Brief Communications

Characterization of DNA Microsatellite Loci From a Threatened Snake: The Eastern Massasauga Rattlesnake (*Sistrurus c. catenatus*) and Their Use in Population Studies

H. L. Gibbs, K. Prior, and C. Parent

We describe the isolation and genetic characterization of six microsatellite DNA loci from a threatened snake species, the eastern massasauga rattlesnake (*Sistrurus c. catenatus*) and document the use of these loci for a variety of population-level analyses. Based on data from more than 70 adults from a single population in Killbear Provincial Park, Ontario, these loci: (1) are highly variable, with numbers of detected alleles per locus ranging between 4 and 12 and expected heterozygosities between 0.15 and 0.85, although observed heterozygosities are lower; (2) generally segregate in a Mendelian fashion based on comparisons between genotypes of mothers and their offspring in two litters; (3) may not evolve via a stepwise mutation model as based on frequency distributions of allele sizes; (4) are sufficiently variable for accurate determination of paternity in this species with a significant probability (P = .979) of detecting multiple paternity in single broods; and (5) amplify similar size fragments in other species of snakes. Further analyses demonstrate a significant heterozygote deficiency at all loci in this population which, for at least three of the six loci, is not likely due to the presence of null alleles. This pattern suggests that this population has experienced local inbreeding and/or is subdivided on a microgeographic scale. These markers will be ideal for further analyses of population variation and differentiation in this species, which will provide useful information for conservation and evolutionary studies of these snakes.

DNA-based genetic markers that exhibit high levels of intraspecific variation are valuable tools for examining a variety of questions at the population level including those related to parentage, estimation of various population parameters, levels of differentiation, and phylogenetic relationships between populations (Avise 1994). Recently developed microsatellite DNA markers (Tautz 1989; Weber and May 1989) seem to be ideal for these types of population studies because they represent loci that are easily distinguishable, have codominant alleles, and the genotypic information is logistically easy to obtain relative to certain other DNA-based markers (Quellar et al. 1993). Allelic variation in microsatellites is due to variation in the number of small (1–6 bp) tandem simple sequence repeats present in different alleles. Microsatellite loci have been isolated from an increasing number of wild animals, including mammals (Amos et al. 1992; Paetkau and Strobeck 1994; Taylor et al. 1994) and birds (Gibbs et al. 1996; Hanotte et al. 1994; McDonald and Potts 1994; Primmer et al. 1995), and used for studies of the ecology, behavior, evolutionary biology, and conservation of such species.

Reports of the isolation and use of microsatellites from another major vertebrate group, the reptiles, are much less common. For snakes, the only study published to date reported the isolation of six loci from a timber rattlesnake (*Crotalus horridus*) genomic library (Villarreal et al. 1996). That study confirmed that snake microsatellites had genetic characteristics similar to those identified in other vertebrates and showed the potential for using such loci for population studies of this species. Here we report the characterization of microsatellites from another snake, the eastern massasauga rattlesnake (*Sistrurus c. catenatus*), and describe their genetic characteristics. In addition we also address topics not dealt with by Villarreal et al. (1996) including the statistical power of these loci for parentage analyses, expected versus observed levels of population heterozygosity, possible existence of null alleles, and the potential for cross-species amplification of these loci. This work is part of a long-term study of the conservation and evolutionary genetics of disjunct populations of this subspecies in eastern North America (cf. Gibbs et al. 1994).

Materials and Methods

Population Samples

In 1994 and 1995 we captured massasaugas opportunistically encountered throughout a 9 km^2 study area within Killbear Provincial Park on Georgian Bay, Ontario (80°12′E, 45°21′N). Massasaugas have been present in this area for no more than 10,000 years, following the last glacial retreat (Johnson and Menzies 1993). Overall we captured 74 adults and 26 neonates (representing the litters of two sampled females). From each snake we obtained 25 to 100 μl of blood drawn from the caudal vein using a 0.5 ml syringe fitted with a 28 gauge needle. Blood samples were then mixed with 800 μl of lysis buffer (Seutin et al. 1991) and stored at 4°C until DNA extraction. Upon capture, all massasaugas were permanently marked with either ventral scale branding or PIT-tags to eliminate the possibility of resampling the same individual. DNA was then extracted from these samples using either standard phenol-chloroform extractions as described in Gibbs et al. (1994), or by using DNA ZOL (GIBCO BRL) and following the manufacturer's protocol. Samples were then resuspended in 1× TE (pH 7.5) and concentrations determined using a fluorometer.

Isolation of Microsatellite DNA Loci

To isolate nuclear microsatellite DNA markers for massasaugas, we followed the

Table 1. Genetic characteristics of six eastern massasauga rattlesnake microsatellite loci

Locus	Repeat motif	Primer sequences (5′–3′)	T_m	Size	Number of alleles (number of individuals)
Scu01	$(AG)_{24}$	F-GTCAACACTTGTGTTCTGC R-CTGTATTAAAGTTGTTTTGTTCA	58	149	12 (73)
Scu05	$(TC)_{15}(AC)_9$	F-GACATTGCTGAACAGACTAT R-TTGTGTAGCATAGTGAAACA	55	194	11 (72)
Scu07	$(TC)_{16}(TG)_{14}TT(TG)_5$	F-CTTTGTGCTATTTTTCCACC R-GCCAAAAAAGTAAAATATGAGC	60	176	8 (72)
Scu11	$(TC)_{12}G(TC)_{14}$	F-AATCAGCATGTGGCTTAAATC R-GCTGCTTGGCTACATATGC	62	154	7 (72)
Scu16	$(AC)_{17}$	F-TATGGGAATCTGGCTTTCTC R-AACTGATTCATATCTGCACTGC	58	167	4 (74)
Scu26	$(AC)_{24}$	F-GAAATTGGTGGAAGAGACCTG R-GTCCAGGATATGAGGGATCTG	62	173	5 (74)

Product sizes (in bp) are based on the size of the clone sequenced for each locus. F is the forward primer sequence and R the reverse primer sequence for each locus. T_m is the annealing temperature (in °C) used to assay variation in the locus.

procedures outlined by Dawson et al. (1997). Briefly a size-selected (350–400 bp) enriched snake genomic DNA library in pUC18 was screened using three radiolabeled dinucleotide polymers (CA, GC, and CT; Pharmacia). Genomic DNA used to make the library came from a pooled sample made up of equal amounts of DNA from individuals from populations in Pennsylvania, New York, Ohio, and Ontario. Primary and secondary screening were used to identify positive clones which possibly contained a microsatellite sequence. These clones were then sequenced using T7 polymerase (Sequenase; USB) and cycle sequencing (Perkin Elmer) kits and this information was used to design primers (using Primer version 0.5) to loci identified in these clones.

Detection of Microsatellite Variation

To assay variation in these loci, PCR amplifications were performed in 10 μl reaction volumes using 50 ng genomic DNA, 0.3 pmol of the forward primer end-labeled with ^{33}P-ATP (Dupont), 0.4 pmol of unlabeled forward primer, 0.8 pmol of unlabeled reverse primer, 200 μM dNTPs, 0.5 U AmpliTaq, 0.1 M Tris-HCl pH 8.3, 0.5 M KCl, and 1.5–2.0 mM $MgCl_2$. Amplifications were run in a Perkin Elmer 480 Thermocycler for 30 cycles, with each cycle consisting of 30 s at 94°C, 30 s at the appropriate annealing temperature, and 30 s at 72°C. Amplification products (3 μl) were then run on 6% denaturing polyacrylamide gels at 55 W for 2.5 h. Gels were then dried and exposed to Biomax (Kodak) X-ray film overnight. Products were sized by reference to a known sequencing reaction of a control template and "hot" amplifications of the known-size clones for each locus, both of which were run on the gel at the same time.

Results

Identification of Microsatellite Loci

We screened approximately 6400 colonies using the procedure outlined above and obtained 46 single-insert positive clones. We sequenced all 46 clones, identified repeat sequences in 30 of these clones, and then designed and synthesized primer sequences which flanked the repeat sequence in 11 of these clones. Only six of these primer pairs successfully amplified polymorphic products in a panel of five snake genomic DNA samples. We then optimized the annealing temperature for these six primer pairs and used these loci for surveys of population variation (see below). The optimal annealing temperature, repeat motif, and primer sequences for each locus are given in Table 1. The sequences of primer pairs which did not amplify polymorphic products are available from the authors. As expected from the probe sequences used to screen the library, all of the detected microsatellites were based on dinucleotide repeat variation. However, some loci were based on uninterrupted strings of a single type of dinucleotide repeat (e.g., *Scu01, 16,* and *26*), whereas the other three loci consisted of mixtures of different two-base repeats combined with single base-pair insertions.

Levels of Variation

Surveys of 72 to 74 adult massasaugas from the Killbear population showed that these loci detected 4 to 12 alleles per locus (Table 1). An example of the variation detected at the locus *Scu07* is shown in Figure 1. Mean allele frequency varied from between 0.083 (*Scu01*) and 0.20 (*Scu16*).

One unexpected result is that allele sizes for three of the loci show strongly disjunct distributions (Figure 2). In particular, *Scu01, 05,* and especially *11* showed gaps between allele sizes ranging in size from 20 to more than 120 bp. The significance of this observation is that the most commonly used stepwise models of mutation in microsatellites (e.g., Valdes et al. 1993) assume that changes occur through either increases or decreases of a single repeat unit. These gaps suggest that mutational events in these loci may involve changes of many more than single repeat units.

Maternal segregation analyses of two litters of massasaugas consisting of 12 and 13 young respectively suggest that the alleles at each locus segregate in a Mendelian manner (data not shown). All offspring share one allele with their mother. Where the female is heterozygous, the data are generally consistent with an

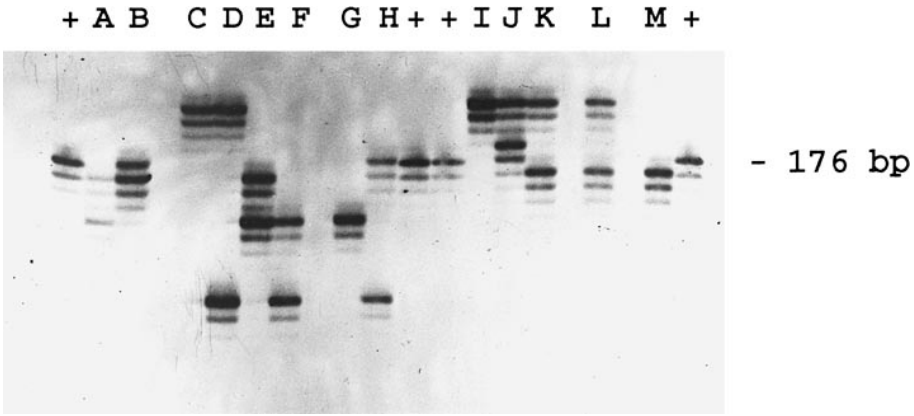

Figure 1. Allelic variation at microsatellite locus *Scu07* among 13 individuals (A–M) from Killbear Provincial Park. Lanes marked with + are the products of amplifications from a plasmid clone containing a known-size (176 bp) allele.

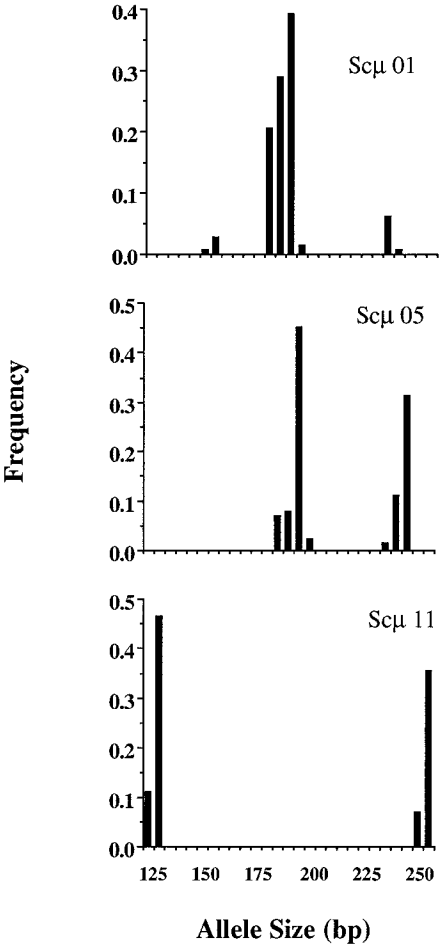

Figure 2. Size distributions for alleles at three microsatellite loci (*Scu01, Scu05,* and *Scu11*) for the Killbear population.

Table 2. Expected and observed heterozygosity and F_{is} values for six microsatellite loci in the Killbear population

Locus	H_{exp}	H_{obs}	Expected number heterozygotes	Observed number heterozygotes (*P*)	F_{is} (*P*)
Scu01	0.867	0.671	63.29	49 (<.0001)	0.227 (<.0001)
Scu05	0.732	0.556	52.04	40 (<.0001)	0.233 (<.001)
Scu07	0.813	0.681	58.52	49 (.0039)	0.164 (<.005)
Scu11	0.664	0.653	47.84	47 (.0224)	0.0177 (>.05)
Scu16	0.456	0.419	40.26	43 (.005)	0.0818 (>.05)
Scu26	0.256	0.176	18.90	13 (.012)	0.314 (<.001)
Overall	0.610	0.437	—	—	0.166 (<.001)

H_{exp} is calculated as described by Nei and Roychoudbury (1974); H_{obs} is the proportion of the individuals in the sample that are heterozygotes. Overall values are means of values for reach locus. F_{is} is calculated as described by Weir and Cockerham (1984). *P* values are for significance of difference between observed and expected number of heterozygotes (*P*) as tested using the exact test described in GENEPOP (Raymond and Rousett 1995), whereas the probability that the F_{is} values were different from zero was tested using the permutation procedure in FSTAT (Goudet 1995).

equal probability of segregation for each maternal allele, since contingency table analyses failed to reject the null hypothesis of equal probability of segregation for seven of eight brood-locus combinations tested.

Parentage Analyses

The usefulness of these loci for parentage analyses was determined by calculating the probability of detecting more than a single father's genotype in offspring [*P*(*E*)] using the formula in Chakravarti and Li (1983). This analysis assumes that the maternity of young is known, which is true for this species given that litters are live born, and random mating within the population, and then uses the population allele frequencies to estimate *P*(*E*) values. The overall *P*(*E*) value is .979, indicating that when the six loci are used for parentage analyses we have a 98% chance of detecting multiple paternity within a litter of this species. The accuracy of these *P*(*E*) values is affected to an unknown extent by the nonrandom associations of alleles documented below. For the two litters used in the segregation analyses (see above), a maximum of two additional alleles other than those observed in the mother were present in the offspring at each locus (data not shown). Given the power of our loci to detect multiple paternity, this suggests that these litters had a single father.

Expected and Observed Levels of Heterozygosity

An initial survey of the levels of heterozygosity in the Killbear population suggested a strong heterozygote deficiency at a number of loci. Results in Table 2 confirm this impression: the mean observed level of heterozygosity across loci (0.437) was more than 28% lower than the mean expected value (0.610), and for all loci there were significantly ($P \leq .012$) fewer of the heterozygotes predicted under Hardy–Weinberg expectations present using the exact test in the program GENEPOP (Raymond and Rousset 1995). F_{is} values, which estimate the extent of nonrandom associations among alleles in a population, are high and significantly different from zero for four of six loci, with a mean value of 0.166, which is also significant (Table 2). Thus these results argue that levels of homozygosity for the microsatellites described here are high in this massasauga population.

There are a number of possible biological explanations for the observed heterozygote deficiency, which we discuss below. However, a recently suggested methodical explanation (e.g., Allen et al. 1995; Paetkau and Strobeck 1994) is that heterozygote deficiencies occur due to the presence of null or nonamplifying alleles which are a result of mutations in the primer sites in different individuals (cf. Pemberton et al. 1995). Thus an individual who is a heterozygote but has a null allele will be scored as a homozygote because only one of the two alleles present will be amplified. We tested this possibility for the three loci (*Scu01, 07,* and *26*) which showed the highest levels of heterozygote deficiency by using the original sequence for each of these loci to redesign sets of new primers that were located 0 to 10 bp upstream or downstream of the primer sequences shown in Table 1. Hence the sets of new primers did not overlap with the locations of the original primer sets (sequences not shown). We then reamplified all individuals that had originally been classified as homozygotes using the new primers for these loci. We predicted that if null alleles were present then at least some of these individuals should be reclassified as heterozygotes. In no case did this occur: all individuals originally scored as homozygotes at a particular locus remained classified as such using the new primers. Thus the presence of null alleles cannot explain the heterozygote deficiency in our population, suggesting that a biological explanation is more plausible (see below).

Cross-Species Amplifications

To test the effectiveness of our primer sets for amplifying homologous loci in other snake species we conducted low stringency amplifications using DNA from a taxonomically diverse set of species including another rattlesnake (*C. viridus*) and a variety of Colubrid species (Table 3). All amplifications were done with a low annealing temperature of 50°C and success of an amplification was scored based on the presence or absence of a fragment of approximately the expected size when

Table 3. Results of cross-species amplification experiments using the rattlesnake microsatellite loci

Species	Locus					
	Scu01	*Scu05*	*Scu07*	*Scu11*	*Scu16*	*Scu26*
Crotalus viridis	+	−	+	+	+	+
Elaphe obsoleta	−	−	−	+	+	+
Elaphe vulpina	−	−	−	+	+	+
Nerodia sipedon	−	?	?	−	+	+
Coluber constrictor	−	−	+	+	+	+

All amplifications were done with an annealing temperature of 50°C. A + indicates that a product of approximately the expected size was produced, whereas a − indicates that either no product or a smear was observed, and ? indicates the species wasn't tested for that locus.

the amplification was run on an agarose gel. The different primer sets gave variable results. For example, *Scu01* appeared to amplify only rattlesnakes, *Scu05* was specific to eastern massasaugas, whereas the remaining primer sets work on both rattlesnake and at least some Colubrid species. Thus some of the primer sets may be useful for detecting variable loci in a variety of snake species, although based on data from birds and other snake species, loci that can be amplified with heterospecific primers are not necessarily polymorphic (Gibbs HL, unpublished data).

Discussion

Our main result is that we have isolated and characterized a set of variable microsatellite loci that should prove useful for a variety of population-level analyses in eastern massasaugas and possibly other snake species. Our results also raise some general issues related to these types of analyses using microsatellites in vertebrates, which we comment on below.

Levels of Heterozygosity

One striking finding was the significant heterozygote deficiency at all loci within the Killbear population of massasaugas. Traditionally such deficiencies have been attributed to some biological phenomenon such as nonrandom mating or undetected population structure (cf. Hartl and Clark 1987). More recently, however, the discovery of null alleles in microsatellites have lead some authors to argue that an alternative explanation for high levels of homozygosity is the presence of nonamplifying alleles within individuals and that this is a sufficient explanation for heterozygote deficiencies in natural populations (cf. Allen et al. 1995; Brookfield 1996; Paetkau and Strobeck 1994). Our use of alternate primer sets for three of the loci described here is one of the first direct tests of the "null allele" hypothesis for heterozygote deficiencies in wild vertebrates. The fact that we detected no additional heterozygotes indicates that (1) the use of comparisons between observed and expected levels of heterozygosity as tests for the presence of null alleles may give misleading results and (2) the reduced levels of heterozygosity in this massasaugas population are probably due to biological phenomena.

Potential biological explanations for the heterozygote deficiency in the Killbear population include (1) a Wahlund effect (cf. Hartl and Clark 1987) due to unrecognized population structure on a microgeographic scale or (2) inbreeding resulting from assortative mating between kin. Additional analysis by Gibbs et al. (unpublished data) indicate that both phenomena may cause heterozygote deficiencies in this and other populations of these snakes.

Microsatellite Evolution

The stepwise mutation model for microsatellite evolution assumes that when a mutation occurs at a locus the allele in question has an equal probability of either increasing or decreasing in size by one repeat unit (Valdes et al. 1993). However, recent analyses using either simulations (Di Rienzo et al. 1994) or analytical predictions (Estoup et al. 1995) suggest that a simple stepwise model does not accurately reflect mutational processes at some microsatellite loci in humans and insects. Based on these results, Estoup et al. (1995) argue that the more traditional infinite alleles mutation model (Ewens 1972) may better match the mutational processes occurring at these loci. Although we did not conduct formal tests of the appropriateness of either model to explain variation in the snake microsatellites described here, the large gaps in allele size distributions for at least three of the loci suggest that, for unknown reasons, at least some mutations may have resulted in changes of allele sizes of many repeat units. Modeling studies of the type conducted by Di Rienzo et al. (1994) and Estoup et al. (1995) are needed to accurately determine which model best describes how mutations occur at these loci, but we feel our data at least raise the issue that the pure stepwise model may not be appropriate. What we assume about how mutational processes operate at microsatellite loci is important because it bears on the accuracy of the different metrics used to measure levels of population differentiation. Our results suggest that measures based on the stepwise models [e.g., R_{st} (Slatkin 1995) and dmμ (Goldstein et al. 1995)] may significantly overestimate the evolutionary divergence between populations and that, at least for the loci described here, more traditional measures of divergence based on the infinite alleles model [e.g., F_{st} and Nei's genetic distance (D; Nei 1972)] will give conservative but possibly more accurate estimates of population differentiation.

There are two other possibilities that could explain the presence of two distinct types of alleles in the Killbear population. First, it could represent a retained ancestral polymorphism that resulted due to introgression between two ancient populations containing different size alleles. A test of this idea would require information on the phylogenetic history of the two allele types. Such data are not currently available. Second, massasaugas collected elsewhere which carry different size alleles may have been translocated into the park by humans. Anecdotal information suggests that small numbers of massasaugas have been released into the park during the past 30 years (Parent C, unpublished data). However, this explanation seems unlikely because the size range of alleles from other Georgian Bay populations is similar to that of the Killbear snakes (Gibbs HL et al., unpublished data) and the survival of translocated massasaugas is extremely low (Johnson B, unpublished data).

From the Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada (Gibbs) and the Department of Biology, Carleton University, Ottawa, Ontario, Canada (Prior and Parent).
We thank Lorie Collins and Lilie DeSousa for expert laboratory assistance, Rob Willson for help with the field work, Lisa Tabak for work on the figures and text, Pat Weatherhead for comments, and especially Barry Hughson (Parks Canada) for his long-term support of this work. This research was funded by Heritage Canada (Parks), NSERC, World Wildlife Fund Canada, and Environment Canada. We also thank Killbear Provincial Park and the Ontario Ministry of Natural Resources for providing logistical support. Address correspondence to Dr. Gibbs at the address above or e-mail: gibbs@mcmail.cis.mcmaster.ca.

The Journal of Heredity 1998:89(2)

References

Allen PJ, Amos W, Pomeroy PP, and Twiss SD, 1995. Microsatellite variation in grey seals (*Halichoerus grypus*) shows evidence of genetic differentiation between two British colonies. Mol Ecol 4:653–662.

Avise JC, 1994. Molecular markers, natural history, and evolution. New York: Chapman and Hall.

Amos B, Schlotterer C, and Tautz D, 1992. Social structure of pilot whales revealed by analytical DNA profiling. Science 260:670–672.

Brookfield JFY, 1996. A simple method for estimating null allele frequency from heterozygote deficiency. Mol Ecol 5:453–455.

Chakravarti A and Li CC, 1983. The effect of linkage on paternity calculations. In: Inclusion probabilities in parentage testing (Walker RH, ed). Arlington, Virginia, American Association of Blood Banks; 411–422.

Dawson RJG, Gibbs HL, Hobson KA, and Yezerniac S, 1997. Isolation of microsatellite markers from a passerine bird, the yellow warbler, *Dendroica petechia,* and their use in population studies. Heredity 79:506–514.

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, and Friemer NB, 1994. Mutational processes of simple-sequence repeat loci in human populations. Proc Natl Acad Sci USA 91:3166–3170.

Estoup A, Garney L, Solignac M, and Cornuet J-M, 1995. Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 140:679–695.

Ewens WJ, 1972. The sampling theory of selectively neutral alleles. Theor Popul Biol 3:87–112.

Gibbs HL, Brooke ML, and Davies NB, 1996. Analysis of genetic differentiation of host races of the Common Cuckoo, *Cuculus canorus,* using mitochondrial and microsatellite DNA variation. Proc R Soc Lond B 263:89–96.

Gibbs HL, Prior KA, and Weatherhead PJ., 1994. Genetic analysis of populations of threatened snake species using RAPD markers. Mol Ecol 3:329–337.

Goldstein DB, Linares AR, Cavalli-Sforza LL, and Feldman M, 1995. Genetic absolute dating based on microsatellites and the origin of modern humans. Proc Natl Acad Sci USA 92:6723– 6727.

Goudet J, 1995. FSTAT ver. 1.2: a computer program to calculate F-statistics. J Hered 86:485– 486.

Hanotte O, Zanon C, Pugh A, Greig C, Dixon A, and Burke T, 1994. Isolation and characterization of microsatellite loci in a passerine bird, the reed bunting, *Emberiza schoeniclus.* Mol Ecol 3:529–530.

Hartl D and Clark AG, 1987. Principles of population genetics, 2nd ed. Sunderland, Massachusetts: Sinauer.

Johnson B and Menzies V (eds), 1993. International symposium and workshop on the conservation of the eastern massasauga rattlesnake. Toronto, Canada: Metro Toronto Zoo.

McDonald DB and Potts WK, 1994. Cooperative display and relatedness among males in a lek-breeding bird. Science 266:1030–1032.

Nei M, 1972. Genetic distance between populations. Am Nat 106:283–292.

Nei M and Roychoudhury AK, 1974. Sampling variances of heterozygosity and genetic distances. Genetics 76: 379–390.

Paetkau D and Strobeck C, 1994. Microsatellite analysis of genetic variation in black bear populations. Mol Ecol 3:489–495.

Pemberton JM, Slate J, Bancroft DR, and Barrett JA, 1995. Non-amplifying alleles at microsatellite loci: a caution for parentage and population studies. Mol Ecol 4:249–252.

Primmer C, Moller AP, and Ellegren H, 1995. Resolving genetic relationships with microsatellite markers: a parentage testing system for the swallow, *Hirundo rustica.* Mol Ecol 4:493–498.

Quellar DC, Strassman JE, and Hughes CR, 1993. Microsatellites and kinship. Trends Ecol Evol 8:285–288.

Raymond M and Rousset F, 1995. GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. J Hered 86:248–249.

Seutin G, White BN, and Boag PT, 1991. Preservation of avian blood and tissue samples for DNA analysis. Can J Zool 69:82–90.

Slatkin M, 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462.

Tautz D, 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res 17:6463–6471.

Taylor AC, Sherwin WB, and Wayne RK, 1994. Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat, *Lasiorhinus krefftii.* Mol Ecol 3:277–290.

Valdes AM, Slatkin M, and Friemer NB, 1993. Alleles frequencies at microsatellite loci: the stepwise mutation model revisited. Genetics 133:737–749.

Villarreal X, Bricker J, Reinert HK, Gelbert L, and Bushar LM, 1996. Isolation and characterization of microsatellite loci for use in population genetic analysis in the timber rattlesnake, *Crotalus horridus.* J Hered 87: 152–155.

Weber JL and May PE, 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44:388–396.

Weir BS and Cockerham CC, 1984. Estimating F-statistics for the analysis of population structure. Evol 38: 1358–1370.

Received July 13, 1996
Accepted May 5, 1997

Corresponding Editor: Stephen J. O'Brien

Microsatellite Markers in Canada Geese (*Branta canadensis)*

J. C. Cathey, J. A. DeWoody, and L. M. Smith

A Canada goose (*Branta canadensis*) genomic library was created and screened for clones containing various microsatellite motifs. Fourteen positive clones were identified and sequenced; five primer pairs were developed and utilized to screen approximately 460 Canada geese for genetic variation. Each of these primer pairs were consistently scorable and polymorphic (average heterozygosity of 55%). DNA was isolated and successfully amplified from 2-year-old goose tail fans which received no special care other than storage at 4°C. Two of the five goose primers also amplified DNA from a partial family of wood ducks (*Aix sponsa; n* = 5), but no variation was detected at either locus. In geese, the number of alleles per locus ranged from 7 to 24. PIC values ranged from 0.41 to 0.91, while average levels of observed heterozygosity varied between 0.34 and 0.78. The high levels of polymorphism exhibited by these markers should be useful in addressing population genetic issues in Canada geese.

Canada geese (*Branta canadensis*) number in the millions across North America. Their populations and natural history have been a source of study for decades (Bellrose 1980; MacInnes 1962; Palmer 1976). Geese are a wildlife management priority from both an economic and biological standpoint. There are 11 putative subspecies of Canada geese based upon morphological differences (Delacour 1954; Johnsgard 1978). These subspecies are grouped according to size into two categories: large- and small-bodied geese. A main objective of North American waterfowl biologists is to identify the population affiliation, if any, of small Canada geese in the Central Flyway (*B. c. parvipes* and *B. c. hutchinsii*).

Initially, analysis of proteins through allozyme electrophoresis was used to detect potential differences among subspecies of Canada geese (Baker and Hanson 1966; Morgan et al. 1977). A major problem with allozyme studies of geese is the low level of variability detectable with conventional protein electrophoresis. From a genetic viewpoint, the available allozyme data suggests that there is no basis for current taxonomic distinctions at the subspecific level. We chose to utilize hypervariable microsatellite loci (which typically have many more alleles per locus than allozymes) to determine if low levels of variability among loci are the primary reason that allozyme studies have failed to delineate populations and subspecies of Canada geese. Our objective was to produce a panel of microsatellite markers that could be used to assess the population structure of Canada geese.

Materials and Methods

Genomic Library

Blood samples were used as a source of high-quality DNA. Our samples came from 13 collecting locales in the Northwest Territories of Canada ranging from Baffin Island in the east to the Mackenzie River delta in the west. Geese were mainly collected on their nesting grounds by driving flightless geese (those that had molted) into an enclosure. Goose blood (0.25–0.50 cc) was drawn from the brachial vein and transferred to a sterile polypropylene tube

Table 1. Molecular properties and population statistics of five Canada goose microsatellites

Locus name	Primer sequence	Product size (bp)	Annealing temperature	Number of geese typed	Average observed heterozygosity	Alleles per locus	PIC values
TTUCG-1F	5′-CCCTGCTGGTATACCTGA-3′						
TTUCG-1R	5′-GTGTCTACACAACAGC-3′	~119	55°C	453	0.4349	7	0.5754
TTUCG-2F	5′-GAGAGCGTTACTCAGCAAA-3′						
TTUCG-2R	5′-TCACTCTGAGCTGCTACAACA-3′	~136	55°C	416	0.7788	24	0.8847
TTUCG-3F	5′-GAGGTCGAATCCAACCTG-3′						
TTUCG-3R	5′-GCACATGATGCATGTGCTG-3′	~79	53°C	377	0.4138	14	0.8562
TTUCG-4F	5′-GGTGTACTCTGCTGAGTGTC-3′						
TTUCG-4R	5′-CTAGAACTAGTGGATCTCTC-3′	~194	55°C	426	0.3427	18	0.4068
TTUCG-5F	5′-GGGTGTTTTCCAACTCAG-3′						
TTUCG-5R	5′-CACTTTCCTTACCTCATCTTG-3′	~206	59°C	402	0.7761	18	0.9090

containing 5 ml of lysis buffer (Longmire et al. 1991). The blood and lysis buffer were incorporated by inversion. Once the tubes arrived at the laboratory, proteinase K was added to the blood-lysis solution to a final concentration of 0.5 μg/ml and incubated at 37°C with rotation overnight. Following a phenol extraction, the aqueous phase was retained and dialyzed against 1× TE (10 mM Tris, 1 mM EDTA) at 4°C overnight. The DNA was then ethanol precipitated and rehydrated with water. DNA concentrations were determined by agarose-gel electrophoresis. Additionally DNA was extracted from feather quills pulled from the tail fan and from muscle associated with the tail fan. These goose tail fans were 2 years old and had no special care given to them other than cold-room storage. The tissues were placed into the lysis buffer and treated in the same manner as the blood samples.

A genomic library was constructed using methods similar to DeWoody et al. (1995). Briefly, genomic DNA from a single goose was digested with *Sau*3A1; small fragments (<500 bp) were collected and cloned into the plasmid vector pBluescript II SK+ (Stratagene Inc., La Jolla, California) and transformed into DH5-alpha competent cells (Stratagene). Different repeat arrays d(GT/CA, CT/GA, GATA/CTAT) were hybridized to resultant colonies according to Janecek et al. (1993); 14 positive clones were isolated and sequenced according to established protocols (Sambrook et al. 1989). Sequences are deposited in GenBank under accession numbers U66089, U66090, U66091, U66092, and U66093.

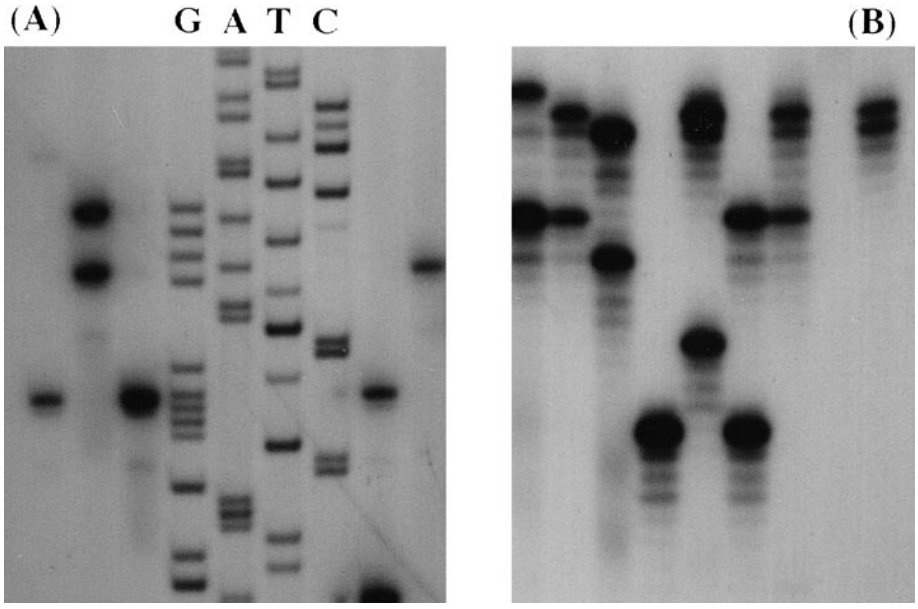

Figure 1. Panels A and B show two microsatellite loci amplified from Canada geese. Panel A shows a pentanucleotide repeat (TTUCG-5) and the sequence of M13. Panel B shows a dinucleotide repeat (TTUCG-2). The numerous slippage bands in the dinucleotide repeat make it more difficult to score than the pentamer, which has fewer and less severe shadow bands. Additionally the size of the dinucleotides are sometimes difficult to discern, as shown in panel B.

DNA Amplification

PCR primers were designed for five of the 14 positive clones. All 14 clones contained repeats; however, 9 of the 14 clones did not allow primer design due to insufficient sequence data. Several of these clones contained microsatellites that were too close to the vector/insert junction to allow primer design. The sequence of each primer, expected product size, and annealing temperature are listed in Table 1.

PCRs were performed in 25 μl volumes containing ~200 ng DNA, 0.25 μM of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 1× buffer, and 1.25 units of *Taq* DNA polymerase. Thermal profile consisted of an initial denaturation of 2 min at 94°C followed by 35 cycles of 94°C for 1 min, primer-specific annealing temperature (Table 1) for 30 s, and 72°C for 30 s. PCR products were visualized using a radioactively labeled primer; the forward primer was labeled with ^{32}P-gamma-ATP using T4-polynucleotide kinase (New England Biolabs, Beverly, Massachusetts) according to manufacturer's instructions.

Radiolabeled PCR products were denatured for 4 min at 80°C and electrophoresed in a 6% polyacrylamide gel (Sequagel, Atlanta, Georgia) at 55 W for up to 6 h. After drying, gels were exposed to X-ray film. Autoradiographs were scored utilizing the DNA sequence of M13 as a molecular size standard. Each allele was discrete and defined by size according to this standard.

Results and Discussion

We isolated and sequenced 14 clones that hybridized to three microsatellite repeat motifs from a Canada goose genomic library. We designed PCR primers to amplify five presumably independent loci that contained microsatellite repeats. Four of the five loci (TTUCG-1–4) are each d(GT/CA)$_n$ repeats, while the fifth locus (TTUCG-5) is a d(GATAA/CTATT)$_n$ pentamer. The clone containing the pentamer repeat also contained a d(CT/GA) repetitive element, but our PCR primers amplify only the pentamer repeat. Our results indicate that the pentamer repeat is much easier to score than the dinucleotide repeats because of fewer slippage bands (Figure 1).

Each of the five PCR primer pairs was highly polymorphic in Canada geese, with an average of 15.4 alleles per locus (Table

1). Average observed heterozygosity ranged from 34 to 78%, with an average of 55% over all five loci (Table 1). PIC values (Botstein et al. 1980) indicate that each marker should be highly informative; values ranged from 0.4068 to 0.9090 (Table 1). Two of the five markers (TTUCG-1 and TTUCG-4) amplified DNA from a small sample of related wood ducks (n = 5; mother and four offspring); however, no genetic variation was observed even though genetic variation is segregating within the family according to a duck microsatellite marker (Cathey et al. 1996).

In summary, we created a goose genomic library and developed a panel of five microsatellite markers that are highly polymorphic in multiple populations of Canada geese. Two of these markers were monomorphic in a small family of wood ducks. Additionally we successfully used all five microsatellite markers to amplify goose DNA from tail fans that were collected in the field and donated by hunters. Goose hunters are often surveyed by the U.S. Fish and Wildlife Service to obtain information about their harvest. These surveys offer a convenient and inexpensive way to gather tissue from wild populations that can later be used for genetic analysis. The tail fans were shipped in an envelope at ambient temperature and transferred to a cold room at 4°C at the earliest convenience. No other care and/or preparation was given to these tail fans until some 2 years later, when DNA was isolated as described above. Therefore the markers described herein should be of value to both wildlife managers who study Canada geese as well as geneticists.

From the Department of Range, Wildlife, and Fisheries Management and the Department of Biological Sciences, Texas Tech University, Lubbock, Texas. Dr. Cathey is now at the Texas Parks and Wildlife Department, Engeling WMA, Rt. 1 Box 27, Tennessee Colony, Texas. This work was supported by the U.S. Fish & Wildlife Service and by the Central Flyway Council. We thank J. Haskins, J. Cornely, and R. Trost for initiating this work. J. Hines, B. Croft, R. Bromley, R. Alisauskas, A. Didiuk, M. Gillispie, and D. Caswell collected blood samples from Canada geese. We thank J. L. Longmire and Los Alamos National Laboratory for technical assistance. R. J. Baker graciously provided guidance, laboratory facilities, and critical comments on an earlier draft of this article. This is manuscript T-9-773, College of Agriculture and Natural Resources, Texas Tech University. Address correspondence to Dr. Cathey, Texas Parks and Wildlife Department, Engeling WMA, Rt. 1 Box 27, Tennessee Colony, TX 75861, or e-mail: ewma@e-tex.com.

The Journal of Heredity 1998:89(2)

References

Baker CMA and Hanson HC, 1966. Molecular genetics of avian proteins—VI. Evolutionary implications of blood proteins of eleven species of geese. Comp Biochem Phys 17:997–1006.

Bellrose FC Jr, 1980. Ducks, geese and swans of North America. Harrisburg, Pennsylvania: Stackpole Books.

Botstein D, White RL, Skolnick M, and Davis RW, 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314–331.

Cathey JC, Smith LM, Baker RJ, and DeWoody JA, 1996. Trinucleotide repeat polymorphism at the alpha-enolase/tau-crystallin locus in ducks. Anim Genet 27:130–131.

Delacour J, 1954. The waterfowl of the world. Vol. 1. London: Country Life.

DeWoody JA, Honeycutt RL, and Skow LC, 1995. Microsatellite markers in white-tailed deer. J Hered 86:317–319.

Janecek LL, Longmire JL, Wichman HA, and Baker RJ, 1993. Genome organization of repetitive elements in the rodent *Peromyscus leucopus.* Mammal Genome 4:375–381.

Johnsgard PA, 1978. Ducks, geese, and swans of the world. Lincoln: University of Nebraska Press.

Longmire JL, Ambrose RE, Brown NC, et al., 1991. Use of sex-linked minisatellite fragments to investigate genetic differentiation and migration of North American populations of the peregrine falcon (*Falco peregrinus*). In: DNA fingerprinting: approaches and applications (Burke T, Dolf G, Jeffreys AJ, and Wolff R, eds). Basil, Switzerland: Birkhauser.

MacInnes CD, 1962. Nesting of small Canada geese near Eskimo Point, Northwest Territories. J Wildl Manage 26:247–256.

Morgan RP, Sulkin ST, and Henny CJ, 1977. Serum proteins of Canada goose (*Branta canadensis*) subspecies. Condor 79:275–278.

Palmer RS, 1976. Handbook of North American birds. New Haven, Connecticut: Yale University Press.

Sambrook J, Fritsch EF, and Maniatis T, 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Received August 14, 1996
Accepted May 5, 1997

Corresponding Editor: Robert Wayne

Short Beak: A New Autosomal Recessive Semilethal Mutation in Japanese Quail

M. Tsudzuki, Y. Nakane, and A. Wada

Short beak (SBK) is a new semilethal mutation of Japanese quail (*Coturnix japonica*). The SBK individuals are characterized externally by short beaks, shanks, and digits. The shank is also thicker than the wild type. The shape of the mutant beak does not show a parrotlike appearance, contrasting with that of other poultry chondrodystrophic mutants reported in the literature. Bones in the fore and hind limbs of the SBK mutant are also shorter than the wild types. The humerus and ulna in the wing are significantly thicker than the wild types. Other bones in the wing and leg show approximately the same thickness as the wild types. The majority of the SBK mutants die at the late embryonic stage. Some chicks can hatch with no assistance, but almost all die around 3 days of age. However, a part of the SBK individuals (5.3%, 8/151) reach sexual maturity and can reproduce. Genetic analyses revealed that the SBK mutation is controlled by an autosomal recessive gene. The proposed gene symbol is *sbk.* The *sbk* gene is not allelic to the previously reported stumpy-limb (*sl*) gene that expresses a somewhat similar phenotype to the SBK.

In August 1994 we found unusual chicks having beaks and shanks reduced in size. These were among the progeny from a mating of a black-at-hatch mutant (Minezawa and Wakasugi 1977) female and a wild-type male Japanese quail (*Coturnix japonica*) kept at the Department of Laboratory Animal Science, College of Agriculture, Osaka Prefecture University. All of these unusual chicks died before reaching maturity. Full-sib matings of their normal siblings also segregated such abnormal individuals. Thus this abnormality seemed to be a recessive lethal mutation. In Japanese quail, there are five mutations that show reduced beak and shank lengths and lethality (Collins et al. 1968; Hermes et al. 1990; Hill et al. 1963; Nichols and Cheng 1991; Tsudzuki 1995b). The present mutant seemed to be phenotypically different from all of them. We named our mutant short beak (SBK) and started genetic analysis. This article describes the features of the SBK character and its mode of inheritance.

Materials and Methods

General care of quail is described elsewhere (Tsudzuki 1995a). We compared external appearances between the SBK and wild-type newly hatched chicks. We measured with a caliper the length of the beak, shank, and third digit and the midshaft thickness of the shank in both mutant and wild-type chicks. Furthermore, we compared SBK and wild-type long bones of 15-day embryos by measuring their length and midshaft thickness under a dissecting microscope. For this purpose we stained the bone with alizarin red S (Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the method described by Tsudzuki and Wakasugi (1988). The resulting data were analyzed by Student's t test or Cochran–Cox test.

Mating Experiments

We separated the female and male of the original pair that produced the first mutants and mated each of them to wild-type controls (UOP-WT line; Ito and Tsudzuki 1994) to produce F_1 progeny. We then mated the F_1 birds among themselves, avoiding sib matings, and identified carrier matings by a progeny test. We mated the progeny from these carrier matings inter se and again identified carrier matings by a progeny test. In these selected proven carrier matings, we examined the incidence of SBK individuals. With the SBK individuals derived from the carrier matings, we examined the viability of this mutant.

Because a few SBK mutants were viable and fertile, we paired SBK mutants with wild-type controls to produce F_1 progeny. We then backcrossed the F_1 birds to the SBK mutants. In these F_1 and backcross progeny, we investigated the incidence of the SBK individuals. All segregation data obtained in the present study were analyzed by the chi-square test.

In addition to the mating experiment with the viable SBK mutants and wild-type controls, we made an allelism test between the viable SBK mutant and stumpy limb (*sl*) mutant (Tsudzuki 1995b; Tsudzuki et al. 1991) that is also characterized by reduced beaks and shanks. For this purpose we paired the SBK male to the SL female and examined the phenotype of their F_1 progeny.

Results and Discussion

Morphology

The SBK chick had a somewhat smaller and more globular head than the wild type. The beak of the mutant was shorter than the wild type. Its shape did not show a parrotlike appearance but was similar to that of the wild-type beak. The shanks and digits of the SBK chick were shorter than those of the wild-type chick. Moreover, the SBK shanks were slightly thicker than the wild type.

Measurement of the Beak, Shank, and Digit

Table 1 shows body weight, length of the beak, shank, and third digit, and thickness of the shank in newly hatched wild-type and SBK mutant chicks. There was no significant difference in the body weight between the mutant and the wild type, nor between the sexes. There was also no sex difference in the length of the beak, shank, and third digit. However, the length of the beak, shank, and third digit of the SBK mutant were all significantly ($P < .001$) shorter than the wild type. The length of the beak, shank, and third digit of the SBK chick was approximately 90% of that of the wild-type chick. The shank was significantly ($P < .05$) thicker in the SBK mutant than in the wild type. The extent to which the SBK shank was thickened was approximately 110%. These data support the correctness of the visual observation mentioned above.

Table 1. Body weight (g), length (mm) of the beak, shank, and third digit, and thickness (mm) of the shank in newly hatched wild-type and short-beak mutant chicks (mean ± SEM)

	Wild type		Mutant	
	Female (n = 15)	Male (n = 15)	Female (n = 15)	Male (n = 15)
Body weight	6.88 ± 0.10[a]	6.94 ± 0.15[a]	6.94 ± 0.13[a]	6.88 ± 0.12[a]
Length				
Beak	8.98 ± 0.07[a]	8.77 ± 0.10[a]	8.09 ± 0.09[b]	8.06 ± 0.10[b]
Shank	16.50 ± 0.19[a]	16.44 ± 0.24[a]	14.81 ± 0.16[b]	14.98 ± 0.14[b]
Third digit	15.61 ± 0.19[a]	15.85 ± 0.15[a]	13.80 ± 0.24[b]	13.71 ± 0.17[b]
Thickness of the shank	1.86 ± 0.04[a]	1.87 ± 0.03[a]	2.00 ± 0.04[b]	2.03 ± 0.04[b]

Means within a line having different superscripts significantly differ by Student's t test or Cochran–Cox test ($P <$ .001 for body weight and length of the beak, shank, and third digit; $P < .05$ for thickness of the shank).

Measurement of Appendicular Bones

Table 2 shows measurements of body weight and appendicular bones of wild-type and SBK mutant embryos at 15 days of incubation. There were no sex differences in all points measured within each group. The body weight of the SBK embryo was significantly smaller than that of the wild type. The length of all the bones that were measured (humerus, ulna, third metacarpus, and first phalanx of the third digit in the wing, and femur, tibia, metatarsus, and first phalanx of the third digit in the leg) was significantly smaller in the SBK embryos (approximately 80–90%) than in the wild-type embryos. On the other hand, the humerus and ulna of the SBK embryos were significantly thicker (approximately 110%) than that of the wild types. The other bones in the wings and all bones in the legs that were measured showed similar values to those of the wild types. The appendicular bones of the SBK mutant had a dumpy appearance in comparison with wild-type bones.

In spite of the similar thickness of the

Table 2. Measurements (mean ± SEM) of body weight (g) and appendicular bones (mm) in wild-type and short-beak mutant embryos at 15 days of incubation

		Wild type		Mutant	
		Female (n = 20)	Male (n = 20)	Female (n = 20)	Male (n = 20)
Body weight		5.90 ± 0.08[a]	5.85 ± 0.10[a]	5.32 ± 0.12[b]	5.25 ± 0.10[b]
Bones[a]					
Wing					
Humerus	L	6.07 ± 0.03[a]	6.09 ± 0.04[a]	4.65 ± 0.16[b]	4.69 ± 0.11[b]
	T	0.54 ± 0.01[a]	0.54 ± 0.01[a]	0.60 ± 0.01[b]	0.60 ± 0.01[b]
Ulna	L	5.09 ± 0.04[a]	5.21 ± 0.05[a]	4.30 ± 0.07[b]	4.34 ± 0.07[b]
	T	0.47 ± 0.01[a]	0.48 ± 0.01[a]	0.52 ± 0.01[b]	0.52 ± 0.01[b]
Metacarpus	L	3.70 ± 0.05[a]	3.63 ± 0.03[a]	3.24 ± 0.06[b]	3.33 ± 0.04[b]
	T	0.45 ± 0.01[a]	0.45 ± 0.01[a]	0.43 ± 0.01[a]	0.43 ± 0.01[a]
Phalanx	L	1.15 ± 0.02[a]	1.16 ± 0.02[a]	0.96 ± 0.02[b]	0.99 ± 0.02[b]
	T	0.54 ± 0.01[a]	0.53 ± 0.01[a]	0.56 ± 0.01[a]	0.56 ± 0.01[a]
Leg					
Femur	L	10.61 ± 0.08[a]	10.55 ± 0.10[a]	8.37 ± 0.24[b]	8.32 ± 0.23[b]
	T	0.83 ± 0.01[a]	0.84 ± 0.01[a]	0.84 ± 0.01[a]	0.84 ± 0.01[a]
Tibia	L	14.24 ± 0.08[a]	14.17 ± 0.11[a]	12.30 ± 0.17[b]	12.22 ± 0.13[b]
	T	0.86 ± 0.01[a]	0.89 ± 0.01[a]	0.85 ± 0.01[a]	0.85 ± 0.01[a]
Metatarsus	L	10.13 ± 0.04[a]	10.00 ± 0.10[a]	8.53 ± 0.15[b]	8.47 ± 0.12[b]
	T	1.21 ± 0.02[a]	1.21 ± 0.02[a]	1.23 ± 0.02[a]	1.24 ± 0.01[a]
Phalanx	L	3.12 ± 0.04[a]	3.08 ± 0.04[a]	2.49 ± 0.04[b]	2.43 ± 0.04[b]
	T	0.65 ± 0.01[a]	0.65 ± 0.01[a]	0.68 ± 0.01[a]	0.67 ± 0.01[a]

L = length; T = thickness; Metacarpus = the third metacarpus; phalanx in the wing = the first phalanx of the third digit; length of the metatarsus = length of the third metatarsus; thickness of the metatarsus = total thickness of the second through fourth metatarsi; phalanx in the leg = the first phalanx of the third digit.

Means within a line having different superscripts differ significantly by Student's t test or Cochran–Cox test ($P <$ .05 for body weight, $P < .01$ for bone thickness, $P < .001$ for bone length).

Table 3. Segregation data for the short-beak mutant (SBK) and wild type (WT) in carrier matings

	No. of eggs set	No. of eggs fertile	No. of dead embryos			No. of chicks hatched		Expected number[a]		
			1–10 days	11–18 days						
				WT	SBK	WT	SBK	WT : SBK	χ^2	P
Carrier matings	705	663	46	40	93	426	58	462.75 : 154.25	0.09	$.80 > P > .70$
Control	284	261	18	11	0	232	0	—	—	—

[a] Based on a 3:1 ratio of simple autosomal recessive inheritance, involving both dead embryos and chicks hatched.

metatarsus between the wild-type and SBK individuals (Table 2), the thickness of the shank was significantly larger in the SBK mutant than in the wild type (Table 1). There is a possibility that the SBK mutant shank might have a greater amount of flesh and/or skin around the metatarsus.

Although the body weight of the 15-day SBK embryos was lighter than the wild type (Table 2), there were no significant differences in body weight between the newly hatched wild-type and SBK mutant chicks (Table 1). The body weight of the 15-day SBK mutant embryos ranged from 4.1 to 6.0 g. As is mentioned later, a considerably larger number of the SBK mutants die before hatching. Thus it is thought that SBK individuals with lower body weight die before hatching and only those with higher weight can hatch, leading to no significant difference in body weight between the wild-type and SBK chicks.

Genetic Analyses

Table 3 shows the incidence of the SBK mutant in proven carrier matings. Before 11 days of incubation, embryonic mortality was similar between the control and carrier matings. Of 617 progeny on and after 11 days of incubation, 466 were wild-types and 151 were SBKs. This segregation ratio was in good agreement ($\chi^2 = 0.09$, $.80 > P > .70$) with the expected 3:1 ratio based on the hypothesis that the SBK character is controlled by an autosomal recessive gene. Among the 466 wild types, 40 (8.6%) died at the late embryonic stage, whereas among the 151 SBKs, 93 (61.6%) died at the same stage. Moreover, of the 58 SBK chicks that hatched, 42 died before maturity, with the peak of death around 3 days of age. The remaining 16 birds (7 females and 9 males) reached adulthood. Of the 7 females and 9 males, a female and a male showed no sexual maturity, 4 females and 2 males showed sexual maturity but died before mating experiments, and 2 females and 6 males could actually reproduce.

Table 4 shows the results of direct matings of the viable SBK mutants to controls. All 182 F_1 birds from reciprocal matings were wild types. The backcross segregation ratio was also in good accordance ($\chi^2 = 0.12$, $.80 > P > .70$) with the expected 1:1 ratio based on the hypothesis mentioned. Thus it is concluded that the SBK character is controlled by an autosomal recessive semilethal gene with variable expressivity in lethality.

The stumpy limb (SL) mutant (Tsudzuki 1995b; Tsudzuki et al. 1991) shows short beaks and shanks and is controlled by an autosomal recessive gene, *sl*. In the test for allelism, the 75 F_1 birds from two pair-matings of the SBK male and SL female showed a wild-type phenotype, which indicates that the two traits are not alleles. We propose the gene symbol *sbk* for the mutant gene controlling the short beak character.

The present mutant is characterized by brachycephaly and short, thick legs and accompanied by lethality. Besides the SL mutation mentioned, there are four chondrodystrophic mutations in Japanese quail that have abnormalities in the beak and leg and show lethality, that is, micromelia (Hill et al. 1963), chondrodystrophy (Collins et al. 1968), chondrodystrophy-2 (Hermes et al. 1990), and micromelia-Nichols (Nichols and Cheng 1991). All of them, however, are easily distinguishable from the present SBK mutant by possessing a parrotlike beak and/or 100% prehatching lethality. The SBK mutant has a shortened beak, but its shape does not show a parrotlike beak appearance. Approximately 40% of the SBK mutants can hatch with no assistance, and a few SBK birds reach sexual maturity.

Several chondrodystrophic mutations have also been reported in chickens and turkeys as well as in Japanese quail (Savage 1990; Somes 1990), but almost all of them are characterized by parrotlike beaks and/or 100% lethality during embryonic stages, apparently differing from the quail SBK mutation. The micromelia-Hays mutant of chickens (Hays 1944) is somewhat similar to the quail SBK mutant in having reduced beaks with normal shape and approximately 30% hatchability. However, mutant chicks have greatly shortened legs and die within 1 week, which differs obviously from the quail SBK mutant. The extent to which the SBK legs are shortened is small, and a few SBK individuals reach sexual maturity and can reproduce. The chondrodystrophy-m mutant of turkeys (Nestor 1978) resembles the present SBK mutant in two points. It has proportionately shortened upper and lower beaks, and some mutants reach adulthood. However, all are sterile, and about half of the mutants show the absence of some terminal phalanges, contrasting with the quail SBK mutant in which phalanges are normal and some individuals are fertile.

Table 4. Incidence of the short-beak (SBK) mutant in F_1 and backcross generations in mating experiments between wild-type (WT) controls and viable SBK mutants

Matings			No. of pairs mated	No. of progeny observed[a]	No. of progeny of		Expected number[b]		
Female	×	Male			WT	SBK	WT : SBK	χ^2	P
WT	×	SBK	4	164	164	0	164:0	—	—
SBK	×	WT	1	18	18	0	18:0	—	—
Total			5	182	182	0	182:0	—	—
F_1	×	SBK	4	144	73	71	72:72	0.03	$.90 > P > .80$
SBK	×	F_1	1	57	30	27	28.5:28.5	0.16	$.70 > P > .60$
Total			5	201	103	98	100.5:100.5	0.12	$.80 > P > .70$

[a] Observation was performed at 15 days of incubation.

[b] Based on simple autosomal recessive inheritance.

From the Laboratory of Animal Breeding and Genetics, Faculty of Applied Biological Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739-8528, Japan (Tsudzuki), and Department of Laboratory Animal Science, College of Agriculture, Osaka Prefecture University, Sakai, Osaka, Japan (Nakane and Wada). Address correspondence to Dr. Tsudzuki at the address above or e-mail: tsudzuki@ue.ipc.hiroshima-u.ac.jp.

The Journal of Heredity 1998:89(2)

References

Collins WM, Abplanalp H, and Yoshida S, 1968. Early embryonic chondrodystrophy in Japanese quail. J Hered 59:248–250.

Hays FA, 1944. Chondrodystrophy in Rhode Island reds. Am Nat 78:54–58.

Hermes JC, Abbott UK, Johnston E, and Owens M, 1990. A new chondrodystrophy mutation in Japanese quail (*Coturnix japonica*). J Hered 81:222–224.

Hill WG, Lloyd GL, and Abplanalp H, 1963. Micromelia in Japanese quail, an embryonic lethal mutant. J Hered 54:188–190.

Ito S and Tsudzuki M, 1994. Orange: a plumage color mutation accompanied by semi-lethality in Japanese quail. J Hered 85:54–56.

Minezawa M and Wakasugi N, 1977. Studies on a plumage mutant (black at hatch) in the Japanese quail. Jpn J Genet 52:183–195.

Nestor KE, 1978. Hereditary chondrodystrophy in the turkey. Poult Sci 57:577–580.

Nichols CR and Cheng KM, 1991. Micromelia-Nichols (*m-N*): an autosomal recessive obligate embryonic lethal mutation in Japanese quail. Poult Sci 70(suppl 1): 88.

Savage TF, 1990. Mutations and major variants in turkeys. In: Poultry breeding and genetics (Crawford RD, ed). Amsterdam: Elsevier; 317–331.

Somes RG Jr, 1990. Lethal mutant traits in chickens. In: Poultry breeding and genetics (Crawford RD, ed). Amsterdam: Elsevier; 293–315.

Tsudzuki M, 1995a. Light down lethal: a new autosomal recessive down color mutation of Japanese quail. J Hered 86:305–307.

Tsudzuki M, 1995b. New findings in the stumpy-limb mutation of Japanese quail. J Hered 86:66–68.

Tsudzuki M and Wakasugi N, 1988. Genetic head-skeleton abnormalities in the ET (ear tuft) strain of Japanese quail: a defect associated with the incomplete closure of the hyomandibular furrow. Jpn Poult Sci 25:207–217.

Tsudzuki M, Wakasugi N, Morioka H, and Esaki K, 1991. Stumpy limb—an embryonic lethal mutation in Japanese quail (*Coturnix coturnix japonica*). Exp Anim 40: 529–536.

Received November 20, 1996
Accepted May 5, 1997

Corresponding Editor: J. James Bitgood

Genetic Variation and Population Genetic Structure in *Trifolium pratense*

M. J. Hagen and J. L. Hamrick

Trifolium pratense (red clover) is a short-lived herbaceous plant native to southeastern Europe and Asia Minor. Widely used in agriculture, *T. pratense* is cultivated as an annual, winter annual, or biennial. It blooms from mid-spring to early fall and is insect pollinated and self-incompatible. Seeds are mammal and bird dispersed. Naturalized populations of *T. pratense* occur along roadsides and in old fields as well as native grasslands. Allozyme diversity and population genetic structure were determined for nine populations of *T. pratense*. Results from 13 allozyme loci indicate that genetic diversity is higher and population divergence is lower than expected based on the life-history characteristics of the species. We conclude that the high levels of genetic diversity found within populations of *T. pratense* suggest that these are not newly established founder populations, and that the low levels of genetic divergence seen among populations are probably due to high rates of gene flow among populations as a result of seed and pollen movement.

Agricultural breeding practices and life-history characteristics of cultivated species effect the levels of genetic diversity maintained within species and their populations. At the species level, high genetic diversity is desirable; thus germplasm collections and seed banks have been established to conserve and maintain the genetic diversity of cultivated species and their relatives (Blixt 1988; Marshall 1989; Marshall and Brown 1975). Genetic diversity within populations of crop species is often reduced, however, when the characteristics of high yield and phenotypic uniformity are selected.

Naturalized populations of cultivated species (i.e., escapes) are ultimately a product of both their biological characteristics and historical cultivation practices. Newly established naturalized populations should have low levels of genetic diversity for several reasons. First, genetic variation in the source population (i.e., a cultivated field) may be limited because the field was planted with a single strain or cultivar. Second, populations of colonizing species are often founded by a small sample of a larger population (i.e., founder effect) and as a result may exhibit reduced genetic diversity (Barrett and Shore 1989; Chakraborty and Nei 1977; Nei et al. 1975; Nei and Tajima 1981). Finally, because the competitive ability of cultivated species is often reduced in natural habitats, the number of successful founders may be small (i.e., founder selection), further reducing genetic variation sampled from the source population. These factors should contribute to low genetic diversity within populations and higher divergence among populations. In contrast, the mixing of strains due to seed movement and pollen flow between established populations should increase intrapopulation diversity and reduce differentiation among populations.

In this study we examined genetic variation within and among naturalized populations of *Trifolium pratense* L. (red clover). A short-lived herbaceous plant, *T. pratense* is cultivated as an annual, winter annual, or biennial. It is widely used in agriculture and is of considerable economic importance. Originating in southeastern Europe and Asia Minor, *T. pratense* was first cultivated in northern Europe around 1650 (Merkenschlager 1934) and was introduced to North America by European colonists (Fergus and Hollowell 1960; Pieters and Hollowell 1937). *T. pratense* is an insect-pollinated, self-incompatible diploid ($2n = 14$) that blooms from mid-spring to early fall (Smith et al. 1985). Seeds are dispersed by horses, cattle, humans and birds (Ridley 1930). *T. pratense* is commonly found along roadsides and in old fields, but it also invades natural and cultivated grasslands.

We compared the genetic diversity and population genetic structure in naturalized populations of *T. pratense* with plant species having similar life-history characteristics. We concluded that the high levels of genetic diversity found within populations of *T. pratense* suggest that these are not newly established founder populations, and that the low levels of genetic divergence seen among populations is probably due to high rates of gene flow among populations as a result of seed and pollen movement.

Materials and Methods

Nine population samples of *T. pratense* were collected, three populations in the southeastern United States in 1992 (Jackson, North Carolina = JNC1; Clayton, Georgia = BMT1; Athens, Georgia = CCR1) and in 1993 (Jackson, North Carolina = JNC2; Clayton, Georgia = BMT2; Athens, Georgia = CCR2) and an additional three populations in 1993 from the northeastern United States (Biddeford, Maine = UNE; Kennebunk, Maine = KBS; Woodstock, Virginia = WHS) (Figure 1). Data from the 1992 and 1993 southeastern populations were pooled for analysis unless stated otherwise. From each population leaf samples were collected from 48 individual plants. Samples were stored on ice after collection and transported to the laboratory for electrophoretic analysis. Each leaf sample was cut finely, frozen with liquid nitrogen, and crushed with a mortar and pestle. Enzymes were stabilized and extracted by the addition of a potassium phosphate buffer (Mitton et al. 1979). The enzyme extracts were absorbed onto filter paper wicks and stored at −70°C.

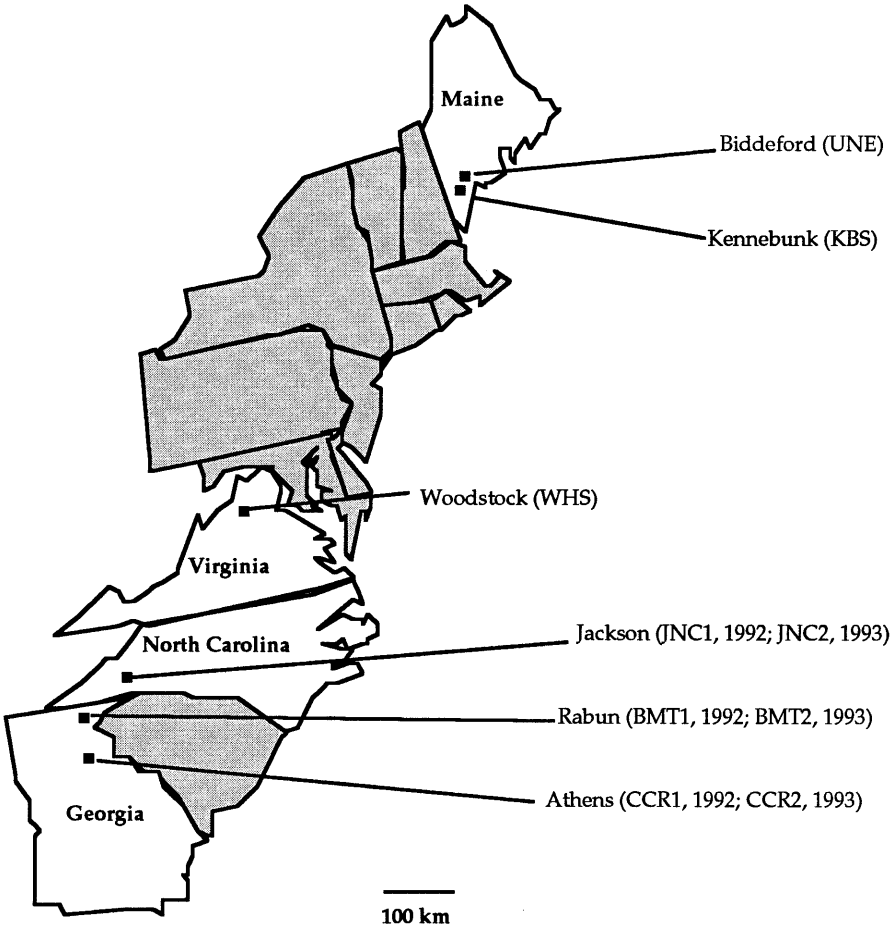

Figure 1. Locations of nine *T. pratense* populations collected in the eastern United States.

Electrophoresis was performed on 10% starch gels according to the gel and electrode buffer systems of Soltis et al. (1983). Twelve enzyme stains resolved 13 loci. The enzymes stained were triose phosphate isomerase (TPI), alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (GDH), peroxidase (PER), malate dehydrogenase (MDH), glucosephosphate isomerase (GPI), diaphorase (DIA), 6-phosphogluconate dehydrogenase (6-PGDH), malic enzyme (ME), β-galactosidase (β-GAL), leucine aminopeptidase (LAP), and fructose biphosphate (FBP). Stain recipes were taken from Soltis et al. (1983), except for DIA and β-GAL, which were taken from Cheliak and Pitel (1984), and FBP, which is from Wendel and Weeden (1989). The genetic basis of the observed allozyme banding patterns was inferred from segregation patterns with reference to typical subunit structure (Gottlieb 1981; Harris and Hopkinson 1976).

Allozyme diversity was calculated for the species as a whole and on a population basis using four standard measures: percent polymorphic loci (*P*), mean number of alleles per polymorphic locus (*AP*), effective number of alleles per locus (A_e), and gene diversity (H_e). Subscripts refer to species (s) or population (p) level parameters. Percent polymorphic loci (P_s) was calculated by dividing the number of loci polymorphic in at least one population by the total number of loci analyzed. The mean number of alleles per polymorphic locus (AP_s) was determined by summing all the alleles observed at polymorphic loci and dividing by the total number of loci. The effective number of alleles was calculated for each locus by $1/\Sigma p_i^2$, where p_i is the mean frequency of the *i*th allele. These values were then averaged across loci to obtain A_{es}. Gene diversity at each locus was calculated as $h = 1 - \Sigma p_i^2$. The mean genetic diversity (H_e) is the arithmetic average of *h* values across all loci. At the population level, the values of P_p, AP_p, A_{ep}, and H_{ep} were calculated using the equations of Hedrick (1985). Genetic parameters at the population level represent population means, whereas at the species level, parameters reflect overall genetic diversity within the species.

Deviations from Hardy–Weinberg expectations were examined for each population by calculating Wright's fixation index (Wright 1922) and testing for significant deviations from the expected value ($F = 0$) by chi-square tests (Li and Horvitz 1953).

Chi-square analyses were also performed to test for heterogeneity in allele frequencies among populations (Workman and Niswander 1970) and among years. Total genetic diversity was partitioned into components using Nei's (1973, 1977) gene diversity statistics. For each polymorphic locus, total gene diversity (H_T) was partitioned into diversity among regions (D_{reg}), among populations within regions (D_{pop}), and within populations (H_{wpop}) as follows: $H_T = D_{reg} + D_{pop} + H_{wpop}$. A temporal component to gene diversity, D_{year}, was examined for the southern populations only; where total gene diversity in the southern sample (H_{ST}) as partitioned into diversity among years (D_{year}), among populations within years ($D_{pop(year)}$), and within populations (H_{swpop}) as follows: $H_{ST} = D_{year} + D_{pop(year)} + H_{swpop}$. A measure of differentiation among populations, relative to total diversity, G_x, as calculated at each locus and averaged over all polymorphic loci to obtain a species value for population divergence. Population divergence was also examined by calculating Nei's genetic distance and identity parameters (Nei 1972) for all pairs of populations. An indirect estimate of gene flow among populations, N_m, was estimated, from the genetic data as

$$N_m = (1 - G_X)/(\alpha 4 G_X),$$

where $\alpha = [n/(n - 1)]$ and n = number of populations sampled (Crow and Aoki 1984).

Results

Ten of the 13 loci (76.9%) were polymorphic in at least one population. β-GAL, 6-PGDH, and ADH were monomorphic enzyme systems. The percent polymorphic loci within populations ranged from 61.5% in population CCR to 76.9% in population BMT, with an overall mean of 68.4%. The number of alleles per polymorphic locus was 2.60 at the species level and averaged 2.52 within populations. Expected heterozygosity for the species was $H_{es} = 0.285$ and at the population level was $H_{ep} = 0.250$ (Table 1).

Hardy–Weinberg expectations were observed in all but 5 of the 117 chi-square tests. Since we would expect to see 5.85

Table 1. Summary of allozyme variation within populations of *T. pratense*

Populations	*P*	*AP*	A_e	H_{obs}	H_e
BMT	76.92	2.60	1.55	0.259	0.299
CCR	61.54	2.75	1.49	0.242	0.266
JNC	69.23	2.56	1.52	0.258	0.277
KBS	63.64	1.92	1.35	0.200	0.198
UNE	69.23	1.91	1.34	0.170	0.201
WHS	69.23	2.00	1.47	0.237	0.259
Mean	68.30	2.52	1.45	0.228	0.250
SD	5.32	0.15	0.09	0.019	0.025
Species	76.9	2.60	1.51	—	0.285

Values are based on 13 loci. Abbreviations: *P,* percent polymorphic loci; *AP,* mean number of alleles per polymorphic locus; A_e, effective number of alleles per locus; H_{obs}, observed heterozygosity; H_e, expected heterozygosity.

Table 2. Gene diversity statistics (Nei 1973, 1977) and 10 polymorphic loci in *T. pratense*

Locus	H_T	G_{reg}	G_{pop}	G_{wpo9}
TPI1	0.265	0.016	0.052	0.932
TPI2	0.258	0.001	0.115	0.884
GDH1	0.002	0.000	0.000	1.000
PER1	0.468	0.001	0.033	0.966
MDH1	0.419	0.001	0.006	0.993
GPI2	0.499	0.057	0.131	0.812
DIA1	0.480	0.021	0.020	0.959
ME1	0.473	0.029	0.038	0.933
LAP1	0.483	0.000	0.032	0.968
FBP2	0.361	0.005	0.063	0.932
Mean	0.371	0.013	0.049	0.938
SD	0.157	0.019	0.044	0.056

Total genetic variation, H_T, was partitioned into the proportion of genetic variation found among regions, G_{reg}, among populations within regions, G_{pop}, and within populations, G_{wpop}.

Table 3. Gene diversity statistics (Nei 1973, 1977) for 10 polymorphic loci in *T. pratense*

Locus	H_{ST}	G_{year}[a]	$G_{pop(year)}$	G_{swpop}
TPI1	0.308	0.051**	0.068	0.881
TPI2	0.268	0.019***	0.184	0.797
GDH1	0.004	0.000n.s.	0.000	1.000
PER1	0.481	0.015**	0.033	0.952
MDH1	0.431	0.013**	0.005	0.982
GPI2	0.496	0.013*	0.230	0.757
DIA1	0.463	0.023***	0.039	0.938
ME1	0.537	0.051***	0.023	0.926
LAP1	0.476	0.002n.s.	0.030	0.968
FBP2	0.390	0.091***	0.060	0.849
Mean	0.385	0.028	0.067	0.905
SD	0.158	0.028	0.077	0.082

Total genetic variation, H_{ST}, estimated in the southern populations BMT, CCR, and JNC, was partitioned into the proportion of genetic variation found among the years 1992 and 1993, G_{year}, among populations within years, $G_{pop(year)}$, and within populations G_{swpop}.

[a] Chi-square test of independence. *** = $P < .001$; ** = $P < .01$; * = $P < .05$; n.s. = not significant.

significant deviations at the $P = .05$ significance level by chance alone, we can conclude that the populations sampled are in Hardy–Weinberg equilibrium. Furthermore, mean observed heterozygosity within populations, $H_{obs} = 0.228$, was close to expectation, $H_{ep} = 0.250$, and the mean F_{IS} value, a measure of the deviation of random mating within populations, was not significantly different from zero ($P < .05$). These results are consistent with the predominantly outcrossing mode of reproduction known for this species (Smith et al. 1985).

Allele frequencies were significantly different among populations for 9 of the 10 polymorphic loci ($P < .001$), the exception being the GDH1 locus which had very little genetic variation. The mean proportion of total genetic variation due to differences among regions, G_{reg}, and among populations within regions, G_{pop}, was 0.013 and 0.049, respectively, indicating that most of the genetic diversity occurs within populations (93.8%) (Table 2). Genetic identity among pairs of populations was relatively high, ranging from 0.952 to 0.989, with a mean of 0.972 (data not shown).

Temporal variation among populations sampled in the southeastern United States, G_{year}, accounted for 2.8% of the total gene diversity. G_{year} values were significant for all loci except GDH and LAP (Table 3). The mean proportion of total genetic variation due to differences among populations within a sampling year, $G_{pop(year)}$, was 0.067. Again, the majority of genetic diversity occurs within populations ($G_{wpop} = 0.905$).

Discussion

Genetic diversity in *T. pratense* is high in comparison to that of most plant species. Mean genetic diversity (H_{es}) was 0.285, mean percent polymorphic loci (P_s) was 76.9, and mean number of alleles per polymorphic locus (AP_s) was 2.60. These same parameters, averaged over many plant species, are $H_{es} = 0.150$, $P_s = 50.5$, and $AP_s = 2.90$ (unpublished results based on data from Hamrick and Godt 1989). A similar pattern was observed at the population level. Mean genetic diversity within *T. pratense* populations (H_{ep}) was 0.250, mean percent polymorphic loci (P_p) was 68.3, and the mean number of alleles per polymorphic locus (AP_p) was 2.52. Averaged over many plant species these values are $H_{ep} = 0.113$, a $P_p = 34.2$, and an $AP_p = 2.55$ (Hamrick and Godt 1989).

The relatively high level of genetic variation in *T. pratense* may reflect this species' wide geographic distribution and obligate outcrossing mode of reproduction. Widely distributed plant species tend to maintain more variation than narrowly distributed species; and predominantly outcrossing species tend to have more genetic diversity overall and also tend to maintain more variation within their populations than species with higher proportions of self-pollination (Hamrick and Godt 1989; Hamrick et al. 1979). The level of genetic variation in *T. pratense* is high even when compared to other widely distributed and outcrossed species. Species with a wide geographic distribution have an H_{es} of 0.202 and H_{ep} of 0.159, a P_s of 58.9 and P_p of 43.0, and an AP_s of 3.2 and AP_p of 2.0. Predominantly outcrossing species have an H_{es} of 0.167 and H_{ep} of 0.124, a P_s of 50.1 and P_p of 35.9, and an AP_s of 3.0 and AP_p of 2.4. The values for *T. pratense* are generally higher than each of these parameters. Because high levels of genetic diversity are often deliberately maintained in crop species (Hamrick and Godt 1989; Marshall 1989; Muona 1989; Weber et al. 1989), the cultivated status of *T. pratense* may have contributed to the high genetic variation observed.

Two observations suggest that the naturalized populations of red clover studied were founded by multiple individuals. First, genetic diversity values among populations of *T. pratense* are uniformly high. Second, genetic differentiation among populations of *T. pratense* within geographic regions and within years is low ($G_{pop} = 0.049$ and $G_{pop(year)} = 0.067$, respectively) compared to values averaged over many plant species and to geographically widely dispersed and outcrossing species. By contrast, G_{pop} values averaged over many plant species, geographically widely dispersed species, and outcrossing animal-pollinated species are 0.224, 0.210, and 0.197, respectively (Hamrick and Godt 1989). Because of the random losses of alleles due to sampling, both low genetic diversity and high population differentiation was predicted. The red clover data do not fit the predictions.

Nei et al. (1975), however, showed that the reduction in average heterozygosity per locus depends not only on the size of a founder population, but also on the subsequent growth rate of the population. If population growth is rapid, reduction in heterozygosity is small, even when the founding population is small. Furthermore, because the subgroups (i.e., 1992, 1993, southern, and northern populations) display similar genetic diversity and population structure, it appears that interpopulation migration has effectively distributed the genetic diversity of the species among populations. Therefore, under the normal conditions of rapid population

growth and high gene flow among populations of *T. pratense,* any founder effect present when the naturalized populations were first established would be ephemeral.

Estimates of gene flow are high among populations. Based on Crow and Aoki's (1984) gene flow equation, an N_m value of 4.75 was estimated among regions. Among populations within regions $N_m = 3.36$ and between years $N_m = 2.17$. Chi-square analysis indicates that the overall G_{year} value, 0.028, is significantly different from zero. Still the genetic identity between populations between years is relatively high (~0.96). These year-to-year differences are probably due to the high turnover of clover plants within naturalized populations (Vescio LV and Hamrick JL, unpublished data).

It appears that the outcrossing nature of *T. pratense,* coupled with strong flying pollinators (e.g., *Bombus* spp.) and dispersal by mammals, birds, farm machinery, and other vehicles has resulted in a high level of gene exchange via pollen between cultivated and naturalized populations of red clover. This conclusion is consistent with indirect estimates of gene flow for plant species with breeding system characteristics similar to that of red clover (Hamrick et al. 1995). Our results are also consistent with genetic marker-based studies of other crop species or their related congeners (e.g., Ellstrand and Hoffman 1985; Kirkpatrick and Wilson 1988). The potential for long-distance seed and pollen movement has maintained high levels of genetic diversity within populations and reduced genetic diversity among populations.

From the USDA/ARS/HCRU, 3420 Orchard Ave., Corvallis, OR 97330 (Hagen) and the Departments of Botany and Genetics, University of Georgia, Athens, Georgia (Hamrick). The work described in this article was supported by an NIH genetics training grant, a University of Georgia Department of Botany grant, and a Sigma Xi research grant awarded to M.J.H.

The Journal of Heredity 1998:89(2)

References

Barrett SCH and Shore JS, 1989. Isozyme variation in colonizing plant species. In: Isozymes in plant biology (Soltis DE and Soltis PS, eds.). Portland, Oregon: Dioscorides Press; 106–127.

Blixt S, 1988. Genetic resources at work: practical examples of the importance of genebank work regarding agriculture, horticulture, and forestry. Hereditas 108: 271–273.

Chakraborty R and Nei M, 1977. Bottleneck effects on average heterozygosity and genetic distance with the stepwise mutation model. Evolution 31:347–356.

Cheliak WM and Pitel JA, 1984. Techniques for starch gel electrophoresis of enzymes from forest tree species. Petawawa National Forestry Institute, Information Report P1-X-42. Chalk River, Ontario, Canada: Canadian Forestry Service, Agriculture.

Crow JF and Aoki K, 1984. Group selection for a polygenic behavioral trait: estimating the degree of population subdivision. Proc Natl Acad Sci USA 81:6073–6077.

Ellstrand NC and Hoffman CA, 1985. Inter-population gene flow by pollen in wild radish, *Raphanus sativus.* Am Nat 126:606–616.

Fergus EN and Hollowell EA, 1960. Red clover. Adv Agron 12:365–436.

Gottlieb LD, 1981. Electrophoretic evidence and plant population. Prog Phytochem 7:1–45.

Hamrick JL and Godt MJW, 1989. Allozyme diversity in plant species. In: Plant population genetics, breeding, and genetic resources (Brown AHD, Clegg MT, Kahler AL, and Weir BS, eds). Sunderland, Massachusetts: Sinauer; 43–63.

Hamrick JL, Godt MJW, and Sherman-Broyles SL, 1995. Gene flow among plant populations: evidence from genetic markers. In: Experimental and molecular approaches to plant biosystematics (Hoch PC and Stephenson AG, eds). St. Louis: Missouri Botanical Garden Press; 215–232.

Hamrick JL, Linhart YB, Mitton JB, 1979. Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. Annu Rev Ecol Syst 10:173–200.

Harris H and Hopkinson DA, 1976. Handbook of enzyme electrophoresis in human genetics. New York: Oxford American Elsevier.

Hedrick PW, 1985. Genetics of populations. Boston: Jones and Bartlett.

Kirkpatrick KJ and Wilson HD, 1988. Interspecific gene flow in *Cucurbita foetidissima* (Cucurbitaceae). Am J Bot 75:519–527.

Li CC and Horvitz DG, 1953. Some methods of estimating the inbreeding coefficient. Am J Hum Genet 5:107–117.

Marshall DR, 1989. Crop genetic resources: current and emerging issues. In: Plant population genetics, breeding, and genetic resources (Brown AHD, Clegg MT, Kahler AL, and Weir BS, eds). Sunderland, Massachusetts: Sinauer; 367–388.

Marshall DR and Brown AHD, 1975. Optimum sampling strategies in genetic conservation. In: Crop genetic resources for today and tomorrow (Frankel OH and Hawkes JG, eds). Cambridge: Cambridge University Press; 53–80.

Merkenschlager F, 1934. Migration and distribution of red clover in Europe. Herb Rev 2:88–92.

Mitton JB, Linhart YB, Sturgeon KB, and Hamrick JL, 1979. Allozyme polymorphisms detected in mature needle tissue of ponderosa pine. J Hered 70:86–89.

Muona O, 1989. Population genetics in forest tree improvement. In: Plantpopulation genetics, breeding, and genetic resources (Brown AHD, Clegg MT, Kahler AL, and Weir BS eds). Sunderland, Massachusetts: Sinauer; 282–298.

Nei M, 1972. Genetic distance between populations. Am Nat 106:283–292.

Nei M, 1973. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323.

Nei M, 1977. F-statistics and analysis of gene diversity in subdivided populations. Ann Hum Genet 41:225–233.

Nei M, Maruyama T, and Chakraborty R, 1975. The bottleneck effect and genetic variability in populations. Evolution 29:1–10.

Nei M and Tajima F, 1981. Genetic drift and estimation of effective population sizes. Genetics 98:625–640.

Pieters AJ and Hollowell EA, 1937. Clover improvement. In: USDA Yearbook. Washington, D.C.: U.S. Government Printing Office; 1190–1214.

Smith RR, Taylor NL, and SR Bowley, 1985. Red clover. In: Clover science and technology (Taylor NL, ed). Madison, Wisconsin: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America.

Soltis DE, Haufler CH, Darrow DC, and Gastony GJ, 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. Am Fern J 73:9–27.

Weber WE, Qualset CO, and Wricke G, 1989. Selection strategies for the improvement of autogamous species. In: Plant population genetics, breeding, and genetic resources (Brown AHD, Clegg MT, Kahler AL, and Weir BS, eds). Sunderland, Massachusetts: Sinauer; 299–316.

Wendel LJF and Weeden NF, 1989. Visualization and interpretation in plant isozymes. In: Isozymes in plant biology, vol. 4 (Soltis DE and Soltis PS, eds). Portland, Oregon: Dioscorides Press; 5–45.

Workman PL and Niswander JD, 1970. Population studies on southwestern Indian tribes. II. Local genetic differentiation in the Papago. Am J Hum Genet 22:24–49.

Wright S, 1922. Coefficients of inbreeding and relationship. Am Nat 56:330–338.

Received November 20, 1995
Accepted May 5, 1997

Corresponding Editor: Norman F. Weeden

Assignment of the *Fr3* Locus to Soybean Linkage Group 9

R. G. Palmer and X. F. Chen

A study was conducted to determine the linkage relationship between the loci *Fr3* (root fluorescence) and *Ap* (acid phosphatase) in the soybean [*Glycine max* (L.) Merr.], as well as to ascertain the linkage relationship of *Fr3* with two other loci in the soybean classical linkage group 9. Exact plant-to-plant reciprocal crosses were made between near-isogenic line Hark-*Fr3* and near-isogenic line Clark-*ti* (Kunitz trypsin inhibitor null) and Hark-*Fr3* with cultivar Norredo. F_2 segregation data, for the *Fr3-Ap* gene pair from reciprocal crosses of Hark-*Fr3* × Clark-*ti,* gave a recombination value of 4.51 ± 0.55 with 1464 F_2 plants. However, reciprocal crosses of Hark-*Fr3* × Norredo gave a recombination value of 10.25 ± 0.83 with 1344 F_2 plants. Finally, the gene order of *Fr3–Ap–Ti–Lap1* (leucine aminopeptidase) was established for classical linkage group 9 in soybean.

Soybean [*Glycine max* (L.) Merr.] linkage maps composed of morphological, protein, RFLP, RAPD, and SSR markers have been published (Akkaya et al. 1995; Palmer and Hedges 1993; Rafalski and Tingey 1993; Shoemaker and Olson 1993; Shoemaker et al. 1995; Shoemaker and Specht 1995). The cultivated soybean is considered as a diploidized tetraploid species ($2n = 2x = 40$ chromosomes) (Hymowitz

and Singh 1987). Thus 20 linkage groups are expected, but the present classical genetic map lists only 19 linkage groups (Palmer and Hedges 1993).

Root fluorescent soybean mutants (a phenomenon in which roots fluoresce when irradiated with ultraviolet light) have been useful in characterizing germplasm diversity (Delannay and Palmer 1982), in tissue culture research (Roth et al. 1982), and in genetic linkage studies (Devine et al. 1993; Griffin et al. 1989). Genetic analyses of these mutants have revealed at least four single recessive genes—*fr1, fr2, fr4,* and *fr5*—that condition the nonfluorescence phenotype. However, the single dominant gene, *Fr3,* also conditions nonfluorescence root phenotype in soybean (Delannay and Palmer 1982; Sawada and Palmer 1987). The *Fr1* locus has been located on the classical genetic map to linkage group 12 (Griffin et al. 1989) and the *Fr2* locus has been located on the soybean molecular map (Devine et al. 1993). The linkage relationships with the other three nonfluorescent loci are unknown.

Near-isogenic lines have been developed in soybean via the backcross breeding method. Near-isogenic lines have been used in mapping classical and molecular markers and in the integration of the classical and molecular linkage maps (Muehlbauer et al. 1988, 1989). Backcrosses of nonfluorescent soybean root mutants (Palmer et al. 1983; Palmer 1994) were done to produce near-isogenic lines that were used in attempts to determine the biochemical pathway. Root fluorescence was not correlated with abundance per se of fluorescent isoflavonoids in roots (Grady et al. 1995).

During the backcrossing it was noticed that the same variant alleles of *Fr3* (root fluorescence) and *Ap* (acid phosphatase) tended to be inherited together, which suggested that the *Fr3* and *Ap* loci might be linked. Soybean linkage group 9 is represented by four protein markers, acid phosphatase, Kunitz trypsin inhibitor, leucine aminopeptidase, and phosphogluconate dehydrogenase. Although some of the same lines were used by Yu and Kiang (1990) and Hildebrand et al. (1980) to determine recombination values between acid phosphatase and Kunitz trypsin inhibitor loci, recombination values varied from 6.1 ± 0.3 (Yu and Kiang 1990) to 16.2 ± 1.5 (Hildebrand et al. 1980). The various recombination values may be due to the fact that plants were grown in different environmental conditions, that distinct electrophoresis procedures were employed to identify isozyme banding patterns, or that different seed sources were used.

The objective of this study was to verify the linkage relationship and the gene order of the *Fr3* locus with other loci in the soybean classical linkage group 9.

Materials and Methods

The genetic stocks used in this experiment and their genotypes were Clark-*ti* (*Ap-b fr3 Lap-b ti*) × Hark-*Fr3* (*Ap-c Fr3 Lap-b Ti-a*) and Norredo (*Ap-b fr3 Lap-a Ti-a*) × Hark-*Fr3* (*Ap-c Fr3 Lap-b Ti-a*). The Norredo cultivar and the near-isogenic line Clark-*ti* (L83–693) were obtained from Dr. R. L. Nelson, USDA, Urbana, Illinois. The near-isogenic line Hark-*Fr3* was developed by the USDA-ARS-FCR at Ames, Iowa (Palmer 1994). Seed of parents and F_2 plants were characterized by using starch-gel electrophoresis for acid phosphatase (*Ap* locus; EC 3.1.3.2) (Cardy and Beversdorf 1984) and by polyacrylamide-gel electrophoresis for Kunitz trypsin inhibitor (*Ti* locus) (Hildebrand et al. 1980) and for leucine aminopeptidase (*Lap1* locus; EC 3.4.1.1) (Kiang et al. 1985). The 4-day-old seedlings were observed in the dark under ultraviolet light and the root phenotypes were nonfluorescent (*Fr3*−) or fluorescent (*fr3fr3*) (Delannay and Palmer 1982). The three lines were planted at the Bruner Farm near Ames, Iowa. One plant was harvested from each of the three lines. Seed were planted at the University of Puerto Rico–Iowa State University Soybean Nursery near Isabela, Puerto Rico. Exact plant-to-plant cross-pollinations with reciprocal cross-pollinations were made for all possible genetic combinations. F_1 seed were harvested individually and planted at the Soybean Nursery near Isabela to generate F_2 seed for linkage determinations.

Recombination values and chi-squares were analyzed for the *Ap* (1:2:1), *Fr3*(3:1), *Ti* (3:1), and *Lap1* (1:2:1) loci by using the computer program Linkage-1, version 90 (Suiter et al. 1983), which uses the maximum likelihood method (Allard 1956).

Results and Discussion

Recombination is one of the mechanisms for generating genetic variability, whereas the lack of recombination conserves genetic variability. Plant breeders depend upon recombinations to create variability from which superior genotypes can be selected. Recombination values for various loci pairs in soybean generally have been consistent and a consensus classical linkage map has been constructed (Palmer and Hedges 1993).

Soybean genetic linkage group 9 has been extensively studied. The parental lines used in our experiment had been maintained by self-pollination for a number of years. One plant from each parental line was selected, and its progenies were used for exact plant-to-plant cross-pollinations (and reciprocals). Stutte et al. (1979) reported that seed from a single homozygous parent minimized variability in physiological studies. Thus pedigree information and seed source are crucial for comparing recombination values.

For the Clark-*ti* × Hark-*Fr3* crosses and their reciprocal crosses, the recombination values for the *Ti-Fr3* (Table 1), *Ap-Fr3* (Table 1), and *Ap-Ti* (Table 2) gene pairs were homogeneous. There was no significant variation among the three loci pairs in recombination values. For the Norredo × Hark-*Fr3* crosses and their reciprocal crosses, the recombination values for the *Ap-Fr3* (Table 1), *Lap1-Fr3* (Table 1), and *Ap-Lap1* (Table 2) gene pairs were homogeneous.

Figure 1 shows both the consensus linkage group 9 recombination values (Palmer and Hedges 1993) and the data from our experiments. The gene order is *Pgd2–Lap1–Ti–Ap–Fr3.*

The recombination values for the *Ap-Fr3* loci (Table 1) from reciprocal crosses of Clark-*ti* × Hark-*Fr3* compared with reciprocal crosses of Norredo × Hark-*Fr3* are not consistent between the two cross combinations. The nonfluorescent root phenotype (*Fr3*−) is easily classified by

Table 1. Tests of linkage between *Fr3* and three loci in soybean

Locus tested	Cross	*N*	χ^2	Recombination values
Ap	Clark-*ti* × Hark-*Fr3*	1,061	826.28	4.47 ± 0.65
Ap	Hark-*Fr3* × Clark-*ti*	403	309.62	4.63 ± 1.07
Ap	Norredo × Hark-*Fr3*	922	496.46	10.20 ± 1.04
Ap	Hark-*Fr3* × Norredo	422	232.32	10.36 ± 1.55
Ti	Clark-*ti* × Hark-*Fr3*	855	425.51	11.83 ± 3.36
Ti	Hark-*Fr3* × Clark-*ti*	154	77.00	12.00 ± 7.92
Lap1	Norredo × Hark-*Fr3*	922	114.26	29.53 ± 1.74
Lap1	Hark-*Fr3* × Norredo	422	57.68	28.69 ± 2.54

Table 2. Tests of linkage between *Ap* and two loci in soybean

Locus tested	Cross	*N*	χ^2	Recombination values
Ti	Clark-*ti* × Hark-*Fr3*	855	480.23	9.93 ± 1.07
Ti	Hark-*Fr3* × Clark-*ti*	154	89.96	9.51 ± 2.46
Lap1	Norredo × Hark-*Fr3*	1,043	455.06	21.80 ± 1.05
Lap1	Hark-*Fr3* × Norredo	581	324.07	19.76 ± 1.30

See Table 1 for recombination values between *Ap* and *Fr3.*

using an ultraviolet light. Acid phosphatase is easily classified by using starch-gel electrophoresis. Both leucine aminopeptidase and Kunitz trypsin inhibitor loci were classified by using polyacrylamide-gel electrophoresis. If the banding patterns were not interpretable, those samples were rerun. All traits classified fit their respective 3:1 or 1:2:1 ratio.

As mentioned in the introduction, recombination values between acid phosphatase and the Kunitz trypsin inhibitor loci varied between 6.1 ± 0.3 (Yu and Kiang 1990) and 16.2 ± 1.5 (Hildebrand et al. 1980). We used self-pollinated progeny from single plants in the cross-pollinations in an attempt to reduce variability in recombination values.

The Clark-*ti* parent is a backcross-6 selection of Clark-*i* (mutation) × USDA PI 157.440 (source of the *ti* allele). PI 157.440 was introduced from South Korea in 1947. Both genotypes are *G. max* species. Hark-*Fr3* parent is a backcross-6 selection of Hark × USDA PI 424.078 (source of the *Fr3* allele). PI 424.078 originally was listed as the wild annual species *G. soja* Sieb. & Zucc. but now is considered *G. max.* The pedigree of Norredo is unknown but is probably from cultivar Laredo (PI 40.658) introduced from China in 1915 (Bernard et al. 1987).

Disparity in estimation of recombination has been reported for linkage group 1, with a recombination value of 4.6 ± 0.9 for the *Aco3* (aconitase) locus with the *Sp1* (β-amylase) locus in the interspecific cross of *G. max* × *G. soja* (Griffin and Palmer 1987). For the same two loci in the intraspecific cross of *G. max* × *G. max,* the recombination value was 30.6 ± 3.0 (Griffin and Palmer 1987). The *G. soja* genotype (PI 342.622A) is homozygous for a chromosomal translocation (Palmer et al. 1987), but linkage with the *Aco3* and *Sp1* loci was not tested. The translocation heterozygosity could have resulted in plants with reduced recombination because of less effective meiotic chromosome pairing.

Pfeiffer and Vogt (1990) found variation in recombination frequencies for three different chromosome regions in soybean. However, a genotype that generally enhanced or repressed recombination was not identified. Recombination rates of soybean varieties from four different release periods were evaluated for their recombination frequencies at three locus pairs. Recombination frequencies were significantly different among varieties within a release period but did not differ among release periods (Pfeiffer 1993).

Webb et al. (1995) reported segregation distortion in molecular mapping of soybean cyst nematode resistance. They attributed the distortion to differential survival of certain genotypes, pleiotropy, linkage of other genes to the soybean cyst nematode-resistance QTL, or a combination of both situations.

The gene order and recombination values for the *Fr3, Ap, Ti,* and *Lap1* loci were established. Acid phosphatase is the most distal marker in linkage group a2 of the USDA-ISU molecular map (Shoemaker and Specht 1995). These additional markers from the classical map can now be placed on the molecular map linkage group a2.

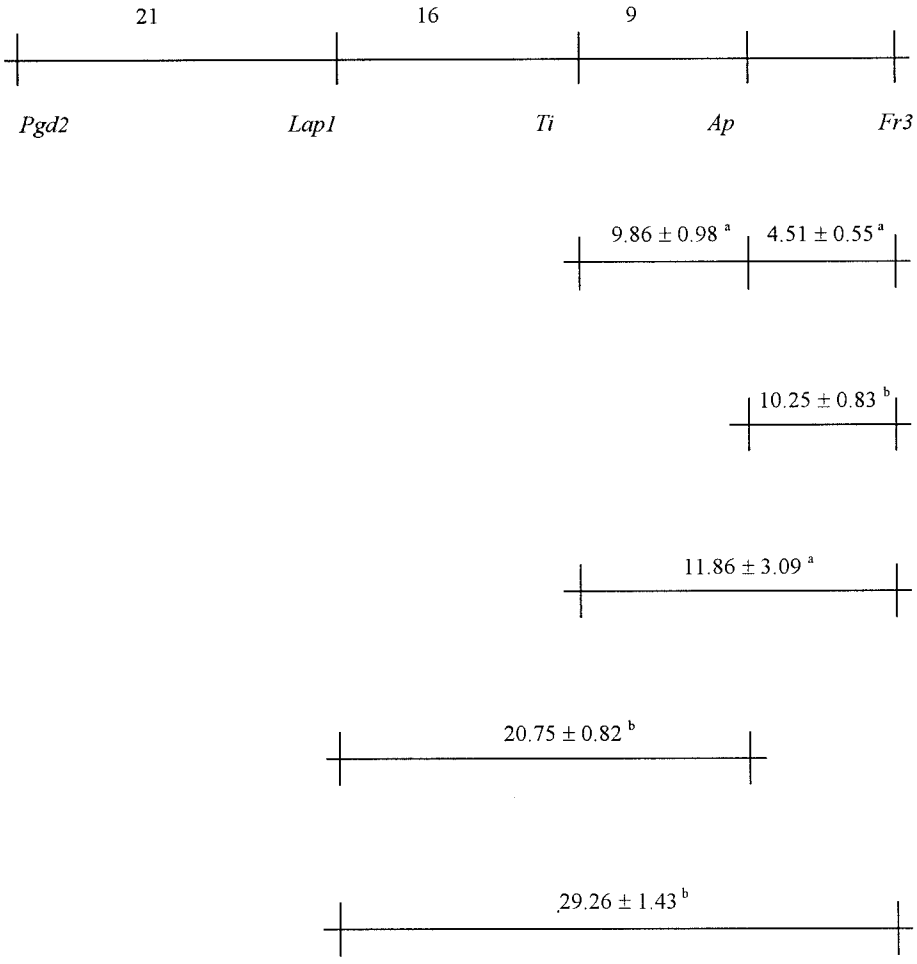

Figure 1. Soybean linkage group 9. The recombination values without standard errors are from the consensus linkage map (Palmer and Hedges 1993). The recombination values with standard errors are from data presented in this article. **(a)** F_2 data from Clark-*ti* × Hark-*Fr3* and reciprocal crosses. **(b)** F_2 data from Norredo × Hark-*Fr3* and reciprocal crosses.

From the USDA-ARS-CICGR and Departments of Agronomy and Zoology/Genetics (Palmer) and the Department of Agronomy and Interdepartmental Genetics Program (Chen), Iowa State University, Ames, Iowa 50011-1010. This is a joint contribution: journal paper J-17070 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa (project 3352) and from the USDA, Agricultural Research Service, Corn Insects and Crop Genetics Research Unit. Names are necessary to report factual or available data; however, the USDA and Iowa State University neither guarantees nor warrants the standard of the product, and the use of the name by the USDA or Iowa State University implies no approval of the product to the exclusion of others that may also be suitable. Address correspondence to Dr. Palmer at the address above or e-mail: rpalmer@iastate.edu.

References

Akkaya MS, Shoemaker RC, Specht JE, Bhgowat AA, and Cregan PB, 1995. Integration of simple sequence repeat DNA markers into a soybean linkage map. Crop Sci 35: 1439–1445.

Allard RW, 1956. Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24:235–278.

Bernard RL, Juvik GA, and Nelson RL, 1987. USDA soybean germplasm collection inventory. Vol. 1. International Agricultural Publication. INTSOY Series no. 30. Urbana, Illinois: College of Agriculture, University of Illinois at Urbana-Champaign; 1–80.

Cardy BJ and Beversdorf WD, 1984. A procedure for the starch gel electrophoretic detection of isozymes of soybean [*Glycine max* (L.) Merr.]. Department of Crop Science Technical Bulletin 119/8401. Guelph, Ontario, Canada: University of Guelph.

Delannay X and Palmer RG, 1982. Four genes controlling root fluorescence in soybean. Crop Sci 22:278–281.

Devine TE, Weisemann JM, and Matthews BF, 1993. Linkage of the *Fr2* locus controlling soybean root fluorescence and four loci detected by RFLP markers. Theor Appl Genet 85:921–925.

Grady H, Palmer RG, and Imsande J, 1995. Isoflavonoids in root and hypocotyl of soybean seedlings [*Glycine max,* Fabaceae]. Am J Bot 82:964–968.

Griffin JD, Broich SL, Delannay X, and Palmer RG, 1989. The loci *Fr1* and *Ep* define soybean linkage group 12. Crop Sci 29:80–82.

Griffin JD and Palmer RG, 1987. Inheritance and linkage studies with five isozyme loci in soybean. Crop Sci 27: 885–892.

Hildebrand DF, Orf JH, and Hymowitz T, 1980. Inheritance of an acid phosphatase and its linkage with the Kunitz trypsin inhibitor in seed protein of soybeans. Crop Sci 20:83–85.

Hymowitz T and Singh RJ, 1987. Taxonomy and speciation. Agronomy 16:23–48.

Kiang YT, Gorman MP, and Chiang YC, 1985. Genetic and linkage analysis of a leucine aminopeptidase in wild and cultivated soybean. Crop Sci 25:319–321.

Muehlbauer GJ, Specht JE, Staswick PE, Graef GL, and Thomas-Compton MA, 1989. Application of the near-isogenic line gene mapping technique to isozyme markers. Crop Sci 29:1548–1553.

Muehlbauer GJ, Specht JE, Thomas-Compton MA, Staswick PE, and Bernard RL, 1988. Near-isogenic lines: a potential resource in the integration of conventional and molecular marker linkage maps. Crop Sci 28:729–735.

Palmer RG, 1994. Development of near-isogenic lines for root fluorescence. Soybean Genet Newslett 21:219–227.

Palmer RG, Broich SL, and Delannay X, 1983. *Fr1* and *fr1* near-isogenic lines. Soybean Genet Newslett 10:41–43.

Palmer RG and Hedges BR, 1993. Linkage map of soybean [*Glycine max* L. Merr.] (2n=40). In: Genetic maps (O'Brien SJ, ed). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 6.136–6.148.

Palmer RG, Newhouse KE, Graybosch RA, and Delannay X, 1987. Chromosome structure of the wild soybean. J Hered 78: 243–247.

Pfeiffer TW, 1993. Recombination rates of soybean varieties from different periods of introduction and release. Theor Appl Genet 86:557–561.

Pfeiffer TW and Vogt SD, 1990. Variability for recombination frequencies in the AP12 soybean population. Crop Sci 30:545–549.

Rafalski A and Tingey S, 1993. RFLP map of soybean [*Glycine max*] 2n = 40. In: Genetic maps (O'Brien SJ, ed). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 6.149–6.156.

Roth EJ, Weber G, and Lark KG, 1982. Use of isopropyl-N (3-chlorophenyl) carbamate (CIPC) to produce partial haploids from suspension cultures of soybean [*Glycine max*]. Plant Cell Rep 1:205–208.

Sawada S and Palmer RG, 1987. Genetic analyses of nonfluorescent root mutants induced by mutagenesis in soybean. Crop Sci 27:62–65.

Shoemaker RC and Olson TC, 1993. Molecular linkage map of soybean [*Glycine max* (L.) Merr.] (2n = 40). In: Genetic maps (O'Brien SJ, ed). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 6.131–6.138.

Shoemaker RC, Polzin KM, Lorenzen LL, and Specht JE, 1995. Molecular genetic mapping of soybean. In: Soybean: genetics, molecular biology and biotechnology (Verma DPS and Shoemaker RC, eds). Wallingford, England: CAB International Publisher; 37–56.

Shoemaker RC and Specht JE, 1995. Integration of the soybean molecular and classical genetic linkage groups. Crop Sci 35:436–446.

Stutte CA, Blem AR, and Weiland RT, 1979. Single plant seed sources for uniform soybean physiology tests. Crop Sci 19:324–326.

Suiter KA, Wendel JF, and Case JS. 1983. LINKAGE-1: a PASCAL computer program for the detection and analysis of genetic linkage. J Hered 74:203–204.

Yu H and Kiang YT, 1990. Re-examination of the recombination frequency between acid phosphatase and the Kunitz trypsin inhibitor loci in soybean. Soybean Genet Newslett 17:106–108.

Webb DM, Baltazar BM, Rao-Arelli AP, Schupp J, Clayton K, Keim P, and Beavis WD, 1995. Genetic mapping of soybean cyst nematode race-3 resistance loci in the soybean PI 437.654. Theor Appl Genet 91:574–581.

Received December 20, 1996
Accepted July 8, 1997

Corresponding Editor: Gary E. Hart

The Use of Confocal Microscopy to Study Chromosome Banding in *Ornithogalum*

R. J. Griesbach

Confocal laser microscopy was used to examine the G-banding pattern of mitotic chromosomes of *Ornithogalum* (Star of Bethlehem) from late prophase. As compared with conventional microscopy, the higher resolution of confocal microscopy allowed for an increase in the contrast between individual bands. The banding pattern of *Ornithogalum dubium* and *O. thyrsoides* was compared. Similarities in banding pattern suggest that the major difference between these two species involves the chromosome containing the nucleolus organizer region. If the NOR is assigned to chromosome 2, then the banding pattern in the region proximal to the centromere of this chromosome in *O. thyrsoides* corresponded to that of chromosome 5 in *O. dubium* and vice versa. During meiosis in the primary hybrid, chromosome 2 of *O. thyrsoides* paired with chromosome 5 of *O. dubium.*

Ornithogalum is an important cut flower and potted plant crop. New hybrids are being created that combine the best characteristics of several species. Two of the most widely used species in breeding are *Ornithogalum thyrsoides* Jacq. and *O. dubium* Hout. Both of these species are classified within the group *Aspasiae* of the subgenus *Aspasia* (Obermeyer 1978). The group *Aspasiae* is characterized by perianth segments that have no dark midrib and bracts that are large, petaloid, and have smooth margins.

There are many biotypes of *O. thyrsoides* ($2n = 12$). Even though these biotypes differ in flower morphology, no distinctions can be made on the basis of karyological characteristics (Roos and Pienaar 1966). *O. thyrsoides* has six different chromosomes that can be separated into three distinct groups based upon total length. Chromosomes 2, 3, and 5 all have the same length; chromosome 1 is slightly larger (5% longer), chromosome 4 is slightly smaller (10% shorter), and chromosome 6 is very small (50% shorter). Chromosome 2 differs from the rest because of its secondary constriction containing the nucleolus organizer region.

There are also many biotypes of *O. dubium* ($2n = 12$). Many of these biotypes have been given species status in the past—*O. alticola, aureum, flavissimum, miniatum,* and *leipoldtii* (Obermeyer 1978). Like the *O. thyrsoides* biotypes, none of the *O. dubium* biotypes can be distinguished based upon karyotype (Pienaar 1963). The karyotype of *O. dubium* is indistinguishable from that of *O. thyrsoides.* In both species, the nucleolus organizer region defines chromosome 2.

Depending upon the biotype used, the interspecific hybrid between *O. thyrsoides* and *O. dubium* can be either partially fertile (Griesbach et. al. 1993) or completely sterile (Niekerk and Pienaar 1968). Even though the karyotypes of the two species superficially resemble each other, there must be major differences in chromosome organization.

During prophase, the chromosomes usually have a banded appearance. However, at this stage of the cell cycle it is nearly impossible to distinguish the individual chromosomes due to their extreme length and narrow width. As the chromo-

somes become more condensed, the bands become more obscured. Occasionally, late prophase chromosomes can be found that are short and banded. The banding pattern of these chromosomes is usually weak, with little contrast between the bands.

This article describes the use of a chromosome banding procedure with confocal microscopy to increase the frequency of late prophase chromosomes showing bands as well as the contrast between the bands. This technique was used to compare the chromosome organization of *O. dubium* and *O. thyrsoides*.

Material and Methods

A single clone each of *O. dubium, O. thyrsoides,* and their interspecific hybrid were compared. The *O. dubium* clone was collected from Plettenberg Bay, South Africa, while the *O. thyrsoides* clone was collected from the Clanwilliam area of South Africa. These clones are maintained at the U.S. National Arboretum.

Actively growing root tips were harvested, pretreated for 3 h at room temperature in 0.05% aqueous colchicine, and fixed for 16 h at room temperature in 75% methanol and 25% propionic acid. The root tips were then stored in fixative at −20°C.

Fixed root tips were softened for 10 min at room temperature in a saturated solution of ammonium oxalate containing 7% hydrogen peroxide (Pienaar 1955). The root tips were then incubated for an additional 10 min in 45% aqueous propionic acid. A single root tip was placed in a drop of 45% propionic acid on a slide coated with formvar resin (EMS, Fort Washington, Pennsylvania). Slides were coated by dipping in 0.3% formvar in ethylene dichloride. The root tip was gently heated, teased apart, and diced with a sharp scalpel. Once the root tip was disassociated into individual cells, the individual cells were squashed beneath a coverslip. The slide was submerged in liquid nitrogen for 30 s. While frozen, the coverslip was removed and the slide plunged into 70% ethanol. The slides were removed after 1 min, flamed, air dried, and stored desiccated at −20°C.

Chromosomes were banded as previously described (Schweizer 1973). Slides were incubated at 65°C for 16 h in 2× SSC (0.3 M NaCl and 0.03 M trisodium citrate, pH 7.0), followed by three washes in cold water, and staining in Giemsa stock solution (Sigma) diluted 50× in phosphate buffer (12 mM Na_2HPO_4 and 4.8 mM NaH_2PO_4, pH 7.0). Bands were visible after 3 to 6 h of staining.

Meiotic chromosomes were not banded and were examined using acid carmine. Flower buds were fixed at −20°C for at least 48 h in 75% methanol and 25% propionic acid. The anthers were then extracted, stained using Snow's procedure (Snow 1955), and squashed in 45% propionic acid.

A confocal laser microscope (Zeiss LSM 410 inverted Laser Scan Microscope) was used. The chromosomes were observed with a Plan-Neofluar 100×/1.3 oil lens (Zeiss) under differential interference contrast (DIC) using the 633 nm band of a helium-neon laser. Contrast between the bands was maximized by setting the pinhole at 12 μm and adjusting both the contrast and brightness. The chromosome preparations were optically sectioned, storing on optical disk each section as the line average of 16 images. Each section was 0.5 μm thick. The stored images were examined and the image with the most distinct banding pattern was selected for image analysis.

The KS 300 Imaging System (Kontron Elektronik) was used to enhance the banding pattern. Threshold analysis was performed using the binary option to set all gray values within a specified range to white. The remaining gray values were set to black. Different values were used to optimize the banding pattern of individual chromosomes.

Results

Microscopy

The standard dry ice technique for removing the coverslip on squash preparations of mitotic cells was not successful (Schweizer 1973). One or two chromosomes were always missing from a mitotic spread. In order to maintain the spreads intact, two modifications were made. First, the slides were coated with formvar. The standard albumen-coated slides did not always retain the complete chromosome complement. Second, the slides were briefly flamed after removal from the 70% ethanol wash. Liquid nitrogen was used to freeze the slides because it was much easier to use than dry ice.

Although the banding pattern could be observed on metaphase chromosomes, it was much easier to distinguish differences in the banding patterns on late prophase chromosomes. The concentration of colchicine and length of treatment was critical for banding. Colchicine treatment for longer than 4 h caused the chromosomes to be too condensed to reveal bands. In addition, concentrations higher than 0.05% also caused excessive condensation.

Incubation in 2× SSC for 16 to 24 h was required for banding. Chromosomes that were incubated for less than 16 h did not band, while chromosomes that were incubated for more than 24 h did not stain at all.

Several hundred root tips and several thousand mitotic spreads were examined for each species. An optical section of a mitotic spread for each species is shown in Figure 1. A comparison of the individual chromosomes from each species, a typical threshold analysis, and their idiograms are presented in Figure 2. Several hundred threshold analyses were performed to gather the data for the idiograms.

Cytology

In this study a G-banding procedure was used to compare the chromosomes of *O. dubium* and *O. thyrsoides*. Of the six different homologous chromosomes, chromosomes 3 and 4 had an identical banding pattern in both *O. dubium* and *O. thyrsoides* (Figures 1 and 2). Chromosomes 1 and 6 were similar in the two species, differing in only a single band. Chromosomes 2 and 5 were the most divergent.

In both species, the end of the long arm of chromosome 2 contained the secondary constriction or the nucleolus organizer region (Figure 2C). Chromosome 2 has been defined by this region and not by the centromere region (Roos and Pienaar 1966). In the region proximal to the centromere of chromosome 2, *O. thyrsoides* contained one band on the short arm and two bands on the long arm; while *O. dubium* contained one band on each of the arms (Figure 2C). In the nucleolus organizer region of chromosome 2, *O. thyrsoides* contained four bands, two on each side of the secondary constriction. *O. dubium* contained three bands, one terminally located and two centrally located.

The banding pattern of chromosome 5 of *O. thyrsoides* and *O. dubium* was very dissimilar, with few if any corresponding bands (Figure 2C). However, the banding pattern within the centromere regions of chromosome 5 of *O. dubium* corresponded with that for chromosome 2 of *O. thyrsoides* and vice versa.

Meiotic pairing in the primary hybrid between *O. thyrsoides* and *O. dubium* was examined. Six bivalents were observed, four of which had chromatids of equal

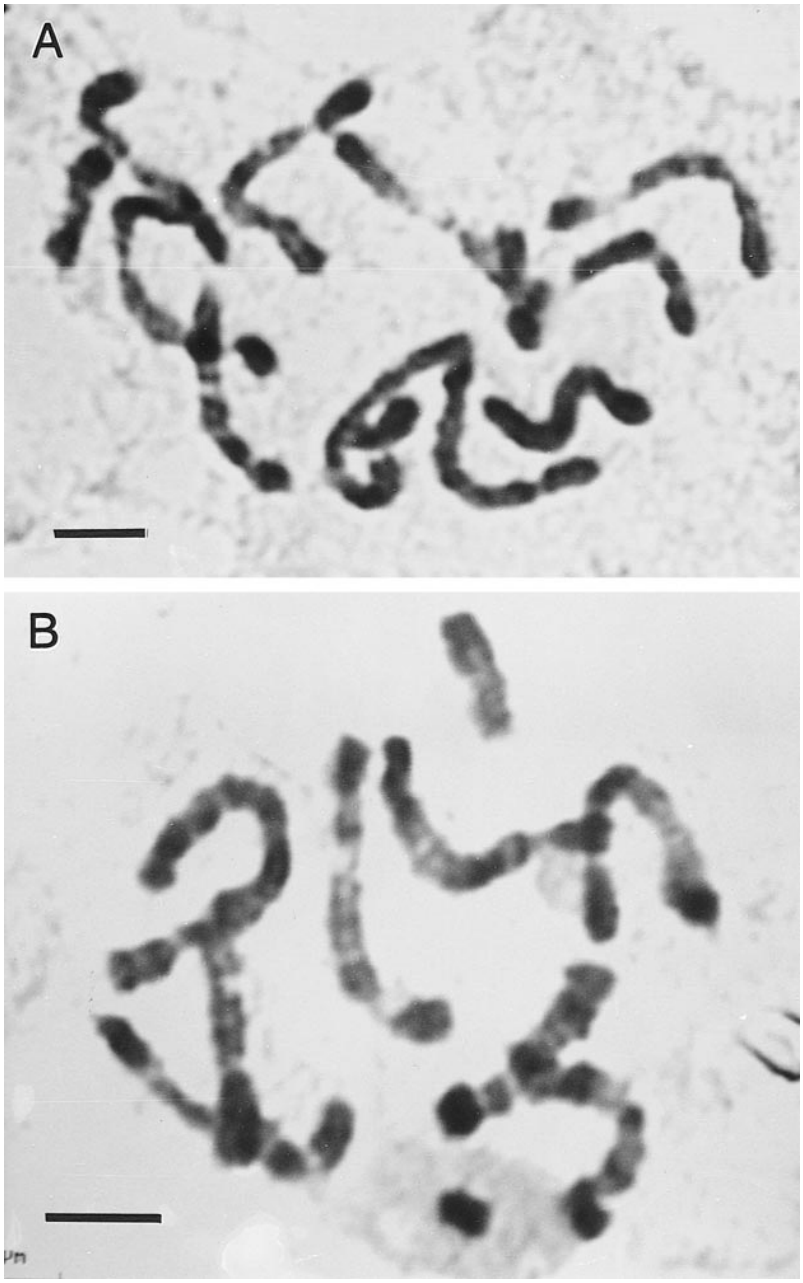

Figure 1. Banded mitotic spread from **(A)** *O. dubium* and **(B)** *O. thyrsoides.* Chromosomes were banded using the Giemsa staining procedure described in the text.

size (Figure 3A). The two nucleolus organizer regions were located on two unequal bivalents. Only one of the chromatids in the bivalent contained a secondary constriction. Both of the unequal bivalents were observed attached to the nucleolus (Figure 3B). During meiosis it appeared that chromosome 2 of *O. thyrsoides* paired with chromosome 5 of *O. dubium* and vice versa.

Discussion

The nuclear genomes of most eukaryotes are a mosaic of isochores of long DNA segments greater than 200 kb that are homogeneous in base composition above a 3 kb level (Bernardi 1995). Most genes are embedded in isochores that are within 1–2% of their GC content. Nearly all the genes in maize were associated with GC-rich isochores that comprised 10–20% of the genome (Bernardi 1995).

Chromosomes that have been denatured in alkali, reannealed in saline-citrate at high temperature, and stained in Giemsa have a banded appearance (Hsu and Arrighi 1971). The bands differentiated by this treatment have been termed C bands. The C-banding procedure removes about 60% of the chromosomal DNA preferentially from the gene-rich isochores (Cummings et al. 1973).

Elimination of the alkali treatment in the C-banding technique produces chromosomes that are also banded (Sumner et al. 1971). The bands differentiated by this treatment have been termed G bands. G-banding patterns of chromosomes from most species correspond exactly with the banding pattern observed on meiotic chromosomes (Cummings 1978). G bands are made up of AT-rich scaffold DNA that is differentially folded and packaged (Saitoh and Laemmli 1994).

Plant chromosomes rarely show G banding. It has been suggested that the reason why plant chromosomes rarely show G bands is because of their condensed shape and contaminating cytoplasm in squash preparations (Greilhuber 1977). The procedure described in this article overcame these problems. First, the effect of shape was reduced by examining chromosomes from late prophase. Second, the effect of contaminating cytoplasm was reduced by first disassociating the root tip into individual cells before squashing. When individual cells were squashed, the cytoplasm became highly dispersed. Finally, the increased resolution of confocal microscopy made it possible to distinguish bands that were not clear using conventional microscopy. In addition, the ability of confocal microscopy to optically section a chromosome greatly increased the clarity of the banding pattern.

Stedje (1988) used a C-banding technique to study two races ($2n = 4$ or 6) of *O. tenuifolium* Delaroche. Based upon the banding pattern, she concluded that the $2n = 4$ race originated from the $2n = 6$ race by a Robertsonian translocation. Azzioui et al. (1990) also used a C-banding technique to study the relationships between several *Ornithogalum* species from Morocco—*O. algeriense, O. kochii, O. narbonense,* and *O. pyrenaicum.* However, nearly all of the bands were restricted to the region adjacent to the nucleolus organizer or centromere and were not very useful.

In this study G banding was used to study the differences between *O. dubium* and *O. thyrsoides.* The banding pattern for all but two of the chromosomes were similar for both species. Similarities in banding pattern suggested that the centromere region of chromosome 2 in *O. dubium* corresponded to the centromere region of chromosome 5 in *O. thyrsoides* and vice versa (Figure 2). Meiotic pairing data in

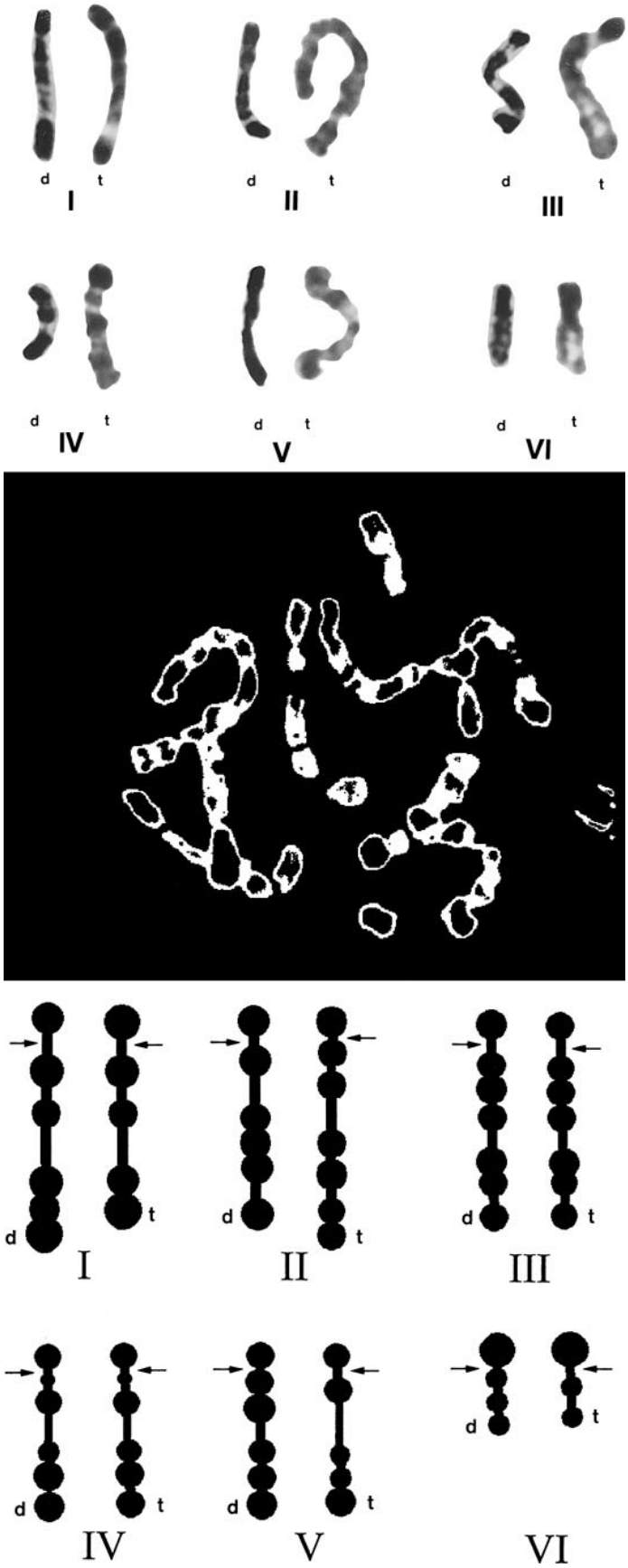

Figure 2. Individual banded chromosomes from *O. dubium* (d) and *O. thyrsoides* (t) (top), a single threshold analysis (middle), and their idiogram (bottom). The individual chromosomes were selected from different spreads based upon the overall uniformity in the clarity of their banding pattern. The arrows show the position of the centromere. Threshold analysis was performed on the chromosome spread shown in Figure 1B, setting the gray values between 65 and 125. The gray values ranged from 0 (black) to 255 (white).

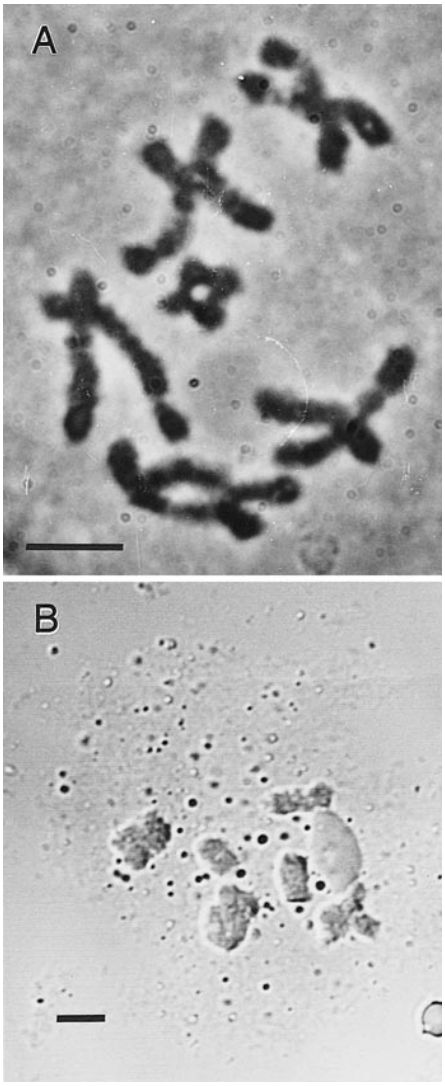

Figure 3. Meiotic diakinesis showing four bivalents with chromatids of equal length and two bivalents with chromatids of different lengths **(A)**. In the unequal bivalents, the nucleolus organizer region was located on only one of the chromatids in pair. The two unequal bivalents were attached to the same nucleolus **(B)**. Bar is 5 μm.

the primary hybrid between *O. thyrsoides* and *O. dubium* confirmed this suggestion. In the hybrid, the homologous chromosome 2's did not pair with each other during meiosis (Figure 3).

Stedje (1989) has suggested that the most primitive *Ornithogalum* species have numerous small chromosomes. By successive unequal translocations, the small chromosomes contribute to the creation of large chromosomes. In a like manner, *O. virens* evolved from *O. tenufolium.* In *O. dubium* and *O. thyrsoides,* there are five large chromosomes and a single small chromosome. This study suggests that the small progenitor chromosome containing the nucleolus organizer region was translocat-

ed to a different large chromosome in *O. dubium* than in *O. thyrsoides*.

The present method used to define chromosome 2 (i.e., presence of the nucleolus organizer region) is probably inappropriate (Roos and Pienaar 1966). The nucleolus organizer region should not be located on chromosome 2 for both species. The nucleolus organizer region should be located on chromosome 2 in one species and on chromosome 5 in the other species.

From Floral and Nursery Plant Research, U.S. National Arboretum, USDA, ARS, BARC- WEST, Bldg. 010A, Beltsville, MD 20705-2350. Mention of a commercial or propriety product does not constitute an endorsement by the USDA.

The Journal of Heredity 1998:89(2)

References

Azzioui O, Moret J, and Guern M, 1990. Giemsa C-banded karyotypes of some *Ornithogalum* L. species in North Africa. Cytologia 55:125–134.

Bernardi G, 1995. The human genome: organization and evolutionary history. Annu Rev Genet 29:445–476.

Cummings DE, 1978. Mechanisms of chromosome banding and implications for chromosome structure. Annu Rev Genet 12:25–46.

Cummings DE, Avelino E, Okada TA, and Wyandt HE, 1973. The mechanism of C- and G-banding of chromosomes. Exp Cell Res 77:469–493.

Greilhuber J, 1977. Why plant chromosomes do not show G-bands. Theor Appl Genet 50:121–124.

Griesbach RJ, Meyer F, and Koopowitz H, 1993. Creation of new flower colors in *Ornithogalum* via interspecific hybridization. J Am Soc Hort Sci 118:409–414.

Hsu TC and Arrighi FE, 1971. Distribution of constitutive heterochromatin in mammalian chromosomes. Chromosoma 34:243–253.

Niekerk HA and Pienaar RD, 1968. Sitogenetiese ondersoek van 'n aantal hibriede in die genus *Ornithogalum.* Proc 3rd Congr South Afric Genet Soc 3:45–51.

Obermeyer A, 1978. *Ornithogalum*: a revision of the southern African species. Bothalia 12:323–376.

Pienaar R, 1955. Combinations and variations of techniques for improved chromosome studies in the *Gramineaea.* J South Afric Bot 21:1–8.

Pienaar R, 1963. Sitogenetiese studies in die genus *Ornithogalum* L. I. Inleidende oorsig. J South Afric Bot 29: 111–130.

Roos T and Pienaar R, 1966. Cytogenetic studies in the genus *Ornithogalum* L. II. Karyotype analyses in some biotypes of *O. conicum* Jacq., *O. lacteum* Jacq. and *O. thyrsoides* Jacq. J South Afric Bot 32:211–227.

Saitoh Y and Laemmli UK, 1994. Metaphase chromosome structure: bands arise from a differential folding path of highly AT-rich scaffold. Cell 76:609–622.

Schweizer D, 1973. Differential staining of plant chromosomes with Giemsa. Chromosoma 40:307–320.

Snow R, 1955. Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 9–13.

Stedje B, 1988. A new low chromosome number for *Ornithogalum tenuifolium.* Plant Syst Evol 161:65–69.

Stedje B, 1989. Chromosome evolution within the *Ornithogalum tenuifolium* complex, with special emphasis on the evaluation of biomodal karyotypes. Plant Syst Evol 166:79–89.

Sumner AT, Evans HJ, and Buckland RA, 1971. A new technique for distinguishing between human chromosomes. Nature 232:31–32.

Received September 6, 1996
Accepted July 8, 1997

Corresponding Editor: Halina Skorupska

Tetraploid Nature of *Sorghum bicolor* (L.) Moench

M. I. Gómez, M. N. Islam-Faridi, M. S. Zwick, D. G. Czeschin Jr., G. E. Hart, R. A. Wing, D. M. Stelly, and H. J. Price

The advent of bacterial artificial chromosomes (BACs) and development of high quality methods for fluorescent in situ hybridization (FISH) to plant chromosomes offer revolutionary tools for plant genome analysis. We discovered a 45 kb sorghum BAC (22B2) that differentially hybridizes to centromere regions of 10 of the 20 chromosomes of sorghum (*Sorghum bicolor*). Moreover, hybridization of this BAC to plants trisomic ($2n = 20 + 1$) for the five available trisomes identified their respective subgenomic affiliations. Plants trisomic for chromosomes E, H, and I displayed 11 signals, indicating that these chromosomes are in the subgenome marked by the BAC-FISH signal, whereas plants trisomic for chromosomes D and G had only 10 signals and therefore belong to the subgenome not displaying the FISH signals. The results provide strong evidence that sorghum is at least of tetraploid origin, and that there are two subgenomes of five chromosomes each in the *S. bicolor* genome.

Sorghum (*Sorghum bicolor*) is widely regarded as a diploid, even though several lines of evidence have suggested that it might be tetraploid (Doggett 1988). The demonstration of polyploidy in sorghum would provide a stronger basis for evolutionary, taxonomic, and genetic research on sorghum and congeneric species. Moreover, ramifications might well extend to other genera of the Gramineae, including those that harbor our most important food and feed crops. Recent comparative analysis of linkage relationships among evolutionarily conserved loci have revealed close genomic relationships among graminous crop species including sorghum, maize, rice, wheat, and sugarcane (Dufour et al. 1996; Hulbert et al. 1990; Moore et al. 1995; Paterson et al. 1995; Pereira et al. 1994; Whitkus et al. 1992; and others).

Chromosome numbers and meiotic behavior inconclusively suggested that sorghum may be tetraploid (Celarier 1956). Whereas *S. bicolor,* a member of the section Eu-sorghum, has $2n = 20$ chromosomes, species in the section Para-sorghum have $2n = 10$ chromosomes. However, the significance of relative chromosome numbers was subject to other considerations, including the apparent large taxonomic distance between the two sections (Garber 1944, 1950). Moreover, Karper and Chisholm (1936) reported that the average somatic chromosome length of the para-sorghum species *S. versicolor* ($2n = 10$) was twice that of *S. bicolor.* Brown (1943) and others (Endrizzi and Morgan 1955; Kidd 1952) found appreciable numbers of bivalents in metaphase I spreads from haploid sorghum plants. Endrizzi and Morgan (1955) also reported translocations among progeny from haploids, which they interpreted as resulting from recombination between homologous duplications in the haploid genome, as might be expected if sorghum is tetraploid. Considered to be less than unequivocal (Doggett 1988), the aforementioned cytogenetic data were not widely embraced as authoritative, so the interpretation of sorghum as a diploid has remained preponderant (Moore et al. 1995).

Although multifactorial inheritance has been observed for certain traits of sorghum, for example, for maturity and height, a compilation of sorghum phenotypic genetic markers by Doggett (1988) did not reveal extensive duplication of genes. Chittenden et al. (1994) detected duplication of three RFLP linkage segments and widely distributed duplicated loci across the genome, but concluded that without further evidence it was not possible to distinguish between the hypotheses of polyploidization and segmental duplication. The frequency of sorghum RFLP probes that mapped to two or more loci was estimated to be 38% by Whitkus et al. (1992) and 11% by Xu et al. (1994). However, Xu et al. (1994) considered their estimate of duplicate loci as likely being an underestimate, since their procedure for clone isolation was designed to isolate a high frequency of clones that hybridized to single or very low copy number sequences.

This article presents results from physical mapping of a bacterial artificial chromosome (BAC) containing centromeric-specific DNA sequence(s) to sorghum

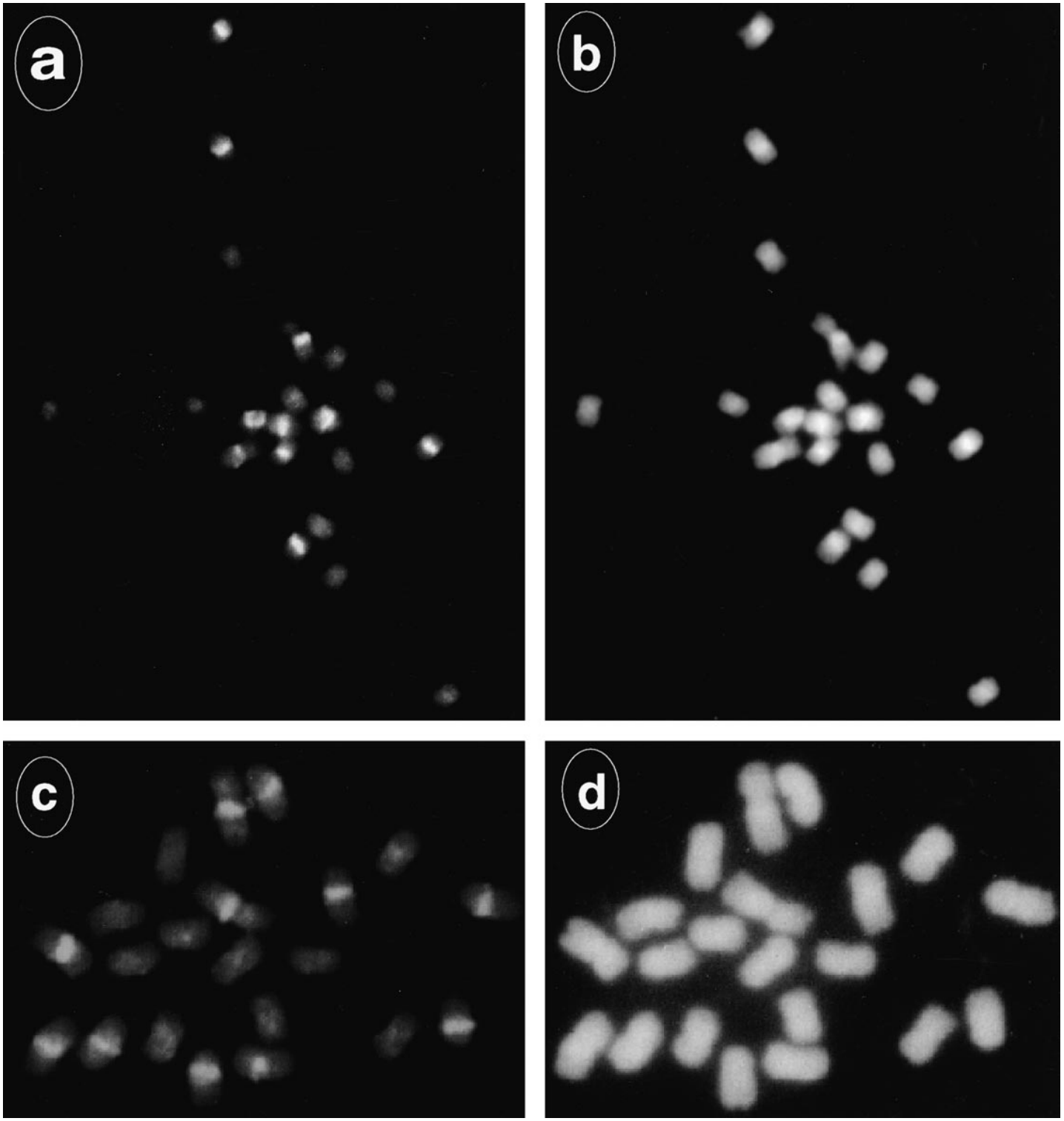

Figure 1. Fluorescent in situ hybridization of *S. bicolor* bacterial artificial chromosome (BAC 22B2) to *S. bicolor* root tip metaphase chromosome spreads. Gray-scale images from paired color photographs show Cy3-detected hybridization sites (left) and DAPI-revealed chromatin distribution (right). **(a, b)** Disomic ($2n = 20$) cell with strong FISH signals on centromeric regions of 10 chromosomes and weak or no signal on the other 10 chromosomes. **(c, d)** A cell trisomic ($2n = 20 + 1$) for chromosome E with strong FISH signals on centromeric regions of 11 chromosomes but weak or no signal on 10 chromosomes.

chromosomes using fluorescent in situ hybridization (FISH). The results show that the *S. bicolor* genome consists of two subgenomes of five chromosomes each, and indicate that sorghum is at least of tetraploid origin.

Materials and Methods

Plant Materials

Seed was germinated from disomic plants of *S. bicolor* cv. Tx 403, and the primary trisomics designated D, E, G, H, and I, which are the only trisomics available today from the set of trisomics defined by Schertz (1966, 1974). Plants grown in pots in a greenhouse with controlled temperature (24°C–25°C day; 18°C–21°C night) provided the root tips used for chromosome preparations.

Preparation of Chromosome Spreads

Mitotic metaphase spreads were prepared following protocols described by Jewell and Islam-Faridi (1994).

DNA Probes

The results presented involve the 45 kb BAC 22B2 from the BAC library produced by Woo et al. (1994). For use as a FISH probe, whole plasmid DNA from BAC 22B2 was labeled with biotin-14-dATP by nick translation according to the standard protocol provided by the manufacturer (Gibco BRL BioNick).

Fluorescence in situ Hybridization (FISH)

Fluorescence in situ hybridization was conducted using procedures from Islam-Faridi and Mujeeb-Kazi (1995) that were adapted for use with BACs by Hanson et al. (1995). Detection involved Cy3-conjugated mouse antibiotin (Jackson ImmunoResearch) system. Chromosomes were counterstained with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI).

Metaphase Observation and Photography

Images were photographed directly on Fuji ASA 400 film with a camera mounted on an Olympus AX-70 epifluorescence microscope using standard Olympus filter sets for single and dual bandpass filtration. Due to the high cost of publishing color photographs, photographic prints were scanned as 8-bit gray-scale images on a conventional flatbed scanner, electronically assembled into a plate, and printed by a dye-sublimation printer.

Results and Discussion

The results indicate that BAC 22B2 contains one or more repetitive elements that are differentially represented in the centromeric regions of 10 of the 20 chromosomes of *S. bicolor* (Figure 1a,b). The signal level on these 10 chromosomes was far stronger than on the other 10 chromosomes. The pattern was consistent across many cells, slides, and root tips. We propose that the subgenome yielding strong BAC 22B2 FISH signal be designated as subgenome A_b and the other subgenome as B_b, where the subscript "b" denotes the genome of origin, that is, the species "*bicolor*." To further test the hypothesis that the strong signals are chromosome specific, BAC 22B2 was FISHed to the available trisomics of the set defined by Schertz (1966, 1974), namely D, E, G, H, and I. Spreads of trisomics E, H, and I displayed strong FISH signals on 11 chromosomes and only weak or no signal on 10 chromosomes (Figure 1c,d). In contrast, spreads of trisomics D and G yielded 10 chromosomes with a strong FISH signal and 11 with weak or no signal (not shown). Therefore, FISH to five trisomic stocks confirmed the chromosome-specific association of the BAC-FISH signal and allowed assignment of three chromosomes (E, H, and I) to subgenome A_b and two chromosomes (D and G) to subgenome B_b.

There has been at least tentative acceptance of the hypothesis that *Zea mays* ($2n = 20$) is a diploidized tetraploid (Ahn and Tanksley 1993; Moore et al. 1995; Paterson et al. 1995). However, there is much less acceptance of a tetraploid origin of sorghum, in spite of its many parallels to maize. The results of the current study provide strong evidence that sorghum is at least of tetraploid ($2n = 4x = 20$) origin, and that there are two subgenomes of five chromosomes each in the *S. bicolor* genome. The results suggest that phylogeny and chromosomal relationships of the genus *Sorghum* may need to be reinterpreted.

From the Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843-2474. R. A. Wing is currently at the Department of Agronomy, Clemson University, Clemson, South Carolina. This research was supported by the Texas Advanced Technology & Research Program (grant 999902-090), Texas Agricultural Experiment Station, and Texas A&M University Office of University Research. Address correspondence to Dr. Price at the address above or e-mail: hjp6300@acs.tamu.edu.

The Journal of Heredity 1998:89(2)

References

Ahn S and Tanksley SD, 1993. Comparative linkage maps of rice and maize genomes. Proc Natl Acad Sci USA 90:7980–7984.

Brown MS, 1943. Haploid plants in sorghum. J Hered 34:163–166.

Celarier RP, 1956. Additional evidence for five as the basic chromosome number of the Andropogoneae. Rhodora 58:135–143.

Chittenden LM, Schertz KF, Lin Y-R, Wing RA, and Paterson AH, 1994. A detailed RFLP map of *Sorghum bicolor* × *S. propinquum,* suitable for high-density mapping, suggests ancestral duplication of *Sorghum* chromosomes or chromosomal segments. Theor Appl Genet 87:925–933.

Doggett H, 1988. Sorghum, 2nd ed. New York: John Wiley.

Dufour P, Grivet L, D'Hont A, Deu M, Trouche G, Glaszmann JC, and Hamno P, 1996. Comparative genetic mapping between duplicated segments on maize chromosomes 3 and 8 and homoeologous regions in sorghum and sugarcane. Theor Appl Genet 92:1024–1030.

Endrizzi JE and Morgan DT, 1955. Chromosomal interchanges and evidence for duplication. J Hered 46:201–208.

Garber ED, 1944. A cytological study of the genus *Sorghum*: subsections Para-sorghum and Eu-sorghum. Am Nat 78:89–93.

Garber ED, 1950. Cytotaxonomic studies in the genus *Sorghum.* Univ Calif Pub Bot 23:283–362.

Hanson RE, Zwick MS, Choi S, Islam-Faridi MN, McKnight TD, Wing RA, Price HJ, and Stelly DM, 1995. Fluorescence in situ hybridization of a bacterial artificial chromosome. Genome 38:646–651.

Hulbert DF, Richter TE, Axtell JD, and Bennetzen JL, 1990. Genetic mapping and characterization of sorghum and related crops by means of maize DNA probes. Proc Natl Acad Sci USA 87:4251–4255.

Islam-Faridi MN and Mujeeb-Kazi A, 1995. *Secale cereale* DNA in wheat germplasm; visualization by fluorescence in situ hybridization and breeding application. Theor Appl Genet 90:595–600.

Jewell DC and Islam-Faridi MN, 1994. Details of a technique for somatic chromosome preparation and C-banding of maize. In: The maize handbook (Freeling M and Walbot V, eds). New York: Springer-Verlag; 484–493.

Karper RE and Chisholm AT, 1936. Chromosome numbers in *Sorghum.* Am J Bot 23:369–374.

Kidd HJ, 1952. Haploid and triploid sorghum. J Hered 43:204–225.

Moore G, Foote T, Helentjaris T, Devos K, Kurata N, and Gale M, 1995. Was there a single ancestral cereal chromosome? Trends Genet 11:81–82.

Paterson AH, Lin Y-R, Li Z, Schertz KF, Doebley JF, Pinson SRM, Liu S-C, Stansel JW, and Irvine JE, 1995. Convergent domestication of cereal crops by independent mutations at corresponding genetic loci. Science 269: 1714–1718.

Pereira MG, Lee M, Bramel-Cox P, Woodman W, Doebley J, and Whitkus R, 1994. Construction of an RFLP map in sorghum and comparative mapping in maize. Genome 37:236–243.

Schertz KF, 1966. Morphological and cytological characterization of five trisomics of *Sorghum vulgare* Pers. Crop Sci 10:519–523.

Schertz KF, 1974. Morphological and cytological characteristics of five additional trisomics of *Sorghum bicolor* (L.) Moench. Crop Sci 14:106–109.

Whitkus R, Doebley J, and Lee M, 1992. Comparative genome mapping of sorghum and maize. Genetics 132: 1119–1130.

Woo S, Jiang J, Gill BS, Paterson AH, and Wing RA, 1994. Construction and characterization of a bacterial artificial chromosome library in *Sorghum bicolor.* Nucleic Acids Res 22:4922–4931.

Xu G-W, Magill CW, Schertz KF, and Hart GE, 1994. A RFLP linkage map of *Sorghum bicolor* (L.) Moench. Theor Appl Genet 89:139–195.

Received February 28, 1997
Accepted July 8, 1997

Corresponding Editor: J. Perry Gustafson