 5.59	4.31	.05–.02	58.70 ± 5.13	4.70	.05–.02
N_1	52.50 ± 6.64	0.11	.80–.70	49.48 ± 5.08	0.01	.95–.90	67.00 ± 5.80	2.76	.01–.05	46.64 ± 5.20	0.36	.70–.50

[a] Estimates of RC% ± SE from Immer (1930).

Research Service, Southern Plains Area, Southern Crops Research Laboratory, Crop Germplasm Research Unit and Cotton Pathology Research Unit, College Station, Texas. This work was done in cooperation with the Texas Agricultural Experiment Station, College Station, Texas. Address correspondence and reprint requests to Dr. R. J. Kohel, USDA-ARS, Crop Germplasm Research Unit, 2765 F&B Road, College Station, TX 77845.

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Received September 5, 1997
Accepted December 13, 1997

Corresponding Editor: Prem P. Jauhar

The Mitochondrial Genome of Allotetraploid Cotton (*Gossypium* L.)

R. L. Small and J. F. Wendel

Knowledge of patterns of organellar inheritance are crucial for analyses of organellar genetics and population or phylogenetic analyses based on organellar genes. While it has previously been shown that chloroplast DNA is maternally inherited in cotton (*Gossypium* L.), no studies have assessed the mitochondrial genome complement of the allotetraploid species of cotton or addressed inheritance of mitochondrial DNA. In this article we report Southern hybridization analyses that indicate that the AD-genome allotetraploid species have an A-genome-like mitochondrial genome and provide evidence that mitochondrial genomes are also maternally inherited in cotton.

Organellar inheritance in plants is of interest from both a theoretical and an applied standpoint. While often generalized as primarily uniparental and maternal (in angiosperms), inheritance of both plastids and mitochondria varies among different plants from strictly uniparental maternal or paternal, to primarily uniparental with trace biparental, to biparental (Mogensen 1996; Reboud and Zeyl 1994). In addition, the same pattern of organellar inheritance may have a different mechanistic basis in different taxa (Birky 1995; Mogensen 1996). Thus it is important to determine empirically the mode of organellar transmission when relevant.

The cotton genus, *Gossypium* L., contains both diploid and tetraploid species. The five tetraploids are a monophyletic assemblage derived from a single polyploidization event that occurred about 1–2 million years ago (Wendel 1989). It has previously been shown that chloroplast DNA is maternally and uniparentally inherited in *Gossypium,* and furthermore that the AD-genome allotetraploid cottons have a chloroplast like that of the African-Asian, A-genome diploid cottons (Galau and Wilkins 1989; Wendel 1989). From this it has been inferred that the A-genome diploid must have served as the seed parent in the initial hybridization event that led to the evolution of the allopolyploids. In this article we use Southern hybridization analyses to show that (1) the allotetraploid species of *Gossypium* also contain an A-genome-derived mitochondrial genome; and (2) like plastomes, mitochondrial genomes are uniparentally and maternally inherited in the crosses we examined.

Table 1. Plant materials surveyed

Species	Genome	Accession(s)
G. herbaceum	A_1	A_1-73
G. arboreum	A_2	A_2-47
G. thurberi	D_1	17
G. davidsonii	$D_{3\text{-}D}$	32
G. raimondii	D_5	D_5-2
G. hirsutum	AD_1	Race Palmeri
		TX303
		TX379
G. barbadense	AD_2	K101
		Tanguis
G. tomentosum	AD_3	WT936
G. mustelinum	AD_4	WM400
G. darwinii	AD_5	PW45

Voucher specimens are deposited at the Ada Hayden Herbarium, Iowa State University (ISC).

Materials and Methods

Plant Materials

Total DNA was isolated from the *Gossypium* accessions shown in Table 1 according to the method of Paterson et al. (1993). These accessions include representatives of both parental diploid lineages (A-genome: *G. arboreum* L. and *G. herbaceum* L.; D-genome: *G. raimondii* L.), as well as accessions of the allotetraploids (AD-genome: *G. hirsutum* L., *G. barbadense* L., *G. tomentosum* Nuttall ex Seemann, *G. mustelinum* Miers ex Watt, and *G. darwinii* Watt). In addition, two synthetic allotetraploids [$2(A_2D_1)$ and $2(A_2D_3)$; A_2 being the maternal parent in both cases] and their diploid parents (A_2: *G. arboreum*; D_1: *G. thurberi* Todaro; D_3: *G. davidsonii* Kellogg) were also surveyed.

Restriction Digestion and Membrane Preparation

Three separate sets of Southern hybridization experiments were performed using two different mitochondrial probes (see below). First, membranes containing DNAs of *G. raimondii, G. hirsutum,* and *G. herbaceum* digested with six different restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I, and *Xho*I), were prepared to identify whether the natural allotetraploid (*G. hirsutum*) RFLP pattern was A- or D-genome-like. Second, membranes containing *Eco*RI-digested DNAs of two synthetic allotetraploids and their diploid parents were prepared to identify whether mitochondrial inheritance is uniparental or biparental. Finally, membranes containing DNAs of all five tetraploid *Gossypium* species digested with 12 different restriction

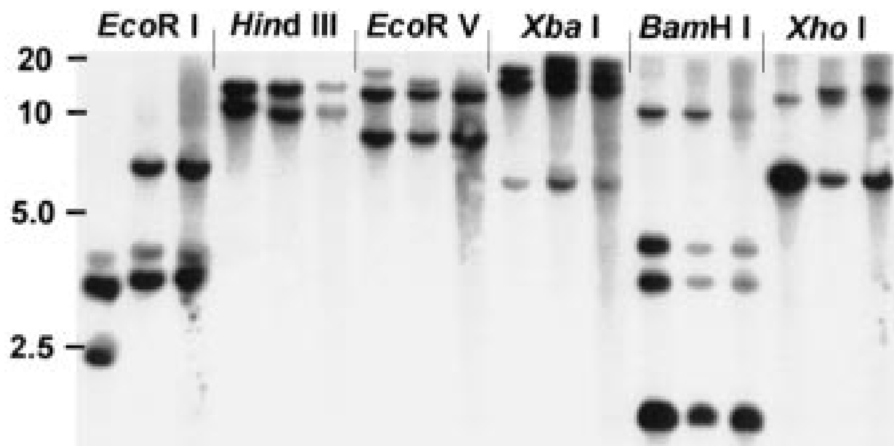

Figure 1. Autoradiograph of Southern hybridization of an *atpA* mitochondrial probe to restriction digested genomic DNAs of *Gossypium* species. Three samples are shown for each enzyme (indicated above the figure): *G. raimondii* (D-genome), *G. hirsutum* (AD-genome), and *G. herbaceum* (A-genome) from left to right, respectively. Note polymorphism exhibited with *Eco*RI, *Eco*RV, *Xba*I, and *Xho*I; in each case the AD-genome allotetraploid, *G. hirsutum,* exhibits the A-genome, *G. herbaceum,* RFLP pattern. Size markers (in kb) are shown to the left.

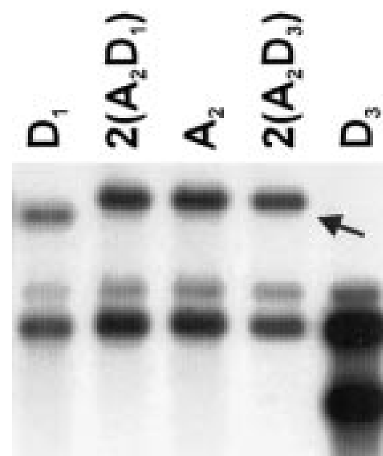

Figure 2. Autoradiograph of Southern hybridization of an *atpA* mitochondrial probe to *Eco*RI-digested genomic DNAs of synthetic allotetraploids of *Gossypium* and their diploid parental species. A_2 = *G. arboreum,* D_1 = *G. thurberi,* D_3 = *G. davidsonii.* In both synthetic tetraploids *G. arboreum* was the seed parent. The arrow indicates the diagnostic restriction fragment which confirms that mitochondrial inheritance is maternal.

enzymes (*Ban*I, *Ban*II, *Bcl*I, *Bst*BI, *Bst*NI, *Cfo*I, *Eco*NI, *Hin*fI, *Nci*I, *Nsi*I, *Rsa*I, and *Taq*I) were probed to determine whether RFLP variation exists among tetraploid *Gossypium* species.

Probe Preparation and Southern Hybridization

Mitochondrial gene probes were prepared by PCR amplification. PCR primers for two mitochondrial genes, *atpA* and *matR,* were generously provided by Y. Qui and J. Palmer (Indiana University). Using *G. hirsutum* DNA as a template, the *matR* primers IP29 (TCCAGACACATCGCAC) and IP30 (TGGTAAAGGTTGTCGC) amplified an approximately 1.7 kb PCR product; the *atpA* primers IP91 (TCAGTTGGAGATGGG) and IP94 (AGAAGCCGTTGACAGC) amplified an approximately 1.3 kb PCR product. These PCR products were cleaned and concentrated with GeneClean II (Bio101). ^{32}P radioactively labeled probes were prepared using a random primer labeling system (Gibco-BRL). Hybridization, washing, and autoradiography conditions were as described (Wendel and Albert 1992).

Results

Mitochondrial Genome of the Natural Allotetraploids

Hybridization of the *atpA* and *matR* probes to membranes containing genomic DNAs digested with six different enzymes revealed different RFLP patterns for *G. raimondii, G. hirsutum,* and *G. herbaceum.* The *matR* probe hybridized either to one or two bands per enzyme and no variation was detected among species. The *atpA* probe, on the other hand, revealed from two to four bands per digestion and gave polymorphic patterns for four of the six enzymes (Figure 1). In all cases where polymorphism was detected, the allotetraploid (*G. hirsutum*) RFLP pattern was identical to that of the A genome (*G. herbaceum*) and different from that of the D genome (*G. raimondii*). These data document the existence of an A-genome-like mitochondrial genome in the natural allotetraploid.

Inheritance of the Mitochondrial Genome

To identify the pattern of mitochondrial inheritance we analyzed the RFLP pattern of two synthetic allotetraploids and their diploid parents. Membrane-bound *Eco*RI-digested DNAs of the synthetic tetraploids and their parents were probed with *atpA,* which showed genome-specific RFLP patterns as described above. This Southern hybridization experiment showed that, in both cases, the synthetic allotetraploid has a mitochondrial genome which is like the maternal A-genome parent and different from the D-genome parent (Figure 2). Given that A_2 (*G. arboreum*) was the maternal parent in both of these crosses, uniparental, maternal inheritance is confirmed for each of these synthetic allotetraploids. To assess the possibility of trace paternal transmission the autoradiograph shown in Figure 2 was overexposed for 10 days, yet no D-genome-specific bands were detected in either allotetraploid lane.

Mitochondrial DNA Diversity in Tetraploid *Gossypium*

Finally, to qualitatively assess interspecific RFLP divergence among the five allotetraploid species we probed membrane-bound digested DNAs of accessions of all five tetraploids using a total of 24 enzyme-probe combinations (2 probes, 12 enzymes). Among the tetraploid species only one enzyme-probe combination yielded a polymorphism.

Discussion

Compared to the widely studied and utilized chloroplast genome, mitochondrial genomes have been relatively ignored in plant genetics, although recent studies are beginning to correct this inequity (reviewed in Levings and Vasil 1995; Palmer 1997). Plant mitochondrial genomes are implicated in agronomically important genetic phenomena, including cytoplasmic male sterility (Levings and Vasil 1995), in many different plants. In addition, the unique (relative to other eukaryotes), variable, and often large size and multipartite structure of plant mitochondrial genomes make them interesting genetic systems in their own right (Andre et al. 1992; Fauron et al. 1995). This may be especially true in *Gossypium,* which has one of the largest plant mitochondrial genomes yet described (Hsu and Mullin 1989). Finally, recent interest has developed in using plant mtDNA for population level and phylogenetic analyses (Hiesel et al. 1994; Luo and Boutry 1995; Paige et al. 1991; Palmer 1992, 1997). Regardless of the application, knowledge of inheritance patterns are essential for studies of organellar genomes.

The data presented here permit conclu-

sions to be drawn regarding the mitochondrial genome complement of the allotetraploid species of *Gossypium* and the pattern of inheritance of mitochondrial DNA. The data demonstrate that the naturally occurring allotetraploid species contain a mitochondrial genome that is derived from the A-genome diploid parent. In addition, available evidence suggests that the primary mode of inheritance of mitochondrial DNA in *Gossypium* is uniparental and maternal. While we are unable to rule out trace biparental inheritance or occasional paternal inheritance in the genus *Gossypium* as a whole, in the crosses we examined inheritance was both uniparental and maternal. This conclusion is supported by two separate lines of evidence. First, in the two synthetic allotetraploids studied, exclusively maternal RFLP patterns were displayed, despite overexposure of the blot in an attempt to discern a possible minority paternal contribution. Second, the naturally occurring allotetraploids, which originated from a single hybridization event with an A-genome diploid maternal parent (Wendel 1989), have RFLP patterns that are A-genome-like and distinct from those of the D-genome diploids. Finally, the fact that only a single polymorphism was detected among the five naturally occurring allotetraploid species out of 24 enzyme-probe combinations qualitatively reinforce the recency of the allotetraploid radiation.

While we have not performed detailed analyses of the *Gossypium* mitochondrial genome, one observation suggests that, like other plants studied thus far, the *Gossypium* mitochondrial genome exists in a multipartite condition. Specifically, Southern hybridization of the *atpA* probe (approximately 1.2 kb) to restriction-digested DNAs revealed multiple bands (3–4), each having a size between 10 and 20 kb (Figure 1, lanes 7–12). If the *atpA* gene exists in a single copy per mitochondrial genome master circle, the most plausible explanation for these high molecular weight bands is that the *atpA* gene exists on multiple mitochondrial subchromosomal configurations, which differ with respect to their spacing of flanking restriction sites. Similar observations have been made regarding the region surrounding *atpA* in maize mitochondria (Small et al. 1987; Wang and Gengenbach 1989).

Further analyses will be necessary to describe the *Gossypium* mitochondrial genome and its patterns of evolution. The data presented here, however, document the presence of A-genome-derived mitochondrial DNA in the allotetraploid species of *Gossypium* and suggest that the mode of mitochondrial inheritance in *Gossypium* is primarily maternal. These data lay the groundwork for additional studies.

From the Botany Department, 353 Bessey Hall, Iowa State University, Ames, IA 50011. The authors thank Y. Qui and J. Palmer for providing PCR primers. This research was supported by a grant from the National Science Foundation. Address correspondence to J. F. Wendel at the address above or e-mail: jfw@iastate.edu.

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Received October 30, 1997
Accepted May 30, 1998

Corresponding Editor: Susan Gabay-Laughnan