Brief Communications

The Chromosomes of *Citrus* and *Poncirus* Species and Hybrids: Identification of Characteristic Chromosomes and Physical Mapping of rDNA Loci Using In Situ Hybridization and Fluorochrome Banding

M. L. Roose, T. Schwarzacher, and J. S. Heslop-Harrison

In situ hybridization of 18S-5.8S-25S rDNA probes labeled with biotin or rhodamine and 5S rDNA probes labeled with digoxigenin was used to locate rDNA sites on root-tip metaphase chromosomes of *Citrus sinensis* L. ($2n = 2x = 18$), *Poncirus trifoliata* L. Raf. ($2n = 2x = 18$), and *Citrus* × *Poncirus* hybrids ($2n = 2x = 18$). Counterstaining with the fluorochromes chromomycin A3 and DAPI uniquely identified many but not all chromosomes. *C. sinensis* had five 18S-25S rDNA sites, *P. trifoliata* had seven, and three different *Citrus* × *Poncirus* hybrids had five or six sites. Four 5S rDNA sites were detected, mostly linked to 18S-25S rDNA sites. Overall we observed high levels of chromosomal heterozygosity in all accessions examined.

The economically important citrus fruits are derived by natural hybridization and backcrossing among *Citrus maxima* [Burm.] Merrill (pummelo), *C. reticulata* Blanco (mandarin), and *C. medica* L. (citron) (Roose et al. 1995). Many of the hybrids reproduce by a form of apomixis—nucellar embryony—and have been given species names by various authorities (see Roose et al. 1995). A related genus, *Poncirus,* produces fertile hybrids with *Citrus* and is an important source of genes for disease resistance in citrus rootstock breeding; most commercial trees are grafted to rootstocks of a different species or hybrid. Although *Poncirus* is quite distinct from *Citrus* in many morphological traits and gene content as measured by isozyme and RFLP markers (Federici and Roose, in preparation; Roose 1988; Torres et al. 1978), meiosis in the hybrid is essentially normal (Agarwal 1987).

Nearly all *Citrus* and *Poncirus* species are diploid, with $2n = 2x = 18$ chromosomes, and most are outcrossing and highly heterozygous. No inbred lines have been developed. The genome size is small at about 600 Mbp (Guerra 1984). Citrus cytogenetics has been limited by the small and relatively similar chromosomes. However, karyotypes based on Giemsa C-banding (Guolu 1988) and staining with the intercalating fluorochromes chromomycin A_3 (CMA) and DAPI (Guerra 1993) show that many chromosome pairs must be heteromorphic, perhaps as a consequence of widespread interspecific hybridization during the evolution of the major domesticated taxa. A key objective of linkage mapping in citrus (Cai et al. 1994; Durham et al. 1992; Jarrell et al. 1992) is map-based cloning of important genes, many of which have been selected as spontaneous mutations (Roose et al. 1995). To better understand genome organization in the citrus group, we wish to develop a corresponding physical map of the chromosomes that will (1) locate genes physically along chromosomes arms and (2) relate identified physical chromosomes to particular genetic linkage groups. Multicolor fluorescent in situ hybridization provides a useful approach to the problem of chromosome analysis and identification. Such technology has been applied in important cereal species (Jiang and Gill 1994; Leitch and Heslop-Harrison 1993; Schwarzacher et al. 1992) and many other species, including those with small chromosomes such as *Arabidopsis thaliana* (L.) Hayn. and its relatives (Kamm et al. 1995), sugar beet (Schmidt et al. 1994), birch (Anamthawat-Jónsson and Heslop-Harrison 1995), and rice (Fukui et al. 1994). In this work we used fluorochrome banding to examine chromosome structure and in situ hybridization with 18S-5.8S-25S and 5S rDNA sequences to locate rRNA genes on chromosomes of *Citrus, Poncirus,* and F_1 hybrids between these two genera.

Materials and Methods

Seeds of Argentina sweet orange (*C. sinensis* L.), Duncan grapefruit [*C. paradisi* (L.)], Flying Dragon trifoliate orange (*Poncirus trifoliata* L. Raf.), Troyer citrange (*C. sinensis* × *P. trifoliata*), Sacaton citrumelo (*C. paradisi* × *P. trifoliata*), and African shaddock × Rubidoux trifoliate (*C. maxima* × *P. trifoliata*) were collected from the University of California Citrus Variety Collection and treated with 0.1% 8-hydroxyquinoline sulfate before drying and storage at 4°C. We germinated seeds at 30°C on agar or filter paper and excised root tips approximately 5 mm long. Additional root tips were obtained from plants grown from seed in hydroponic culture at 25°C in continuous light. For the accessions studied here, genetic analysis shows that at least 90% of all seedlings are of nucellar (i.e., apomictic) origin (Xiang and Roose 1988; our unpublished observations). The only exception is Sacaton citrumelo, which produces about 50% sexual seedlings, but most of these can be identified by morphology (and were excluded from analysis here).

Methods for chromosome-spread preparation, probe labeling, and hybridization followed Schwarzacher et al. (1994). Briefly, root tips were treated with 0.1% 8-hydroxyquinoline for 45 min at 17°C and 45 min at 4°C, then fixed in methanol:acetic acid (3:1). Root tips were washed in enzyme buffer (0.01 M sodium citrate, pH 4.6), digested in 1% macerozyme, 1% Onozuka cellulase RS, 0.2% pectolyase Y23, 6 mM $CaCl_2$ in enzyme buffer, washed, and squashed in 45% acetic acid. The quality

of the spreads was checked by staining with 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole). Satisfactory preparations were destained by washing with methanol:acetic acid and dehydration in a 70-90-100% ethanol series. Prehybridization treatments were digestion with 100 μg/ml pancreatic RNase, digestion with 20–40 units/ml pepsin, treatment with 4% paraformaldehyde, and dehydration in ethanol. The rDNA probe was pTa71, a 9 kb *Eco*RI fragment containing the 18S, 5.8S, and 25S rRNA genes and intergenic spacers (the 18S-25S rDNA) of *Triticum aestivum* (Gerlach and Bedbrook 1979). The whole plasmid was labeled with digoxigenin-11-dUTP (Boehringher Mannheim), biotin-11-dUTP (Sigma), or rhodamine-4-dUTP (Fluorored, Amersham) by nick translation. The 5S rDNA probe was a 410 bp fragment from the clone pTa794 (Gerlach and Dyer 1980) containing the 5S rDNA repeat unit of *T. aestivum.* It was labeled with digoxigenin-11-dUTP or biotin-11-dUTP by polymerase chain reaction using primers flanking the cloning site with standard buffers and a ratio of 4 labeled nucleotides:1 dTTP. Chromosomes were denatured in a temperature cycler (Heslop-Harrison et al., 1991) at 70°C–75°C and slides were hybridized overnight at 37°C with 30 μl of hybridization mix consisting of 35–50% formamide, 10% dextran sulfate, 0.33% SDS, 2× SSC, 165 μg/ml sheared salmon sperm DNA, and 2 μg/ml denatured probe. Hybridization stringency was varied from 67 to 76% by adjusting formamide concentration in the hybridization mix. Following hybridization, slides were washed in 2× SSC at 42°C and then given a 10 min stringent wash in 0.75× or 0.1× SSC-20% formamide at 42°C, equivalent to washing stringencies of 70 and 84%, respectively. Hybridization sites of digoxigenin and biotin labeled probes were detected by antidigoxigenin antibody conjugated with FITC, or avidin-conjugated Texas Red, or streptavidin-Cy3 (Sigma) (Schwarzacher et al. 1994). Preparations were counterstained with DAPI**,** and sometimes with chromomycin A_3 (CMA; 1 mg/ml), mounted in antifade (Citifluor, AF1), and examined with a Zeiss epifluorescence microscope using single band-pass and long-pass filters. Where chromosomes could not be seen on the same photograph as probes, probe locations were determined by preparing overlay drawings from probe and DAPI or CMA-DAPI photographs, or by scanning negatives and forming composite images using Adobe Photoshop. Unless otherwise noted, all descriptions are based on analysis of at least four complete metaphase spreads from at least two seedlings.

Results

Fluorochrome Banding

DAPI banding rarely provided sufficient contrast for reliable differentiation among metaphase chromosomes of the *Citrus* and *Poncirus* species and hybrids analyzed (Figure 1a,c,f, and i). Centromeres were difficult to identify in many spreads; although often evident as constrictions, they were not associated with DAPI-positive heterochromatin. All chromosomes were metacentric or submetacentric. Conspicuous large DAPI-negative bands were observed at some paracentromeric and subtelomeric regions of metaphase and prometaphase chromosomes. Many chromosomes had a single, terminal CMA-positive band, generally on the long arm of the chromosome. Two long and several shorter chromosomes had no CMA bands in any of the taxa studied. Large DAPI-negative regions were normally CMA-positive (compare Figure 1c,d, and also f and h). In *C. sinensis,* two chromosomes, presumably homologs, had CMA bands at both ends. Such chromosomes were not detected in *P. trifoliata,* and only one chromosome of this type was detected in hybrids between *Citrus* and *Poncirus.*

18S-25S rDNA Sites

Counts of 18S-25S rDNA sites on metaphase chromosomes or interphase and prophase nuclei suggested that *Citrus* and *Poncirus* had 3–5 major and one or more additional minor 18S-25S rDNA sites per spread. Three strong and two minor sites were detected in *C. sinensis* (Figure 1b), while *Poncirus* had 18S-25S rDNA sites on six chromosomes, and a seventh, minor site was occasionally detected (Figure 1e). Three sites were subterminal (including the minor site) and four were submedial. Troyer citrange (*C. sinensis* × *P. trifoliata*) had four major and usually one (occasionally two) minor sites in all complete metaphases examined (Figure 1g). Sacaton citrumelo had six sites and a *C. maxima* × *P. trifoliata* hybrid had five sites in two complete metaphases. In *C. sinensis,* two characteristic chromosomes had distal CMA bands on both arms and an 18S-25S rDNA site on one arm. This type of chromosome was not detected in *P. trifoliata,* but was detected in two of eight CMA-DAPI stained metaphases of Troyer citrange, but the 18S-25S rDNA site was minor (Figure 1h, upper asterisk). This type of chromosome also occurred in two metaphases of the *C. maxima* × *P. trifoliata* hybrid, along with another novel chromosome type having an 18S-25S rDNA site on the long arm adjacent to a CMA-positive site. Troyer citrange also had a characteristic chromosome with a large, often extended 18S-25S rDNA site at the end of the long arm and distal to a 5S rDNA site (asterisks in Figure 1g,h, and k) that we did not observe in either parental accession. 18S-25S rDNA sites usually appeared as DAPI-negative, CMA-positive sites (Figure 1c versus 1d, 1f versus 1h) and were often extended (Figure 1b,g). These sites presumably included genes that were active at the previous interphase and had not fully condensed.

5S rDNA Sites

In both Troyer citrange (three metaphases) and *Poncirus* (two metaphases), the 5S rDNA sites were detected at four sites (Figure 1g,j) of which three sites were adjacent to 18S-25S rDNA sites on longer chromosomes (Figure 1g,k). In *C. sinensis,* three sites were detected (two metaphases, and one or two sites in others; Figure 1b). Overall, signal intensity was low for consistent detection of this probe, but at least four sites exist, and some are close to 18S-25S rDNA sites.

Discussion

The data presented here show that using a combination of chromosome morphology, fluorochrome banding, and fluorescent in situ hybridization with rDNA probes enables many chromosome types in *Citrus* and *Poncirus* to be distinguished. We were able to identify significant marker chromosomes, with their variants, in several species, accessions of one species, and hybrids. The analysis starts to build a picture of the overall organization and evolution of the *Citrus*-group genomes. In all the species analyzed, we observed 18 submedian chromosomes ranging in size by about twofold. More than half of the chromosomes have prominent terminal CMA-positive bands, indicating the location of GC-rich DNA. Most major 18S-25S rDNA sites are intercalary on the larger chromosomes, corresponding to CMA sites. The 5S rDNA loci were mostly located near the 18S-25S loci at both distal and proximal locations.

C. sinensis and, to a lesser extent, *P. trifoliata* are characterized by chromosomal heterozygosity as evident by the lack of

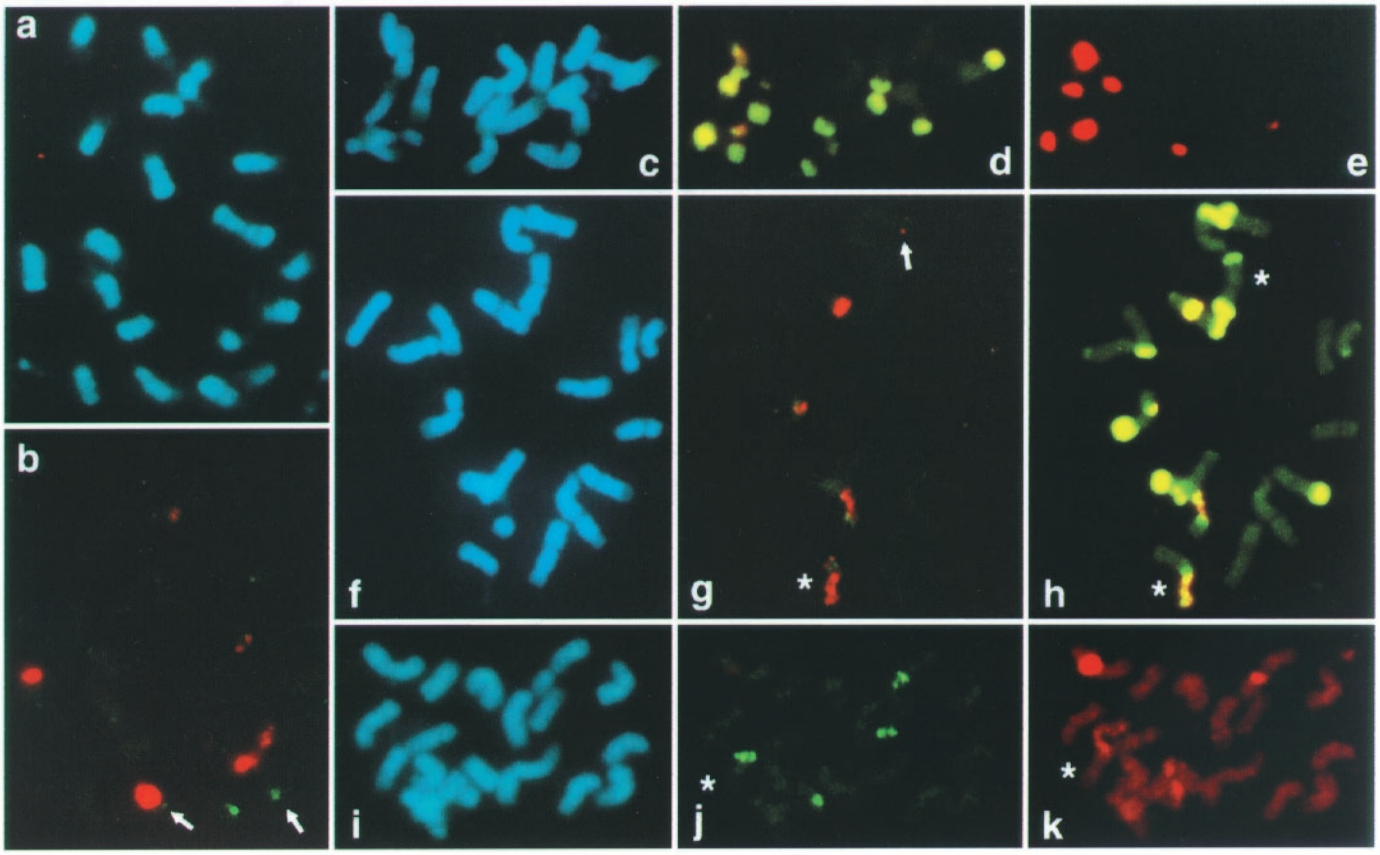

Figure 1. Fluorescent in situ hybridization using 18S-25S and 5S rDNA probes to root-tip metaphase chromosomes from seedlings of *Citrus* and *Poncirus* cultivars and hybrids. **(a,b)** Chromosomes of Argentina sweet orange (*C. sinensis*; $2n = 2x = 18$) counterstained with DAPI **(a)** and in a composite micrograph of in situ hybridization signals **(b)**. Two sites of 5S rDNA (green, arrows, further green sites are background) were detected and three major and two minor sites of 18S-25S rDNA (red). **(c–e)** Flying Dragon trifoliate orange (*P. trifoliata,* $2n = 2x = 18$) chromosomes counterstained with DAPI **(c)** and CMA **(d)** showing seven sites of 18S-25S rDNA (red, **e**). The green signal of the 5S rDNA are not clearly distinguishable from the yellow-green CMA **(d)**. **(f–k)** Metaphase chromosomes from two seedlings (**f–h** and **i–k**) of Troyer citrange (*C. sinenesis* × *P.trifoliata,* $2n = 2x = 18$) counterstained with DAPI **(f, i)**. In situ hybridization shows up to four 5S rDNA sites (green, in the composite **g** and **j**) and three major and two smaller 18S-25S rDNA sites (red, **g** and **k**). **(h)** the same metaphase as in **(f)** and **(g)** after restaining with CMA. Staining with the second fluorochrome after hybridization allows two probes to be localized, but often leads to loss of chromosomes, as here where two chromosomes are missing in **(h)**. The chromosome marked by asterisks in the lower part of **(g)** and **(h)**, and in **(j)** and **(k)** was not found in the accessions that represent Troyer's parental species. The chromosome in the upper part of **(h)** that is marked with an asterisk had a minor 18S-25S rDNA site in some metaphases. Magnification 2300×.

clear pairs of homologs after DAPI-CMA banding and 5S and 18S-25S rDNA localization. High levels of heteromorphism were also reported by Guerra (1993) in *Citrus* using fluorochrome banding. Factors contributing to chromosomal heterozygosity in citrus include the origin of many accessions by interspecific hybridization and clonal propagation which allows accumulation of karyotypic rearrangements. Other accessions of these taxa may have small variations from the karyotypes presented here, since chromosomal rearrangements are often involved in selection for seedlessness and other traits within cultivated citrus (Gmitter et al. 1992).

The number and location of 18S-25S rDNA and 5S rDNA sites and CMA bands in Troyer differs somewhat from the additive pattern expected based on locations in its parental species, *C. sinensis* and *P. trifoliata.* The cultivars of these species that we examined have diverged from the parents of Troyer by mutation over hundreds of years. 18S-25S rDNA copy number is known to evolve quickly (see Leitch et al. 1993), and it is possible that some sites are lost or below the limit of detection with fluorescent in situ hybridization.

The molecular cytogenetic methods described here enable certain characteristic chromosomes to be identified in species and hybrids. Comparative studies of various citrus taxa can allow detection of cytological abnormalities such as translocations resulting in chromosomes with two rDNA sites, or that produce chromosomes with submedial 18S-25S rDNA sites that are not accompanied by a CMA-positive band on the other chromosome arm. Such rearrangements are relatively frequent within vegetatively propagated species and may be difficult to analyze by marker technology. Previously, meiotic analysis was required, but flowering often takes 5–8 years and may occur only once per year. Thus the methods described here represent a substantial advance in citrus cytogenetics because they allow quick and accurate detection of many chromosomal rearrangements. They should also be useful for following individual chromosomes during breeding programs or reconstructing phylogeny.

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A Recessive Gene for Revolute Cotyledons in Cucumber

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An experiment was conducted to determine the genetics of the revolute cotyledons trait in the cucumber inbred NCG-093 (short petiole mutant). NCG-093 was crossed with inbred WI 2757 to produce F_1, F_2, and BC_1 generations for evaluation. The F_1 progeny had normal cotyledons, and the segregation of the F_2 progeny fit a ratio of 3 normal:1 revolute cotyledons. The $BC_{1A}(F_1 \times$ WI 2757) progeny had normal cotyledons, and the segregation of the $BC_{1B}(F_1 \times$ NCG-093) fit a ratio of 1 normal: 1 revolute cotyledons. We concluded that revolute cotyledons in NCG-093 was conferred by a single recessive gene, revolute cotyledons-2, for which we propose the symbol *rc-2.* A mutant from Burpless Hybrid was previously described as having revolute cotyledons, controlled by the *rc* gene. However, that mutant was apparently lost, making it impossible to test allelism with the gene in NCG-093.

Cotyledon mutants are useful for linkage studies in cucumber (*Cucumis sativus* L.) because they can be identified in the early stages of plant growth (Pierce and Wehner, 1990). Three single-gene mutations of cotyledon shape have been reported previously: *rc,* revolute cotyledons (Whelan et al. 1975), where the cotyledons are cupped downward and are shorter and narrower than normal; *sc,* stunted cotyledons (Shanmugasundarum et al. 1971), where the cotyledons are small and concavely curved; and *wy,* wavy rimed cotyledons (Iida and Amano 1990), where the cotyledons have wavy rims and white centers. The revolute cotyledons mutant apparently has been lost, but the other two are available in germplasm collections.

We recently discovered a revolute cotyledons mutant in the self-pollinated progeny of line NCG-093, which was derived from an unknown line obtained from Russia. The mutant can be observed reliably as soon as the cotyledons are fully expanded, and it has cotyledons whose edges are curved partly downwards (revolute), especially when the plants are

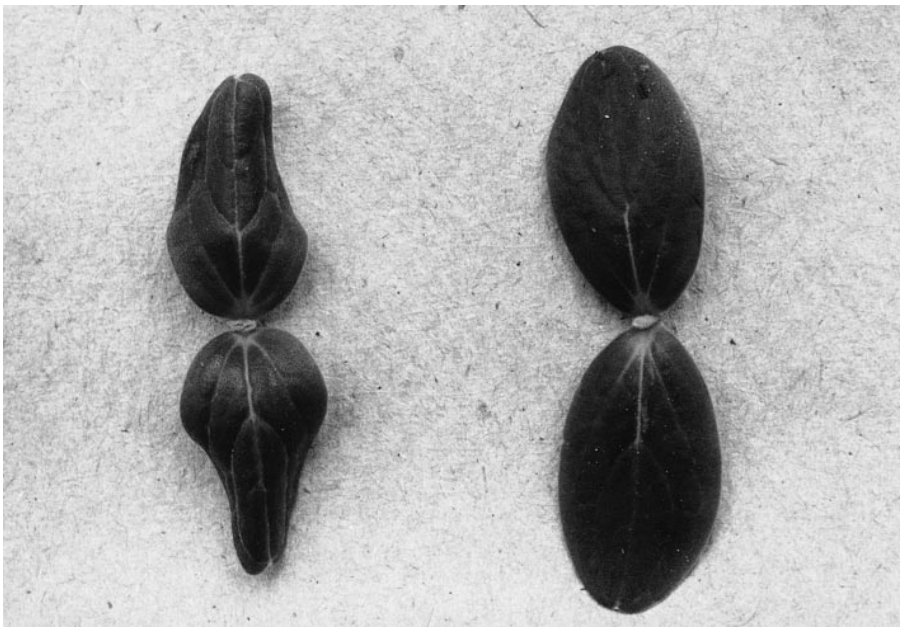

Figure 1. Cucumber seedlings from the F_2 progeny of WI 2757 × NCG-093, 6 days after seeding in a greenhouse flat, showing revolute cotyledons (*rc-2*) (left) and normal cotyledons (right).

Table 1. Inheritance of revolute cotyledons (*rc-2*) in the cucumber cross WI 2757 × NCG-093

Generation	No. observed (N:R)[a]	No. expected (N:R)	Expected ratio (N:R)	χ^2	*P* value
WI 2757 (P_A)	44:0	44:0	1:0	—	—
NCG-093 (P_B)	0:16	0:16	0:1	—	—
F_1	54:0	54:0	1:0	—	—
F_2	122:41	122:41	3:1	0.002	.96
BC_{1A}	79:0	79:0	1:0	—	—
BC_{1B}	47:33	40:40	1:1	2.45	.11

[a] R = revolute; N = normal cotyledons.

young (Figure 1). The size and green color of the mutant cotyledons are the same as the normal cotyledons of WI 2757 (Peterson et al. 1982). The true leaves of the seedlings are also normal, and the cotyledons become difficult to distinguish from normal cotyledons when the plants reach the four true-leaf stage.

In order to study the inheritance of the revolute cotyledons mutant, NCG-093 and WI 2757 were increased by self-pollination and checked for uniformity of cotyledon type to develop parental inbred lines. The two inbreds were crossed using hand-pollination in a greenhouse. The F_1 progeny were self-pollinated to produce the F_2 generation and also backcrossed to each parent to produce the BC_{1A} (F_1 × WI 2757) and BC_{1B} (F_1 × NCG-093).

Seedlings were grown in flats of vermiculite on benches in the greenhouse (temperature 20°C–35°C with a 13–14 h photoperiod). Six days after seeding, plants were evaluated for cotyledon phenotype and classified as revolute or normal.

The cross of normal cotyledons WI 2757 with revolute cotyledons NCG-093 produced all normal F_1 progeny (Table 1). Segregation in the F_2 progeny fit the 3:1 expected ratio ($P > .96$), assuming the trait was controlled by a single recessive gene. Progeny segregation in the BC_{1A} and BC_{1B} generations verified the inheritance pattern for a single recessive gene observed in the F_2 progeny. The BC_{1A} (to NCG-093) segregated in a 1:1 ratio, with an adequate fit to expected values ($P > .05$). No revolute cotyledons seedlings were observed in BC_{1B} (to WI 2757).

We concluded that there was a single recessive gene for revolute cotyledons-2 in NCG-093 for which we propose the symbol *rc-2*. The mutant of Burpless Hybrid having revolute cotyledons described by Whelan et al. (1975) was lost, so it was impossible to compare the mutants for similarity or to cross them to test for allelism. Seeds of NCG-093 can be obtained from T.C.W.

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Preferential Male Transmission of an Alien Chromosome in Wheat

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A telocentric chromosome 5H^{t}L from *Elymus trachycaulus* (Link) Gould ex Shinners was transferred into common wheat (*Triticum aestivum* L.). This chromosome was assigned to the homoeologous group 5 of Triticeae species by restriction fragment length polymorphism (RFLP) analysis. Chromosome 5H^{t}L was transmitted in 20% of the female gametes and 97% of the male gametes in the genetic background of wheat, although the expected transmission frequencies of 5H^{t}L through female and male gametes are 25% and 0–5%, respectively. It is likely that a gene located on 5H^{t}L promotes male gamete competition. We suggest that the long arms of homoeologous group 5 chromosomes in Triticeae species carry genes that affect their transmission through male gametes.

Genes from a number of wild species have been successfully transferred into cultivated wheat (Islam and Shepherd 1992; Jiang et al. 1994a). Production of wheat-alien chromosome addition lines is usually the first step to do such transfers. One common character for most of the wheat-alien monosomic addition lines (42 wheat chromosomes plus one alien chromosome) is the low transmission of the alien chromosome through pollen, often less than 5%. Thus pollen with the additional alien chromosome can hardly compete with normal ones in fertilization. During the past few years we have isolated several wheat-*Elymus trachycaulus* ($2n = 4x = 28$, genomically S^{t}S^{t}H^{t}H^t) chromosome addition lines (Jiang et al. 1994b; Morris et al. 1990). In the selfed progenies of a monotelosomic 5H^{t}L addition line, the telocentric chromosome 5H^{t}L was detected almost exclusively in all the plants analyzed. This article reports on this rare case of preferential male transmission of 5H^{t}L in the genetic background of wheat.

Materials and Methods

Wheat-*E. trachycaulus* monosomic addition line 5H^t (designated as MA 5H^t, chromosome constitution is 21″ + 5H^t′), monotelosomic addition line 5H^{t}L (MTA 5H^{t}L, 21″ + 5H^{t}L′; 5H^{t}L represents a telocentric chromosome derived from the long arm of 5H^t) and monoisosomic addition line 5H^{t}L·5H^{t}L (MIA 5H^{t}L·5H^{t}L, 21″ + 5H^{t}L·5H^{t}L′; 5H^{t}L·5H^{t}L represents an isochromosome derived from the long arm of 5H^t) were isolated from the backcrossed progenies of an *E. trachycaulus* × Chinese Spring (CS) wheat hybrid. These lines were used for male and female transmission studies in crosses with CS wheat. Chromosomes 5H^{t}L and 5H^{t}L·5H^{t}L were identified by N-banding analysis (Endo and Gill 1984).

For RFLP analysis, genomic DNA was isolated from young leaf tissue of wheat and the monosomic addition lines. DNA samples were digested with restriction enzyme *Hin*dIII and blotted to MSI membrane. Prehybridization and hybridization were done at 65°C in 5× SSC, 100 mM $NaPO_4$ (pH 6.5), 20 mM EDTA, 0.5% SDS, and 0.2 mg/ml denatured salmon sperm DNA. The membranes were washed at 50°C in 0.1× SSC and 0.1% SDS, and exposed to X-ray film. The two group 5-specific RFLP markers, PSR118 and PSR128 (Sharp et al. 1989), were kindly provided by Dr. M. D. Gale, John Innes Center, Norwich, England.

Results

Designation of Chromosome 5H^t

Chromosome 5H^t has two characteristic N-bands near the centromere on the long

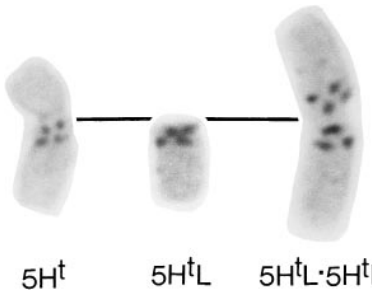

Figure 1. N-banding patterns of complete chromosome 5H^t, telocentric chromosome 5H^{t}L, and isochromosome 5H^{t}L·5H^{t}L.

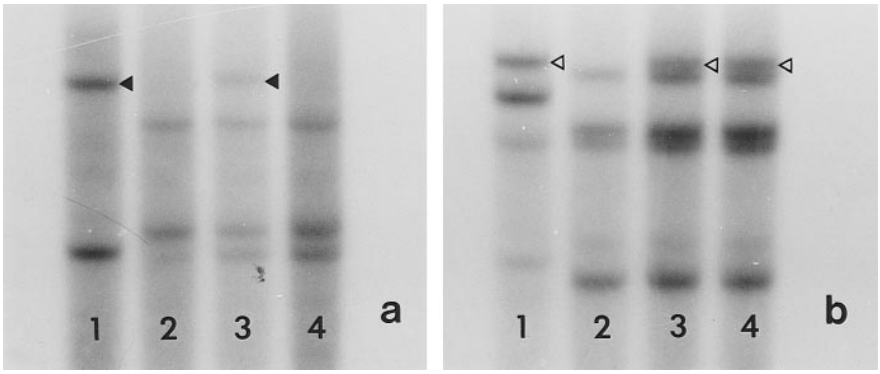

Figure 2. *Hin*dIII-digested DNA samples from *E. trachycaulus* (lane 1), Chinese Spring wheat (lane 2), monosomic 5H^t addition line (lane 3), and monotelosomic 5H^{t}L addition line (lane 4) probed with **(a)** a group 5 short arm marker, PSR118; **(b)** a group 5 long arm marker, PSR128. Solid arrowheads point to the bands specific to the short arm of 5H^t. Open arrowheads point to the bands specific to the long arm of 5H^t.

arm (Figure 1). Therefore chromosomes 5H^t, 5H^{t}L, and 5H^{t}L·5H^{t}L can be distinguished from all the wheat chromosomes. RFLP analysis with wheat homoeologous group 5-specific probes indicated that 5H^t belongs to group 5. The RFLP marker, PSR118, assigned to the short arms of group 5 chromosomes, hybridized to a specific DNA fragment derived from the short arm of 5H^t (Figure 2a). Similarly the group 5 long arm marker, PSR128, hybridized to a DNA fragment from the long arm of 5H^t (Figure 2b).

Chromosome Transmission

The telocentric chromosome 5H^{t}L from a monotelosomic addition line showed male and female transmission frequencies of 0.97 (29/30) and 0.20 (2/10), respectively (Table 1). Based on the previous estimates of transmission of alien chromosomes in the genetic background of wheat (Hyde 1953; Riley 1960), the expected transmission frequencies of 5H^{t}L through male and female gamete are 0.00–0.05 and 0.25, respectively. The present data indicate a preferential transmission of 5H^{t}L through the male gametes.

Preferential male transmission was also observed in progeny of selfed MTA 5H^{t}L. Assuming 0.20 female and 0.97 male transmission of 5H^{t}L, we expected five ditelosomic addition (21″ + 5H^{t}L′) and 21 monotelosomic addition plants from a total of 27. This is close to the 6 and 21 plants observed for the respective classes (Table 1). We also analyzed 20 seeds from a selfed population of a double monosomic 5B and 5H^t (20″ + 5B′ + 5H^t′). Nineteen plants had at least one copy of 5H^t. This result indicated that the complete chromosome 5H^t has a similar male transmission behavior as its long arm 5H^{t}L.

A population of selfed MIA 5H^{t}L·5H^{t}L was analyzed for the transmission of the isochromosome 5H^{t}L·5H^{t}L. If an alien chromosome has no preferential transmission in the wheat genetic background, 25% monosomic additions and 75% euploid plants will be expected from selfed progenies of a monosomic addition line. While assuming 5H^{t}L·5H^{t}L has 20% female and 97% male transmission, similar to 5H^{t}L, we expected about 20% diisosomic addition (21″ + 5H^{t}L·5H^{t}L″) and 80% monoisosomic addition plants. The observed transmission of 5H^{t}L·5H^{t}L was in the middle of these expectations (Table 1). Therefore the isochromosome 5H^{t}L·5H^{t}L showed reduced preferential transmission as compared to the telocentric chromosome 5H^{t}L. About 38% (8/21) of the plants in the selfed progenies of MIA 5H^{t}L·5H^{t}L contained a 5H^{t}L telosome. These telocentric chromosomes may have been derived from misdivision of the isochromosome 5H^{t}L·5H^{t}L.

The meiosis of MTA 5H^{t}L was studied. No abnormal meiotic behavior except lagging 5H^{t}L was observed at anaphase I and anaphase II. More than 95% of the pollen grains of MTA 5H^{t}L were of normal size, with two visible sperm nuclei and one vegetative nucleus.

Discussion

In plants, euploid male gametes generally have a distinct competitive advantage over male gametes with extra chromosomes. The frequency of disomic wheat-alien chromosome additions among the progenies of monosomic additions is usually very low because of the low transmission of the alien chromosome through male gametes. In this study, the transmission of 5H^{t}L through the female gametes (20%) is within the range (15–45%) of other *E. trachycaulus* chromosomes in the genetic background of wheat. However, 5H^{t}L was transmitted in 97% of the male gametes, suggesting that the male gametes

Table 1. Transmission of chromosomes 5H^t, 5H^{t}L, and 5H^{t}L·5H^{t}L in the genetic background of wheat

Cross[a] (♀/♂)	Chromosome constitution	No. of plants
CS/21″ + t′	21″ + t′	29
	21″	1
		Total 30
21″ + t′/CS	21″ + t′	2
	21″	8
		Total 10
21″ + t′, self	21″ + t″	6
	21″ + t′	21
	21″	0
		Total 27
20″ + 5B′ + 5H^t′, self	20″ + (0–2)5B + (1–2)5H^t	19[b]
	20″ + 5B″	1
		Total 20
21″ + i′, self	21″ + i″	2
	21″ + i′ + t′	2
	21″ + i′	5
	21″ + t′	6
	21″	6
		Total 21

[a] t: 5H^{t}L; i: 5H^{t}L·5H^{t}L.

[b] Four plants had a 5H^{t}L instead of 5H^t.

with $5H^tL$ had a competitive advantage over euploid male gametes. Telocentric chromosome $5H^tL$ was frequently detected in the selfed progenies of MIA $5H^tL{\cdot}5H^tL$ and MA $5H^t$ (Table 1). Most of these telocentric chromosomes probably originated in the pollen because they can be preferentially transmitted through male gametes. In contrast to $5H^tL$, the short arm of $5H^t$ ($5H^tS$) was rarely recovered in the selfed progenies of MA $5H^t$. The frequent recovery of $5H^tL$ from the rare misdivision of $5H^tL{\cdot}5H^tL$ and $5H^t$ again indicates a strong selective advantage of male gametes with $5H^tL$.

The genetic mechanism of the preferential male transmission of $5H^tL$ is not known, but it seems different from that reported for the gametocidal chromosomes from *Aegilops* species (Endo 1990; Endo and Tsunewaki 1975; Maan 1975; Miller et al. 1982). A gametocidal chromosome will cause abortion of the gametes lacking it, resulting in partial sterility and exclusive transmission of such chromosomes through both male and female gametes (Endo 1990). In the present case, more than 95% of the pollen from MTA $5H^tL$ appeared normal, and both MA $5H^t$ and MTA $5H^tL$ are fully fertile. In addition, no chromosomal structural changes were observed in the progenies derived from MA $5H^t$, MTA $5H^tL$, and MIA $5H^tL{\cdot}5H^tL$, while chromosomal mutations were frequently detected in the progenies of wheat plants containing gametocidal chromosomes (Endo 1990). Another distinct characteristic of $5H^tL$ is its preferential transmission only through male gametes. Gametocidal chromosomes are transmitted preferentially through both male and female gametes (Endo 1990).

A possible genetic mechanism is that a gene located on $5H^tL$ may be responsible for its preferential male transmission. This gene makes male gametes more competitive than the gametes lacking it. Supporting evidence for this hypothesis is that another homoeologous group 5 chromosome, $5S^t$, from *E. trachycaulus* may have a similar gene that enhances male gamete transmission. Although this chromosome is not preferentially transmitted through male gametes like $5H^tL$, it was detected more frequently in the backcrossed derivatives of the *E. trachycaulus* × CS hybrids than the other six S^t genome chromosomes.

The long arms of wheat chromosomes 5A, 5B, and 5D may also carry important genes for transmission of male gametes. Ditelosomic 5AS, 5BS, and 5DS, in which the long arm is absent, are among the few unavailable ditelosomic lines of CS wheat (Sears and Sears 1978). It has been impossible to recover ditelosomic 5AS, 5BS, and 5DS plants from progenies of selfed plants with the chromosome constitution of 20″ + 5AS″ + 5AL′, 20″ + 5BS″ + 5BL′, and 20″ + 5DS″ + 5DL′, respectively. It is most likely that male gametes of 20′ + 5AS′, 20′ + 5BS′, and 20′ + 5DS' are not functional or they cannot compete with those of 20′ + 5AS′ + 5AL′, 20′ + 5BS′ + 5BL′, and 20′ + 5DS′ + 5DL′, respectively.

In the progenies of heterozygous 5A (or 5B or 5D) deletion lines, homozygous deletion plants were never recovered if the deleted 5A (or 5B or 5D) had less than 50% of the long arm (Endo and Gill 1996). This result suggests that male gametes with deleted 5A (or 5B or 5D) are not functional or are unable to compete with those having normal 5A (or 5B or 5D). The genes that are critical for male gamete transmission are possibly located on the proximal half of chromosomes 5A, 5B, and 5D.

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Low Allozyme Diversity in *Schwalbea americana* (Scrophulariaceae), an Endangered Plant Species

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Schwalbea americana, a hemiparasitic member of the Scrophulariaceae, is an early successional, fire-dependent species of the eastern coastal plain of North America. We sampled 13 populations across the range of this endangered perennial herb to describe allozyme diversity at 15 presumptive loci. Genetic diversity was low for the species, within populations, and for polymorphic loci (H_{es} = 0.006; H_{ep} = 0.005; H_T = 0.028). Three of the 15 loci (20%) were polymorphic across the species' range, but frequencies of uncommon alleles were uniformly low (mean P = .05). No polymorphism was detected in seven populations. Population extinctions and decreases in population sizes, coupled with habitat fragmentation, may account for the low genetic diversity. The fugitive life-history characteristics of this shade-intolerant species presumably have also contributed to the loss of genetic diversity by predisposing the species to founder effects and population extinctions.

Schwalbea americana comprises a monotypic genus in the figwort family, Scrophulariaceae. Like many members of this family, *S. americana* is a hemiparasite, ob-

taining a portion of its nutritional needs from other plants via root haustoria. *S. americana* is not host specific and forms haustorial connections with several species. Commonly called American chaffseed, *S. americana* was federally listed as endangered in 1992 (U.S. Fish and Wildlife Service 1992). Largely a coastal plain species, *S. americana* historically occurred in the eastern coastal states of the United States from Massachusetts to Louisiana, as well as in Kentucky and Tennessee. The species has always been considered rare and local in distribution (U.S. Fish and Wildlife Service 1994). At the time of listing, 19 occurrences of the species were documented; 11 of these were in South Carolina, with the remaining eight spread among five states. Recent (1994) detailed searches for the species in appropriate habitats in South Carolina and delineation of distinct occurrences at Fort Bragg, North Carolina, have led to the recognition of 71 total occurrences (U.S. Fish and Wildlife Service 1994). The distribution of these occurrences is: New Jersey (1), North Carolina (17), South Carolina (42), Georgia (10), and Florida (1).

S. americana is shade intolerant and occurs in subclimax fire-maintained communities once widespread in the southeastern United States. Fire suppression is likely to have been one of the causes of the species' decline, and may yet represent a major threat to the species; extant populations of the species are found in habitats that are burned regularly. These include privately owned plantations managed for bobwhite quail, impact areas of Fort Bragg, North Carolina, that experience fires due to the use of live ammunition, a national forest (Francis Marion National Forest, South Carolina) and a privately owned research area (Jones Preserve, Georgia) managed for long-leaf pine (*Pinus palustris*), a fire-adapted tree.

A perennial herb with unbranched stems, American chaffseed grows to a height of 30 to 60 cm. Its subtle purplish-yellow flowers are borne singly in the axils of the uppermost, reduced leaves and form a spikelike raceme. Bumblebees are probably the primary pollinators (Kirkman K, personal communication). Fruits produce copious minute wind-dispersed seeds. Germination rates of *Schwalbea* seeds are reported to be high, and evidence of a seed bank is lacking (U.S. Fish and Wildlife Service 1994).

The object of this study was to describe genetic diversity and its distribution within and among *S. americana* populations.

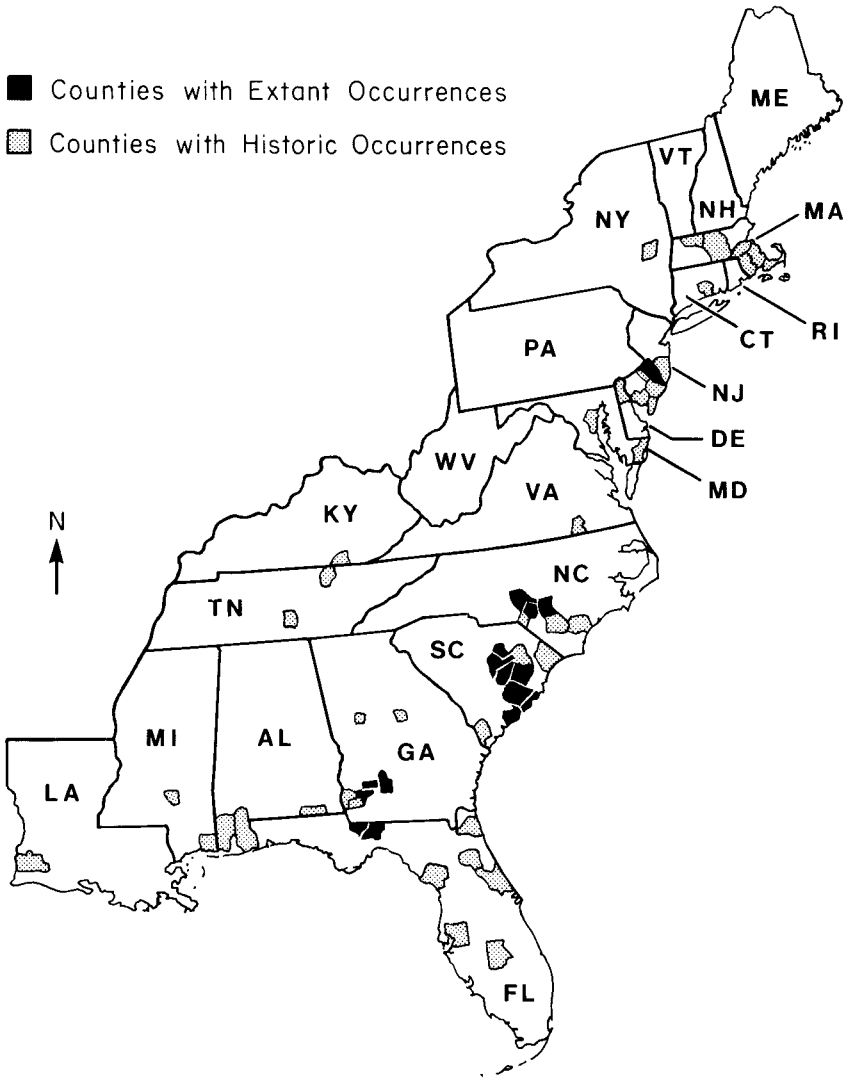

Figure 1. Distribution of *Schwalbea americana* (adapted from U.S. Fish and Wildlife Service 1994).

The identification of populations with high genetic diversity is useful because such populations are preferred sources of propagules for restoration and ex situ conservation (Godt et al. 1995). In addition, knowledge of the distribution of genetic diversity is essential for the formulation of sampling strategies that capture variation within species (Hamrick et al. 1991).

Materials and Methods

Thirteen populations of *S. americana* were sampled throughout its range (Figure 1; Table 1). Forty-eight individuals were sampled per population except in the small populations Fort Bragg 1 and New Jersey, where 13 and 10 individuals were sampled, respectively. Several leaves were removed from each sampled individual, placed in a zip-lock bag, and transported to the laboratory on ice where they were pulverized under liquid nitrogen using a mortar and pestle. An extraction buffer (Mitton et al. 1979) was added to the leaf powder to solubilize and stabilize the enzymes. Enzyme extracts were absorbed onto chromatography paper wicks that were stored at −70°C until analyzed.

Allozyme diversity was analyzed using standard starch-gel electrophoretic techniques. These enzymes were stained following recipes in Soltis et al. (1983): fluorescent esterase (FE), malic enzyme (ME), mannose phosphate isomerase (MPI), 6-phosphogluconate dehydrogenase (6PGDH), peroxidase (PER), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), tetrazolium oxidase (TO), and triose phosphate isomerase (TPI). Amino acid transferase (AAT) and diaphorase (DIA) were stained following recipes in Cheliak and Pitel (1984). DIA, PGM, PER, and TPI were resolved on buffer 6 (numbers refer to Table 1 in Soltis et al.

Table 1. Estimates of genetic diversity within 13 populations of *Schwalbea americana*

Population	*P*	*AP*	*A*	A_e	H_o	H_e
Georgia (Baker County)						
King's Creek	6.7	2.0	1.07	1.01	0.010	0.009
Jericho	0.0	—	1.00	1.00	0.000	0.000
North Carolina (Hoke County)						
Fort Bragg 1	0.0	—	1.00	1.00	0.000	0.000
Fort Bragg 2	0.0	—	1.00	1.00	0.000	0.000
Fort Bragg 3	0.0	—	1.00	1.00	0.000	0.000
Fort Bragg 4	6.7	3.0	1.13	1.00	0.004	0.004
New Jersey (Burlington County)						
New Jersey	0.0	—	1.00	1.00	0.000	0.000
South Carolina [Berkeley County (A–C) and Williamsburg County (D–F)]						
A-Halfway Creek	13.3	2.0	1.13	1.03	0.022	0.026
B-Hellhole	6.7	3.0	1.13	1.01	0.007	0.009
C-Watson	20.0	2.3	1.20	1.02	0.018	0.019
D-Cade's	0.0	—	1.00	1.00	0.000	0.000
E-Salter's	0.0	—	1.00	1.00	0.000	0.000
F-Friendly Field	6.7	2.0	1.07	1.00	0.001	0.001
Mean	4.6	2.4	1.06	1.01	0.005	0.005
Species total	20.0	2.3	1.27	1.01	—	0.006
Endemics[a]	26.3	—	1.39	1.09	—	0.063
Endemics[b]	40.0	—	1.80	1.15	—	0.096

P is the percent polymorphic loci; *AP* is the mean number of alleles per polymorphic locus; *A* is the mean number of alleles per locus; A_e is the effective number of alleles per locus; H_o is the observed heterozygosity; and H_e is the heterozygosity expected under Hardy–Weinberg.

[a] Mean within-population statistics for 100 endemic taxa (Hamrick and Godt 1989).

[b] Mean species-level genetic diversity for 81 endemic taxa (Hamrick and Godt 1989).

1983); MPI, 6PGDH, and TO on buffer 4; AAT on buffer 7; and FE, ME, and PGI on a modified buffer 8. Calculation of genetic diversity statistics was as described in Hamrick and Godt (1989).

Results

Fifteen loci (*Aat-1*, *Aat-2*, *Dia-1*, *Fe-1*, *Fe-2*, *Me*, *Mpi-1*, *Mpi-2*, *6Pgdh-1*, *Pgi-1*, *Pgi-2*, *Pgm-1*, *Pgm-2*, *To-2*, and *Tpi-1*) were resolved. Three loci (*Aat-2*, *Fe-1*, and *Pgi-1*) (20%) were polymorphic. *Fe-1* and *Pgi-1* had two alleles, whereas *Aat-2* had three alleles. Diversity was low at polymorphic loci (H_T = 0.028), with a mean frequency of the less common alleles of 0.05. Percent polymorphic loci ranged from 0 to 20% among populations, with a mean of 4.6% (Table 1). Thus genetic diversity was low within populations (H_{ep} = 0.005) and for the species overall (H_{es} = 0.006). No polymorphism was detected in 7 of the 13 populations. The South Carolina population at Halfway Creek maintained the highest genetic diversity (H_e = 0.026). Observed heterozygosity conformed closely with Hardy–Weinberg expectations. The majority of the genetic diversity observed at the three polymorphic loci occurred within individual populations (G_{st} = 0.107).

Table 2. Genetic diversity in hemiparasitic plants as measured by allozyme analyses

Species	Populations analyzed	No. of loci	P_s	P_p	A_p	H_{ep}
Agalinis strictifolia[a]	2	11	45%	45%	2.2	0.220
Arceuthobium campylopodum[b]	9	9	90%	81%	2.8	0.263
Arceuthobium occidentale[b]	9	9	90%	86%	1.4	0.277
Arceuthobium littorum[b]	9	9	90%	82%	1.3	0.226
Arceuthobium tsugense[c]	21	13	100%	84%	2.6	0.297
Phoradendron californicum[d]	10	5	100%	86%	2.4	0.339
Schwalbea americana[e]	13	15	20%	5%	2.4	0.005
Striga asiatica[f]	2	32	0%	0%	0.0	0.000
Striga hermonthica[g]	3	9	67%	56%	2.5	0.317

P is the percent polymorphic loci; *A* is the mean number of alleles per locus; and H_e is the heterozygosity expected under Hardy–Weinberg. Parameters subscripted with a p indicate population means; those subscripted with an s indicate species values.

[a] Dieringer and Werth (1994).

[b] Nickrent and Butler (1990).

[c] Nickrent and Stell (1990).

[d] Glazner et al. (1988).

[e] This study.

[f] Werth et al. (1984).

[g] Bharathalakshmi et al. (1990).

Discussion

Genetic diversity tends to be low in endangered and endemic plant species (e.g., Godt et al. 1997; Hamrick and Godt 1989; Lesica et al. 1988; Soltis et al. 1992) and *S. americana* is no exception. The absence of genetic diversity in a species or the occurrence of very low genetic diversity can be due to events in the phylogenetic history of the species, its recent evolutionary history, or both. The relative contribution of events in evolutionary or ecological time to the reduction of genetic diversity in a species is often difficult to ascertain. However, the simultaneous determination of genetic diversity in an endemic species and in one of its more widespread congeners (e.g., Karron et al. 1988) has sometimes led to insights into the evolutionary history of the endemic. For example, it has been hypothesized that the geographically restricted and genetically depauperate species *Cirsium pitcheri* and *Erythronium propullans* were derived from more widespread congeners (Loveless and Hamrick 1988; Pleasants and Wendel 1989). The low genetic diversity in these endemics has been attributed, in part, to the loss of genetic diversity during the speciation process. Unfortunately genetic diversity comparisons with closely related species are precluded for monotypic genera such as *Schwalbea*. Nonetheless, it is worth noting that the family Scrophulariaceae as a whole is not genetically depauperate, as evidenced by the allozyme diversity found in *Mimulus caespitosus* (Ritland 1989).

To address whether hemiparasitism is associated with low genetic diversity we reviewed the plant allozyme literature for data on other hemiparasites. Allozyme diversity has been examined in several hemiparasites (Table 2), including witchweed (*Striga hermonthica* and *S. asiatica*) and *Agalinis strictifolia*, members of the Scrophulariaceae, and dwarf (*Arceuthobium* spp.) and desert mistletoes (*Phoradendron californicum*), members of the Viscaceae. With the exceptions of *S. americana* and *S. asiatica*, genetic diversity is high in these hemiparasites (Table 2), indicating that hemiparasitism per se cannot account for *S. americana*'s low genetic

diversity. The low allozyme diversity found for the weed *Striga asiatica* was attributed to the probable founding of the introduced population by one or two individuals of this obligate selfer (Werth et al. 1984).

Like *Schwalbea*, a number of federally endangered and threatened species constitute monotypic genera (e.g., *Harperocallis flava*, *Helonias bullata*, and *Howellia aquatilis*). Some conservation biologists (e.g., Holsinger and Gottlieb 1991) suggest that taxa with a high degree of genetic and morphological distinctness, particularly monotypic genera and families, should be given high conservation priority. Although monotypic genera are unique taxonomic entities, they may represent the remnants of relatively unsuccessful lineages that have had little opportunity to evolve because of limited genetic diversity. In this regard it would be interesting to determine whether monotypic endangered species maintain less genetic diversity than other endangered species. If so, it would suggest that the occurrence of low genetic diversity in these genera may be, in large part, a function of their phylogenetic history. The low genetic diversity found within *Schwalbea* as a species and the low level of population differentiation suggest that dispersal into its present range may have occurred following a genetic bottleneck, since reductions in population size and numbers across its range would be expected to fix different alleles in different populations from a richer gene pool. Nonetheless, loss of populations and reductions in population sizes cannot be discounted as having further reduced genetic diversity in this species.

The habitat requirements of *S. americana* may predispose the species to the loss of genetic diversity. A shade-intolerant species adapted to early successional habitats opened by fire, *Schwalbea* may always have been a fugitive species. Certainly its copious numbers of lightweight, wind-dispersed seeds are features characteristic of fugitive plant species (Baker 1974). Prior to modern man's intervention, fires ignited by lightning strikes occurred frequently in the southeastern United States. Irregular fires in the pine flatwoods and open savannahs where *Schwalbea* was typically found almost certainly created new habitats and opened old ones in a patchy manner. It is likely that *Schwalbea* and other herbs in these fire-maintained communities existed as metapopulations, continually colonizing newly opened habitats and being extirpated from areas in more advanced states of succession. Fire suppression probably disrupted these metapopulation dynamics. As newly opened habitats became more scarce, many *Schwalbea* populations may have crashed without effectively migrating by colonizing new sites. The lack of a seed bank and the probable requirement for mineral soil for germination (U.S. Fish and Wildlife Service 1994) may have further compounded *Schwalbea*'s difficulties.

The existence of *S. americana* remains precarious even though numerous populations were found during recent searches of appropriate habitats. Its low genetic diversity suggests low potential for adaptation to changing environmental conditions and increased susceptibility to pests and diseases (Barrett and Kohn 1991). Furthermore, the continued existence of the species is contingent on fire management regimes that favor its persistence. The frequency and season of fires that best benefit growth and reproduction of *Schwalbea* (in Georgia) are currently under investigation (Kirkman K, personal communication). Recently instituted fire management in the Southeast is unlikely to restore the species to its former abundance and distribution given the lack of a seed bank and the irregular distribution of the remaining fire-maintained communities. Hence population restoration may be required to expand the number of viable populations.

We recommend that local seed sources be used in *S. americana* population restoration, since the species is found over a large geographic range and may be adapted to local environmental conditions. Modification of this recommendation may be indicated if further insights are gained into the species' genetic structure. Sampling for ex situ conservation should include representatives of the species from across its geographic range.

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Microphthalmia: A Morphogenetic Lethal Mutation of the Campbelli Hamster (*Phodopus campbelli*)

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Microphthalmia is a new mutation of the campbelli hamster (*Phodopus campbelli*) that is controlled by an incomplete dominant autosomal gene *Mi.* The dorsal coat of the heterozygote has dark markings on a white background. The dark markings appear on the head, back, and rump. Their color is very similar to that of the wild type, but slightly lighter. The pupil and iris of the heterozygote are black with a reddish tinge. The homozygote has pure white fur all over the body and shows a smaller body size than the wild type and heterozygote. Moreover, the homozygote is characterized by small eyes with unopened eyelids and loss of the incisors. The pupil and iris are colorless and transparent or show a faint reddish tinge when the eyelid is artificially opened. In addition to these abnormalities, individual bones of the homozygote show dwarfism. The parietal and frontal region of the skull are thin and their symphysis is incomplete. The distal regions of the vertebral ribs show swelling. The homozygote is basically lethal within 3 weeks of age. A very few survivors are sterile.

A campbelli hamster (*Phodopus campbelli*) is a small hamster that inhabits Transbaikalia in Russia, Mongolia, and adjacent China from Heilungkiang through Nei Mongol to Xinjiang (Musser and Carleton 1993). The campbelli hamster resembles a sungorus hamster (*P. sungorus*), which is distributed in east Kazakhstan and southwest Siberia (Musser and Carleton 1993), in body shape and body size. Thomas (1905) first gave the scientific name *Cricetulus campbelli* to this species; Corbet (1978) classified it as *P. sungorus campbelli*; then Pavlinov and Rossolimo (1987) named it *P. campbelli.*

This species is apparently different in some points from the Syrian hamster (*Mesocricetus auratus*) and Chinese hamster (*C. griseus*), which are commonly used as laboratory animals. The campbelli hamster has the karyotype of $2n = 28$ (Magalhaes 1969), but those of the Syrian and Chinese hamsters are $2n = 44$ and $2n = 22$, respectively (Gamperl et al. 1976; Magalhaes 1969). The campbelli hamster has the sole covered with fur (Thomas 1905), and the systemic coat color turns to white under a short photoperiod (Hamann 1987). In contrast, the Syrian and Chinese hamsters have bare soles and their coat colors are constant through both short and long photoperiods.

The campbelli hamster has rarely been used as a laboratory research animal. However, we recently determined that the campbelli hamster is a good candidate for a laboratory animal, because it has small body size and good reproductive ability under laboratory conditions. In our unpublished data, this species reaches sexual maturity at about 50 days of age and the body weight is approximately 50 g. It can reproduce once in 16–30 days, with a maximum litter size of eight. A laboratory animal should be capable of many mutations for various uses in varied research fields, as is typically seen in the mouse (Doolittle et al. 1995). However, in the campbelli hamster, only two coat color mutations have been described in the scientific literature (Robinson 1996), and no disease model mutation has so far been reported.

In 1994 we acquired from a fancier a pair of unique campbelli hamsters showing spotted coat color in the dorsal region. We then propagated the spotted hamsters to perform a genetic analysis of this trait. Matings of the spotted hamsters among themselves produced unusual individuals that have pure white fur all over the body and eye abnormalities, along with spotted and wild-type animals. Almost all of the white animals died within 3 weeks of age. Thus the character was suspected to be controlled by an incompletely dominant autosomal gene with homozygous lethality. This article describes this mutant trait and its mode of inheritance.

Materials and Methods

General Care of Animals

We paired animals in a polycarbonate cage (182 × 260 × 128 mm, Clea Japan, Inc., Osaka, Japan) that was bedded with sterilized wood chips (Clea Japan, Inc.). We set the cages in a temperature- and light-controlled room (21 ± 3°C, 14 h/10 h light/dark cycle) and supplied the animals with water and commercial pellets for small rodents (CE-2, Clea Japan, Inc.) for ad libitum consumption.

Morphology

We compared external appearances of 8-day-old and adult (2 months) mutants with those of the wild-type individuals. Moreover, we compared the skeleton of 13-day-old mutants with that of the wild types.

Prior to the skeletal observation, we examined bone and cartilage differential by staining with alizarin red S and alcian blue 8GX (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The method was the same as that of McLeod (1980) .

Mating Experiment

To determine the mode of inheritance of the character, we first made reciprocal matings of spotted and wild-type hamsters. Second, we mated spotted hamsters among themselves. The segregation ratios of their progeny were analyzed by the chi-square test.

Results and Discussion

Morphology

The dorsal surface of the adult wild-type campbelli hamster showed agouti coat color, with a black spinal stripe from the top of the head to the rump, whereas that of the spotted mutant had dark markings on a white background (Figure 1). The dark markings appeared on the head, back, and rump, coloration which was near to that of the wild type but slightly lighter than the wild type. The size and shape of the dark markings were variable from individual to individual. The belly was whitish in both wild-type and spotted hamsters. However, the individual hairs of the mutant belly were pure white, in contrast to the wild-type hairs in which the basal part was gray. The sole, instep, and tail were covered with short white fur in both wild-type and spotted animals. The pupil and iris of the wild-type animals were black. Those of the spotted individuals were also black, although a careful

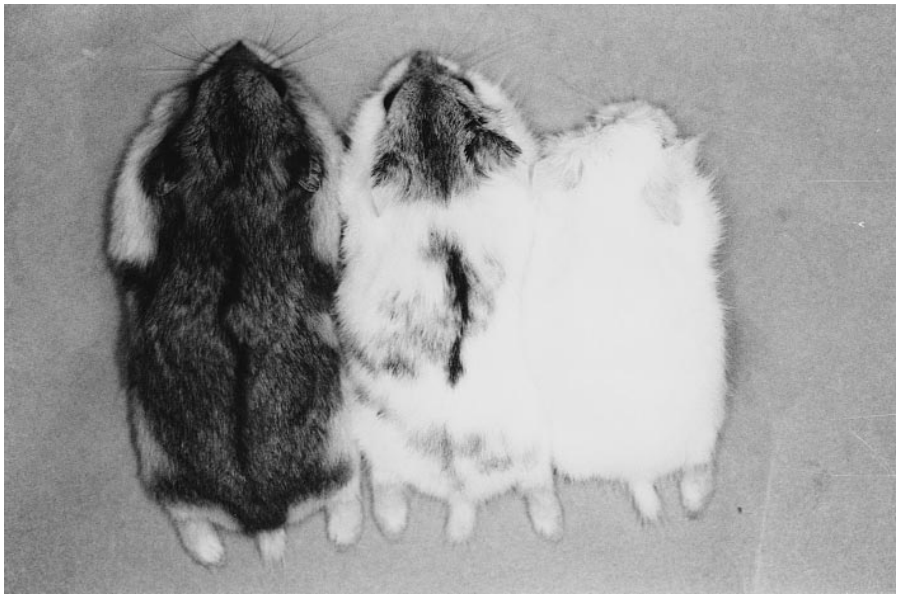

Figure 1. Dorsal view of wild-type (left), spotted (middle), and white (right) hamsters at 2 months of age. The spotted hamster shows a combination of dark and white markings on the dorsal region. The coloration of the dark marking is approximately the same as that of the wild type, but slightly lighter than the wild type. The white hamster has a smaller body than the wild-type and spotted individuals, and shows pure white coat color all over the body.

observation revealed that they had a reddish tinge.

Matings of the spotted female and spotted male segregated the third phenotype of coat color—white (Figure 1). The white animals showed smaller body size than the wild-type and spotted individuals and had pinkish skin and pure white fur all over the body. Of particular interest were the eye and mouth of the white animals. In the wild-type and spotted hamsters, the incisors started growing at 6 days of age and the eye opened at 9 days. In contrast, the white hamster had no incisors (Figure 2) and small eyes with unopened eyelids (Figure 3) throughout its life. When the eyelid was opened artificially, the pupil and iris were colorless and transparent or had a faint reddish tinge. Of 37 white animals we obtained, 36 died within 3 weeks of age. Only one female survived to adulthood, but she could not reproduce when mated to a wild-type male.

Skeletal Abnormalities

In addition to the external abnormalities mentioned, the white hamster possessed skeletal abnormalities (Figure 4). The whole skeleton of the white animal was smaller than the wild type in size, and individual bones showed dwarf appearance. Furthermore, in the skull, the parietal and frontal bones were thin, and the closure of their boundaries was incomplete. In the trunk, the distal regions of the vertebral ribs showed swelling.

Mating Experiments

Table 1 shows the results of mating experiments with the spotted mutants and wild-type hamsters. Reciprocal matings of the wild type and spotted mutant produced 97 wild-type and 94 spotted mutant hamsters. There was no difference in their segregation ratio in the reciprocal matings. This segregation ratio was in good agreement ($\chi^2 = 0.047$, $P = .83$) with the expected 1:1 ratio based on the hypothesis that the spotted hamster is a heterozygote for a single autosomal dominant gene. In the matings of the spotted animals among themselves, unique individuals that showed white coat color and abnormalities in the mouth and eye were produced, in addition to wild-type and spotted animals. The segregation ratio of wild-type, spotted, and white animals was 34:78:37, in good accordance ($\chi^2 = 0.450$, $P = .80$) with the expected 1:2:1 ratio on the basis of the hypothesis that the present character is controlled by an incomplete dominant autosomal gene, homozygotes for which show white coat color. As mentioned, the white animals were basically lethal. Thus it is concluded that (1) the character is controlled by a semidominant autosomal gene, (2) the heterozygote for the gene is viable and shows a spotted pattern in coat color, and (3) the homozygote is lethal with pure white coat color, small eyes, and no incisors. We named the mutation microphthalmia, based on the small-eyed phenotype of the homozygote, and propose the gene symbol *Mi* for the mutant gene controlling the trait.

So far hereditary microphthalmia has been found in chickens (Somes 1992; Somes et al. 1990), dogs (Collins et al. 1992; Shastry and Reddy 1994), sheep (Jackson 1990), mice (Silvers 1979), rats (Hedrich 1990), and Syrian hamsters (Robinson 1968). However, in chickens, dogs, and sheep, the mutants show no abnormalities in the pigmentation of the body, contrasting well with this hamster mutant. On the other hand, microphthalmia of mice (Silvers 1979), microphthal-

Figure 2. The incisor of wild-type (left), spotted (middle), and white (right) hamsters at 8 days of age. The incisor has already erupted in the wild-type and spotted animals, but it is missing in the white animal.

Figure 3. Lateral view of spotted (left) and white (right) hamsters at 2 months of age. The eye of the white hamster is obviously smaller than that of the spotted animal, with an unopened eyelid.

mia of rats (Hedrich 1990), and anophthalmic white of Syrian hamsters (Robinson 1968) are very similar to this hamster mutant, that is, all of them show a partial defect of skin and hair pigmentation in the heterozygous state and lack all pigmentation in the homozygous condition, with abnormalities in eye and skeleton. In addition to these rodent mutants, the silver mutant of Japanese quail (Homma et al. 1969) is also phenotypically similar to this hamster mutant. The silver mutant quail shows partially white plumage in the heterozygous state and pure white plumage with defective eyes under the homozygous condition. Although the eye abnormality of the silver mutant does not show microphthalmia, but partial lack of retinal pigmentation with normal or larger eye size (Fuji and Wakasugi 1993), the silver mutation has been revealed to be caused by the gene homologous to the mouse microphthalmia gene (Mochii M, personal communication).

The microphthalmia of mice and the anophthalmic white of Syrian hamsters were suggested by Asher and Fiedman (1990), based on the chromosomal position of the mutant genes controlling these characters, to be homologous to human disease Waardenburg syndrome type 2. Then Tassabehji et al. (1994) revealed at a molecular level that the Waardenburg syndrome type 2 is controlled by the human microphthalmia gene *MITF* and actually is a homologue of the mouse microphthalmia. The microphthalmia (*mi*) gene of mice encodes a basic helix-loop-helix leucine zipper structural protein as does the *MITF* gene of humans (Hodgkinson et al. 1993; Tassabehji et al. 1994). It would be of interest to determine at a molecular level whether the *Mi* gene of the campbelli hamster is a homologue of the microphthalmia gene of the mouse and human. This hamster mutant may be an excellent animal model to study morphogenesis of the eye, tooth, and skeleton, especially in comparative studies with similar mutants of the mouse and other species. It would also be of interest to reveal the evolutionary aspect of the microphthalmia gene possessed by various species.

Figure 4. Lateral view of the whole skeleton of wild-type (left) and white (right) hamsters at 13 days of age. The whole skeletal size of the mutant is smaller than that of the wild type. The parietal and frontal bones of the mutant skull are thin, and their symphysis is incomplete. The mutant ribs show swelling in their distal regions (arrows).

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Table 1. Segregation data for coat color phenotypes in mating experiments with spotted mutant and wild-type hamsters

Matings (dam × sire)	No. of matings set	No. of offspring examined	No. of offspring with phenotypes of: Wild-type ♀	Wild-type ♂	Spotted ♀	Spotted ♂	White ♀	White ♂	Expected ratio[a]	χ^2	*P*
Wild-type × spotted	6	80	20	18	21	21	0	0		0.200	0.65
Spotted × wild-type	9	111	26	33	28	24	0	0	1:1:0	0.441	0.51
Total	15	191	46	51	49	45	0	0		0.047	0.83
Spotted × spotted	18	149	20	14	40	38	24	13	1:2:1	0.450	0.80

[a] Based on the hypothesis that the mutation is controlled by an autosomal semidominant gene and the spotted and white phenotypes are expressed in heterozygous and homozygous states, respectively.

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Centric Fusion Polymorphisms in Waterbuck (*Kobus ellipsiprymnus*)

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Twenty-six captive individuals of the *ellipsiprymnus* subspecies group of *Kobus ellipsiprymnus* were found to have chromosomal complements of $2n = 50–52$ (FN = 61–62), and 26 of the *defassa* subspecies group, including three specimens from Lake Nakuru National Park, Kenya, had complements of $2n = 53–54$ (FN = 62). G-banded karyotypes that were numbered according to the standard karyotype of *Bos taurus* revealed that variation in diploid number was the result of polymorphism for two independent centric (Robertsonian) fusions. The *ellipsiprymnus* group was polymorphic for a 7;11 centric fusion. Both elements of chromosome pairs 7 and 11 were fused in fusion homozygotes ($2n = 50$); in fusion heterozygotes ($2n = 51$), only one element of each pair was fused. The 7;11 fusion was lacking in specimens with $2n = 52$. The *defassa* group was polymorphic for a 6;18 centric fusion; individuals were either heterozygous for the fusion ($2n = 53$) or lacking it ($2n = 54$). There were no *defassa* group individuals that were homozygous for the 6;18 fusion ($2n = 52$), but this may be a sampling artifact. The 6;18 fusion was fixed in the *ellipsiprymnus* group, whereas the 7;11 fusion was absent in the *defassa* group. In G- and C-banded karyotypes, all autosomal arms and the X chromosomes of the two subspecies groups appeared to be completely homologous. However, the Y chromosome was acrocentric in the *ellipsiprymnus* group and submetacentric in the *defassa* group, possibly the result of a pericentric inversion. Fixed chromosomal differences between the two subspecies groups reflect a period of supposed geographic isolation during which time they diverged genetically and phenotypically, and the centric fusion polymorphisms raise the possibility of reduced fertility in hybrids. These data, in conjunction with phenotypic and mitochondrial DNA data, suggest to us that populations of the *ellipsiprymnus* and *defassa* groups should be managed separately.

Chromosomal evolution in the mammalian family Bovidae is believed to have been predominated by centric (Robertsonian) fusions. For example, the diploid number of 48 species of bovids varies widely ($2n = 30–60$), but the fundamental number is relatively constant (FN = 58–62) in all but three of these taxa (Wurster and Benirschke 1968). Among species representing four of the five subfamilies of Bovidae, banded karyotypes demonstrate that chromosome-arm homologies are extensive, and that shared homologous biarmed chromosomes are rare, further indicating the importance of centric fusions in bovid chromosomal evolution (Buckland and Evans 1978; Bunch and Nadler 1980; Gallagher and Womack 1992). Monobrachially homologous biarmed chromosomes also distinguish karyotypes within certain bovid genera, notably *Damaliscus* (Kumamoto et al. 1997) and *Gazella* (Effron et al. 1976; Kumamoto et al. 1995; Vassart et al. 1995).

The degree to which centric fusions have resulted in monobrachial homologies, particularly between closely related species, raises the possibility that speciation (i.e., reproductive isolation) in bovids has occurred by monobrachial centric fusions, a model advanced by Baker and Bickham (1986). A prerequisite to the establishment of monobrachially homologous biarmed chromosomes between populations is the fixation of single centric fusions within populations. It is generally accepted that heterozygotes for single centric fusions often encounter minimal meiotic problems because trivalents are able to segregate normally (Baker and Bickham 1986), and among bovids this has been demonstrated in goitered gazelles (Kingswood et al. 1994). Thus it is not surprising that a number of bovid taxa are polymorphic for centric fusions, including African buffalo (Buckland and Evans 1978), Arabian oryx (Cribiu et al. 1990), Guenther's dik-dik (Ryder et al. 1989), blackbuck (Effron et al. 1976), impala (Wallace 1980), and several species of gazelles (Arroyo Nombela et al. 1990; Benirschke et al. 1984; Kingswood and Kumamoto 1988; Kumamoto et al. 1995).

In this article we document centric fusion polymorphisms in another bovid, the waterbuck (*Kobus ellipsiprymnus*). Taxonomically, *K. ellipsiprymnus* is comprised of two subspecies groups, the *ellipsiprymnus* group (common waterbuck) and the *defassa* group (defassa waterbuck). Ansell (1971) lists four subspecies in the *ellipsiprymnus* group and nine in the *defassa* group, but within these two groups the validity and distribution of many subspecies are not well established. Previously, chromosomes of waterbuck have been reported as $2n = 50$ (Gallagher and Womack 1992; Wallace 1980; Wurster and Benirschke 1968). The purpose of this study is to describe G- and C-banded karyotypes of common and defassa waterbuck, each subspecies group being polymorphic for one of two centric fusions.

Materials and Methods

We studied the chromosomes of 49 specimens of *K. ellipsiprymnus* from five zoos and three individuals from Lake Nakuru

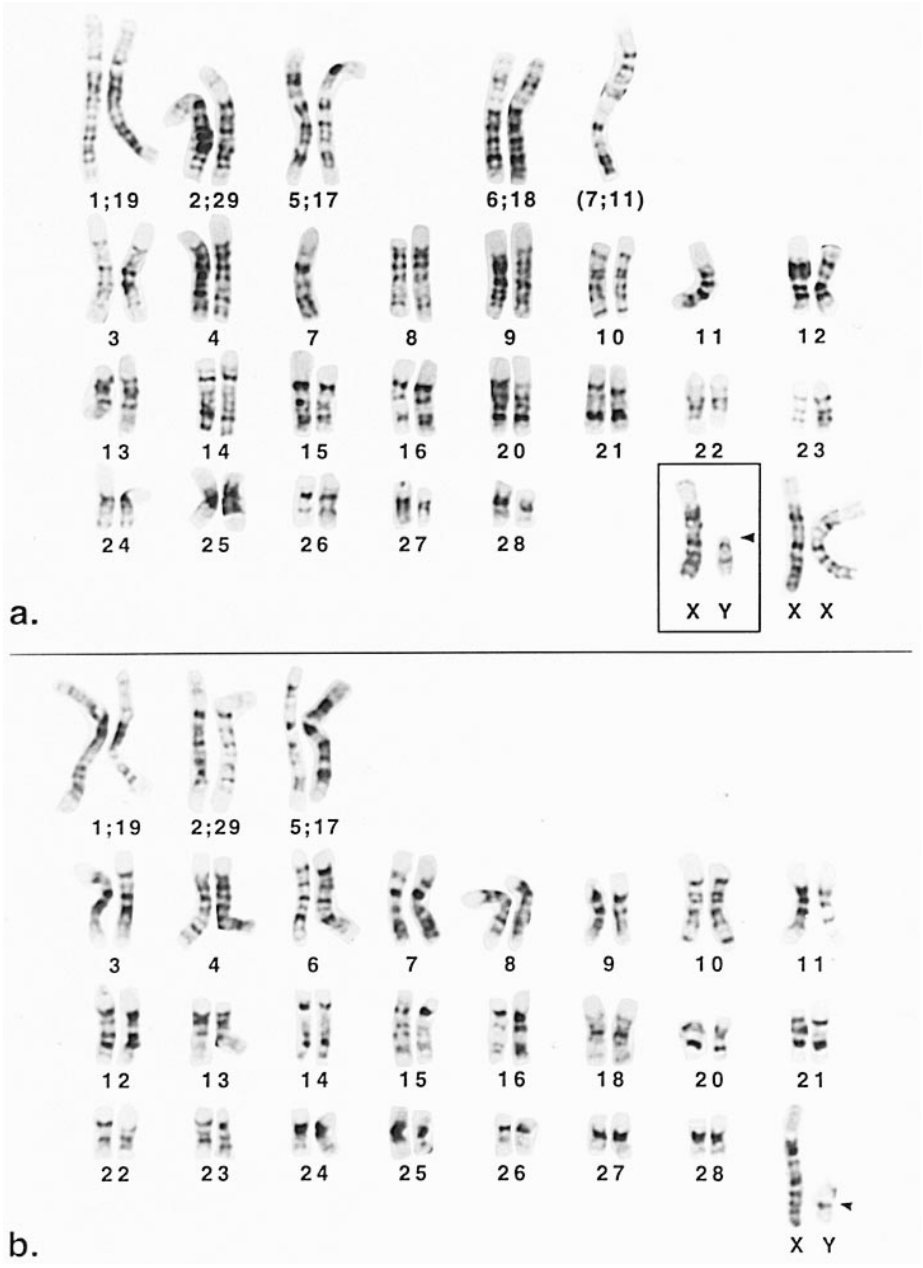

Figure 1. G-banded karyotypes of *K. ellipsiprymnus*: **(a)** karyotype of a female ($2n = 51$) from the *ellipsiprymnus* subspecies group with a boxed inset showing the G-banded sex chromosomes of a male; **(b)** karyotype of a male ($2n = 54$) from the *defassa* subspecies group. In **(a)** the polymorphic centric fusion (7;11) is in parenthesis to distinguish it from fixed fusions. Arrowheads indicate centromeric positions.

Table 1. Summary of chromosomal data for *Kobus ellipsiprymnus*

				Autosomes				Centric fusions	
Subspecies group	*N*	2*n*	FN	m/s	a	X	Y	6;18	7;11
ellipsiprymnus	4:2	50	61–62	10	38	lm	sa	++	++
	9:6	51	61–62	9	40	lm	sa	++	+−
	3:2	52	61–62	8	42	lm	sa	++	—
defassa	2:6	53	62	7	44	lm	ss	+−	—
	8:10	54	62	6	46	lm	ss	—	—

N = sample size (females : males); 2*n* = diploid number; FN = fundamental number; m/s = metacentric/submetacentric; a = acrocentric; lm = large metacentric; sa = small acrocentric; ss = small submetacentric; ++ = fusion homozygote; +− = fusion heterozygote; — = lacking fusion.

National Park, Kenya; these included 16 females and 10 males of common waterbuck and 10 females and 16 males of defassa waterbuck. Origins of the zoo animals were unknown, but differences between the rump coloration of common and defassa waterbuck (see Kingdon 1982) made it possible to phenotypically identify the subspecies group of each specimen. Metaphase chromosomes were obtained from cell cultures derived either from skin biopsies (ca. 5 mm^2) or lymphocytes from 5–10 ml of heparinized whole blood.

For short-term lymphocyte culture, we followed a modified technique of Moorhead et al. (1960) and Wiley and Meisner (1984) using pokeweed mitogen (0.3 ml) and comitogen phorbol 12-myristate 13-acetate-4-0-methyl ether (final concentration 6 μg/ml). Blood cultures were harvested after a 94 h incubation period followed by a 1 h exposure to colcemid (final concentration 0.025 μg/ml). For fibroblast culture of skin biopsies we used a collagenase disaggregation technique, harvesting cells according to the general protocol for monolayer cultures (Barch 1991). At peak mitotic activity, monolayer cultures were exposed to colcemid (final concentration 0.025 μg/ml) for 10–30 min, and cultures were then exposed to 0.075 M KCl for 10 min prior to fixation of cells.

From the mitotic cell harvests we studied the chromosomes of all 52 animals by nondifferential Giemsa staining. In addition, 30 of the specimens were G-banded using the method of Verma and Babu (1989), and 24 were C-banded following the method of Sumner (1972). Because of the difficulty in comparing G-band homologies between taxa without a standardized nomenclature, G-banded chromosomes were numbered according to the standard karyotype of cattle (*Bos taurus*) presented by the Reading Conference (1980) and Iannuzzi and Di Meo (1995). Gallagher and Womack (1992) demonstrated extensive arm homologies among several species of bovids using the cattle standard. Because waterbuck chromosomes could be identified to cattle equivalents, we followed this convention to facilitate comparisons between our specimens.

Results

Chromosomal complements were $2n = 50–52$ (FN = 61–62) in the *ellipsiprymnus* group and $2n = 53–54$ (FN = 62) in the *defassa* group of *K. ellipsiprymnus* (Figure 1; Table 1). Pericentromeric heterochromatin was pronounced on all acrocentric

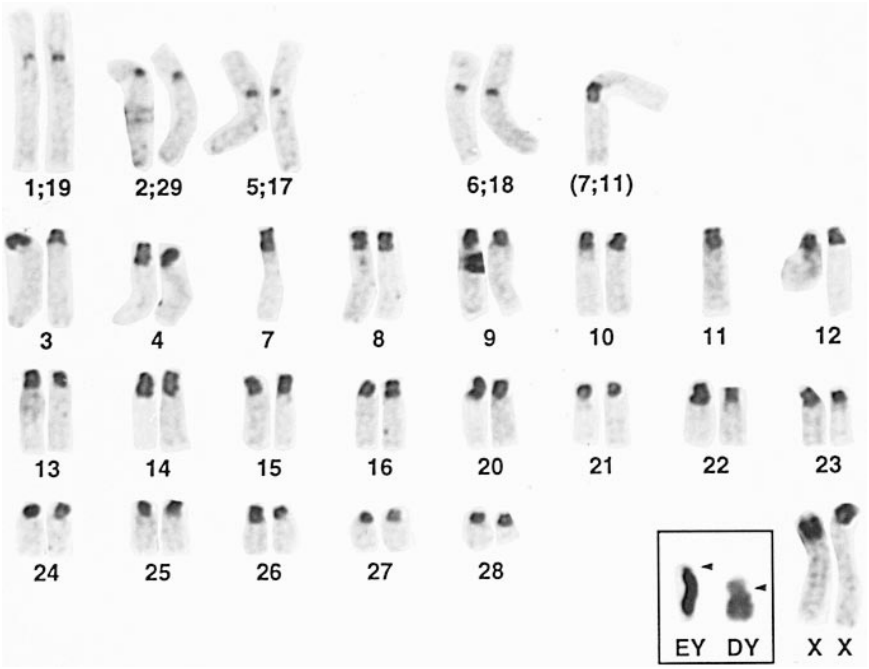

Figure 2. C-banded karyotype of a female *K. ellipsiprymnus* ($2n = 51$) with a boxed inset showing the C-banded Y chromosomes of a male of the *ellipsiprymnus* subspecies group (EY) and a male of the *defassa* subspecies group (DY). The polymorphic centric fusion (7;11) is in parenthesis to distinguish it from fixed fusions. Arrowheads indicate centromeric positions.

autosomes, but heterochromatic staining of metacentrics was faint and restricted to the centromeric region (Figure 2). In both subspecies groups the Y chromosome and short arm of the X chromosome appeared to stain entirely heterochromatic; Xp was G-band negative, corresponding to the area that was C-band positive.

Variation in the diploid number of *K. ellipsiprymnus* was the result of polymorphism for two independent centric fusions (Figure 3; Table 1). The *ellipsiprymnus* group was polymorphic for a 7;11 centric fusion. Both elements of chromosome pairs 7 and 11 were fused in fusion homozygotes ($2n = 50$); in fusion heterozygotes ($2n = 51$), only one element of each pair was fused. The 7;11 fusion was lacking in *ellipsiprymnus* group specimens with 2n = 52 and in all *defassa* group specimens. The *defassa* group was polymorphic for a 6;18 centric fusion; individuals were either heterozygous for the fusion ($2n = 53$) or lacking it ($2n = 54$). There were no waterbuck of the *defassa* group that were homozygous for the 6;18 fusion ($2n = 52$). However, this possibly reflects a sampling artifact since selection against the homozygous form of the fusion seems unlikely. The 6;18 fusion was fixed in all specimens of the *ellipsiprymnus* group.

It is worth noting that the 6;18 polymorphism was found in the one wild population of waterbuck that we studied. Among the three specimens of defassa waterbuck from Lake Nakuru National Park, one male was heterozygous for the 6;18 fusion ($2n = 53$), but the fusion was absent in the other two males ($2n = 54$). However, the extent to which the 6;18 and 7;11 polymorphisms occur naturally is unknown because the captive populations of waterbuck that we sampled may represent a mixture of individuals from different natural populations (Jones ML, in literature). We cannot rule out the possibility that the common waterbuck that were heterozygous for the 7;11 fusion ($2n = 51$) were the result of captive breeding between animals taken from two natural populations, for example, one population where individuals were homozygous for the 7;11 fusion ($2n = 50$) and the other where individuals did not have the fusion ($2n = 52$).

In G- and C-banded karyotypes, all autosomal arms and the X chromosomes of common and defassa waterbuck appeared to be completely homologous. The Y chromosomes of the two subspecies groups were of similar size, suggesting that their morphological differences (see Table 1) were not due to short-arm additions or deletions. The Y chromosomes may differ by a pericentric inversion; however, it is difficult to interpret the G-banding patterns of these small elements. All autosomal arms of both groups of *K. ellipsiprymnus* could be identified with *Bos* homologues, but the pericentromeric banding patterns of chromosomes 9 and 14 of *K. ellipsiprymnus* were more similar to those of *Capra hircus* and *Ovis aries* (see Iannuzzi and Di Meo 1995). The sex chromosomes of *K. ellipsiprymnus* and *B. taurus* differed; the cattle X was C-band negative, but the waterbuck Xp was entirely heterochromatic. Xq of *K. ellipsiprymnus* appeared homologous to the acrocentric X of *C. hircus* and *O. aries*, which was reported to differ from *B. taurus* by para- and pericentric inversions (see Iannuzzi and Di Meo 1995). The Y chromosomes of cattle and defassa waterbuck were similar in morphology (i.e., both were small submetacentrics) and both were G-band positive at the distal end of Yp. However, G-bands of the q arms differed; cattle Yq was entirely G-band positive (see Iannuzzi and Di Meo 1995), but in defassa waterbuck, Yq was G-band negative around the centromere.

Discussion

Chromosomal complements of $2n = 50–54$ (FN = 61–62) for *K. ellipsiprymnus* found in this study compare with previous reports of $2n = 50$ (FN = 61–62) for the species (Gallagher and Womack 1992; Wallace 1980; Wurster and Benirschke 1968). Each of these previous studies were based on sample sizes of only one or two specimens. Wallace (1980) studied *ellipsiprymnus* group waterbuck from a natural population in Kruger National Park, South Africa, but the other studies involved captive populations. Chromosome-arm banding patterns of G-banded karyotypes (Figure 1) correspond with those of the QFH-banded karyotype in Gallagher and Womack (1992: Figure 8), including waterbuck chromosomes 9 and 14, which are banded like the chamois, *Rupicapra rupicapra* (Gallagher and Womack 1992: Figure 5). As in our waterbuck which had complements of 2n = 50, the Gallagher and Womack (1992) waterbuck had five metacentric pairs of autosomes: 1;19, 2;29, 5;17, 6;18, and 7;11. Wallace (1980) and Wurster and Benirschke (1968) did not present karyotypes of *K. ellipsiprymnus*, but for 2n = 50 waterbuck, their data were consistent with this study.

Karyotypic differences between the *ellipsiprymnus* and *defassa* groups of *K. ellipsiprymnus* reflect differences in pelage coloration and nucleic acid sequences that are believed to have evolved during their geographic isolation (Kat 1993). Waterbuck are found in savannas throughout much of sub-Saharan Africa, but their dis-

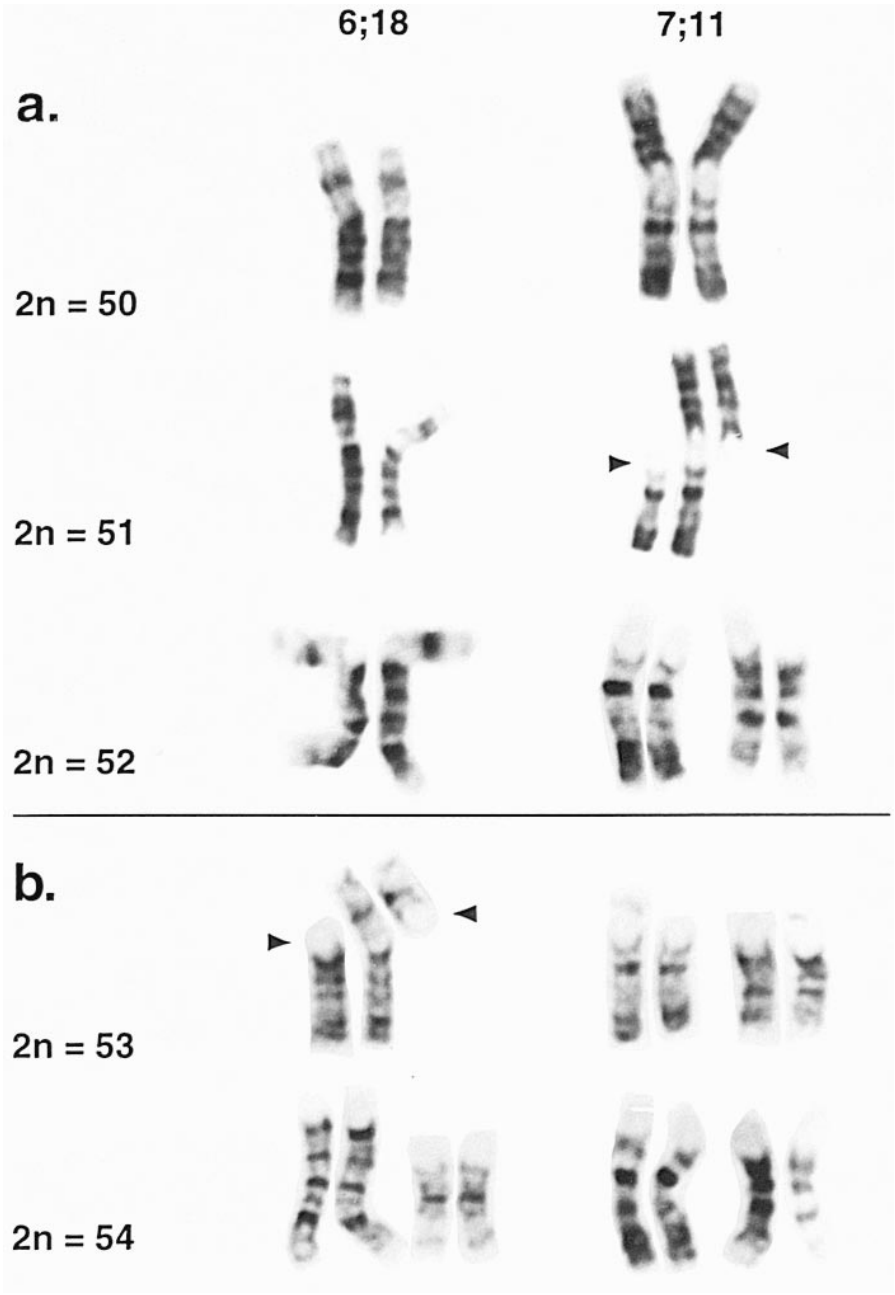

Figure 3. G-banded preparations of autosomes involved in the 6;18 and 7;11 fusion polymorphisms of *K. ellipsiprymnus*: **(a)** *ellipsiprymnus* subspecies group (2*n* = 50–52); **(b)** *defassa* subspecies group (2*n* = 53–54). Arrows indicate centromeric positions.

tribution can be rather localized owing to their preference for mesic habitats (Ansell 1971). For the most part, common waterbuck occur east of the Gregory (eastern) Rift Valley and defassa waterbuck are found west of the Albertine (western) Rift Valley. These two forms are distinguished by a difference in rump coloration; typically, common waterbuck have an elliptical white ring on a dark rump, and defassa waterbuck lack the ring because of their white rump (Kat 1993; Kingdon 1982). This difference was the basis for classification of the *defassa* group as a separate species, *K. defassa* (Ellerman et al. 1953). Based on sequence variation in the mitochondrial DNA control region, common and defassa waterbuck populations in Kenya are genetically different from each other (Kat 1993). However, the two groups are sympatric in areas of Kenya and Tanzania, probably as a result of secondary contact. Intergradation of the rump pattern and relationships based on genetic distances (i.e., nucleic acid sequence differences) among populations indicate that common and defassa waterbuck hybridize, and they are now considered conspecific (Ansell 1971; Kat 1993; Kingdon 1982). These two subspecies groups have also hybridized in captivity, and at least one individual lived several years (Gray 1972), but reproduction by hybrids has not been reported.

How is reproduction affected by the chromosomal differences between the *ellipsiprymnus* and *defassa* groups of *K. ellipsiprymnus*? Karyotypes of common and defassa waterbuck (2*n* = 50–52 and 53–54, respectively) vary largely because of two centric fusions (6;18 and 7;11), each rearrangement being polymorphic in either one subspecies group or the other. The 6;18 fusion, which is polymorphic in defassa waterbuck, is fixed in common waterbuck. The 7;11 fusion is polymorphic in common waterbuck, but this fusion is completely absent in defassa waterbuck. These differences and those involving the Y chromosomes indicate a period during which there was no gene flow between the two groups. However, putative zones of secondary contact and hybridization (see Kat 1993) suggest that common and defassa waterbuck were not separated for a period sufficient to result in reproductive isolation. In a captive population of Soemmerring's gazelles, polymorphisms for three centric fusions were suspected as being related to poor reproduction (Benirschke et al. 1984). Although it is speculative to infer possible segregational difficulties from other bovid taxa, the centric fusion polymorphisms distinguishing common and defassa waterbuck theoretically could lead to reduced fertility in their hybrids or aneuploid offspring.

In conclusion we believe it is appropriate to consider the cytogenetics of waterbuck in the management of their natural and captive populations. The importance of cytogenetics in wildlife management and conservation has been advanced by Benirschke and Kumamoto (1991) and Robinson and Elder (1993). If wildlife conservation is not simply directed toward conservation of species, but also toward the preservation of unique populations, then chromosomal data in conjunction with phenotypic and mitochondrial DNA data suggest to us that populations of common and defassa waterbuck should be managed separately. However, in order to address the conservation of waterbuck more effectively, future genetic research should be directed toward natural populations of this antelope throughout Africa.

From the Center for Reproduction of Endangered Species, Zoological Society of San Diego, PO Box 551, San Diego, CA 92112-0551 (Kingswood, Kumamoto, Charter,

and Ryder) and the National Museums of Kenya, Nairobi, Kenya (Aman). We thank Brookfield Zoo (Chicago), Busch Gardens (Tampa), Fossil Rim Wildlife Center (Glen Rose, Texas), San Diego Wild Animal Park, and Utah's Hogle Zoo (Salt Lake City, Utah) for providing samples from animals in their collections; specific thanks go to N. Abou-Madi, J. Allen, R. Anderson, E. Blumer, T. De Maar, P. Ensley, C. Miller, G. Nachel, J. Olsen, J. Oosterhuis, A. Petric, S. Schofield, J. Zdziarski, and J. Zuba. In Kenya, samples were collected by J. Jonyo and P. Kat. M. Houck and B. Kiawa helped with laboratory work. M. Jones and International Species Information System shared their extensive records of animals in captivity. K. Kelley and F. Schmidt helped produce the figures. K. Benirschke, D. Gallagher, and M. Houck reviewed the manuscript and suggested helpful revisions. This study was supported through grants from the Pew Charitable Trusts, John and Beverly Stauffer Foundation, and Zoological Society of San Diego. We dedicate this article to the memory of our friend, John Jonyo.

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Head Spot and Dilute Mutations in the Norway Rat

R. Robinson

A mutant allele of a new white spotting locus in the Norway rat is described, which is designated as head spot (*hs*). The allele is inherited as a recessive to normal. The expression is regularly manifested and has the form of a white spot of variable size in the middle of the forehead just above the eyes. A probable reoccurrence of blue dilution coat color is also reported. Tests for genetic linkage for *hs* indicate that it is independent of the agouti, dilute, and hooded loci.

A white spotting locus, *H,* has been known for the Norway rat since the earliest days of mammalian genetics and is featured prominently in the classic selection experiments of W. E. Castle (see summary in 1951). The locus has been designated hooded after its best known mutant allele (*h*). The allele produces a characteristic colored head and white body, with a colored spinal stripe that is often, if not usually, incomplete from head to tail. The normal gene is incompletely dominant since the heterozygote *Hh* typically shows a variable white patch on the stomach. A second allele for the locus is known as Irish spotting (h^i), which induces a white spot or an inverted V blaze on the forehead and variable amounts of white on the stomach. The order of dominance is $H > h^i > h$, although this is usually incomplete. In particular, the heterozygote *Hh* can be phenotypically similar to Hh^i.

It should be noted that it is not uncommon for individual rats to possess a variable white patch on the stomach independent of the hooded alleles. This appears to arise by chance ineffective migration of melanoblasts from the neural crest. The size of the patch can be readily increased by selection and is probably mediated by polygenes. The h^i allele can increase the size of the patch, while the *h* allele produces a fully white stomach.

The present blue mutant rat was discovered in a shop that sells rodents as pets by a fancier who was sufficiently knowledgeable to realize that the color was novel. The coat is slate blue and agrees closely with the description given by Roberts (1929) for a previously discovered blue dilution mutant.

Materials and Results

The head spot trait was observed as a segregant from a stock of agouti animals that were of wild origin (Greaves 1981; Robinson 1988). Interbreeding of the mutant showed that head spot was consistently expressed. The size of the spot was variable, ranging from a small spot of a few hairs to roughly 1 cm in diameter. Over 200 individuals were examined. The stomach frequently had a variable white patch that did not differ in size from belly spots commonly found in rats.

To determine the mode of inheritance of the head spot, agouti spot rats were mated to nonagouti (black) rats. The F_1 prog-

eny were agouti without head spots and the F_2 assorted into 40 agouti, 11 agouti head spot, 9 black, and 4 black head spot, indicating simple recessive inheritance of the head spot. The symbol *hs* is proposed for the mutant allele. The data also indicate that *hs* assorts independently of nonagouti, the linkage χ^2 of 0.95 for one degree of freedom being insignificant. Almost all of the F_2 rats had a variable white stomach patch. Little or no difference of patch size could be seen between those individuals with head spot and those without.

A stock was developed from the above F_2 in which selection was continued for five generations to increase the size of the head spot. The size of the spot became larger in a small number of individuals. Eventually the spot became a blaze in the form of an inverted V extending from the nose to the top of the head. Quite often the blaze was irregular in that it was off-center from the midline of the face. Breeding rats with the blaze increased the proportion of those with the blaze over succeeding generations but failed to extend the area. The area of white on the stomach remained variable but did not increase in size, or if it did increase, the increase was not quantifiable.

To test for possible allelism of *hs* to hooded and to ascertain the mode of inheritance of the blue mutant, a cross was made between agouti head spot and nonagouti blue hooded. The F_1 was agouti without head spots and with a small enlargement of the stomach patch, which is to be expected due to the heterozygosity for the hooded allele. More notable, 20 animals, both male and female, of the F_1 were devoid of a stomach patch.

The 350 F_2 assorted into the 12 phenotypes as shown by Table 1. All of the phenotypes were to expectation except for the *h/h hs/hs* combination. Here the amount of white in the coat was increased, especially on the head. A few of the individuals were typical hooded with a head spot, a larger number had a small blaze but many had a large blaze that covered most of the forehead. The colored spinal strip was reduced in expression, shorter than usual and less continuous. The two alleles *h* and *hs* interacted to break up the semiregular expression of the hooded pattern. The expectations for entries in the table are 9:3:3:1 for each row, but 9:3:4 for each column, because separating the dilute class as either agouti or nonagouti proved to be unreliable. The dilute allele has been provisionally symbolized as *d.*

Statistical assessment of assortment of the genes, both singly and in pairs, for possible linkage is shown by Table 2. None of the monogenic segregations depart significantly from expectation. The largest departure is for *hs,* and the χ^2 value falls just short of being significant. It is probable that some dilute progeny with very small head spots could have been missed due to the light coat. Not one of the tests of association between the loci is significant. It may be assumed that either the genes are loosely linked or they are on different chromosomes. The test between *A* and *D* could not be calculated because of the recording difficulty noted above. However, Roberts and Quisenberg (1936) found no evidence of linkage between *A* and a blue dilution mutant that appeared to be a repeat of an earlier mutant of Roberts.

Table 1. Phenotypic classification of the F_2 progeny from the cross of agouti head spot with nonagouti dilute hooded

	Normal	Head spot	Hooded	Hooded spot
Agouti	129	31	32	10
Nonagouti	41	12	14	3
Dilute	51	11	11	5

Discussion

The discovery of a second locus for white spotting in the Norway rat should be recorded because only one locus for white spotting has been reported; in spite of several apparent repeated occurrences of h^i and *h* (reviewed by Robinson 1965), the reporting of a third allele known as notch (Castle 1951), and the description of a fourth allele (H^{re}) which appears to be a complex entity (Gumbeck and Stanley 1971).

The blue dilution mutant allele was first discovered by Roberts (1929). A repeat of the dilution allele was described by Curtis and Dunning (1940) and the identity was confirmed by crosses between the two mutants. Breeding experiments to establish the probable identity of the present dilute mutant to that of Roberts were impossible because stock with the original allele was unobtainable. The dilution allele is included in this report because of the linkage test with head spot.

Table 2. Statistical analysis of monogenic assortment of genes and tests for independent assortment

Monogenic assortment		Independency	
Gene	χ^2	Loci	χ^2
a	0.08	*a–h*	0.37
d	1.38	*a–hs*	0.04
h	2.38	*d–h*	0.05
hs	3.67	*d–hs*	0.58
		h–hs	0.87

Gene symbols as in text except for nonagouti (*a*). All χ^2 have one degree of freedom and are insignificant.

From St. Stephens Nursery, St. Stephens Rd., London, W13 8HB, UK. This article is being published posthumously. Dr. Robinson died June 16, 1996.

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Inheritance Mode of *Drosophila simulans* Female Mating Propensity With *D. melanogaster* Males

M. C. Carracedo, A. Asenjo, and P. Casares

The genetic factors involved in the sexual isolation between *Drosophila simulans* females and *D. melanogaster* males are hardly known. We present a study of female mating propensity of *D. simulans* from different geographic origins with *D. melanogaster* males. Our results reveal the existence of genetic variability in *D. simulans* for this trait. Female discrimination is dominant against high female interspecific mating, which differs from that detected in other *Drosophila* species crosses.

Drosophila melanogaster and *D. simulans* are sibling species that often coexist in nature. Gene flow between them is prevented by the occurrence of partial inviability

and total sterility of the hybrids. Hybridization between *D. melanogaster* females and *D. simulans* males occurs with relative ease in the laboratory (Carracedo and Casares 1985; Das et al. 1995; Parsons 1972; Sturtevant 1920; Watanabe et al. 1977) and in nature (Inoue et al. 1990; Kamping and Van Delden 1988; Mensua and Perez 1977; Sperlich 1962; Tracey et al. 1973). The success of this heterospecific cross is under a polygenic system with additive effects (Carracedo et al. 1989; Eoff 1975; Izquierdo et al. 1992). In *D. melanogaster* females, the genes involved in it appear located in all the three major chromosomes (Carracedo et al. 1995).

The reciprocal cross, *D. simulans* females and *D. melanogaster* males, is very rare (Biddle 1932; Carracedo and Casares 1985; Manning 1959; Parsons 1972; Sturtevant 1929) and only occasional heterospecific matings involving some strains or populations have been reported (Barker 1962, 1967; Ronen 1957). In the last few years, however, several articles have shown that hybridization in this direction of mating sometimes occurs more frequently than previously expected. Strain-dependent differences in the success rate of interspecific matings indicate that the frequency of hybridization between *D. simulans* females and *D. melanogaster* males also has a genetic basis (Uenoyama and Inoue 1995; Welbergen et al. 1992). Welbergen et al. (1992) revealed the existence of an important component of dominance for high hybridization frequency in *D. melanogaster* males. However, they were unable to determine the genetic nature of such a trait in females of *D. simulans,* since no differences were detected among the inbred lines they used.

Cytoplasm effects on sexual isolation in the *D. melanogaster* species group have not been reported. However, this possibility in the *melanogaster-simulans* pair cannot be ruled out, since several sorts of cytoplasmic incompatibility between strains and populations of *D. simulans,* which appear as variable degrees of progeny inviability, have been found (Hale and Hoffmann 1990; Hoffman and Turelli 1988; Hoffmann et al. 1990; Louis and Nigro 1989; Montchamp-Moreau et al. 1991; O'Neill and Karr 1990).

In this article we used five *D. simulans* populations from a wide geographic range: Athens (A), Tel-Aviv (T), New Caledonia (N), Seychelles (S), and Spain (P). We performed diallel crosses between them to obtain 20 types of F_1 which, together with the five pure populations, were used to investigate the inheritance mode of the interspecific female mating propensity of *D. simulans.* This design also permits us to analyze the possible influence of the cytoplasm in sexual isolation. Males from two *D. melanogaster* strains, MC and ML, caught in Asturias (Spain) in 1992, were used in the experiments.

Table 1. Female hybridization percentages of five *D. simulans* populations obtained from dissection of females and from offspring appearance

D. simulans female population	*D. melanogaster* male strain	Method				
		Dissection (%)	Offspring (%)	χ^{2a}	Pooled (%)	χ^{2b}
A	ML	20.00	31.43	1.19	25.71	18.16**
	MC	62.86	60.00	0.06	61.43	
T	ML	22.86	11.43	1.61	17.14	18.26**
	MC	54.29	48.57	0.23	51.43	
N	ML	5.71	11.43	0.73	8.57	9.26*
	MC	34.29	22.86	1.12	28.56	
S	ML	100.00	91.43	3.13	95.71	0.59
	MC	94.29	91.43	0.22	92.86	
P	ML	0.00	0.00	0.00	0.00	3.07
	MC	2.86	5.71	0.35	4.29	

Each population was tested with two different *D. melanogaster* male strains. Percentages are based on 35 females. Statistical analyses were done by 2×2 contingency chi squares with 1 df.

[a] Between-methods comparison.

[b] Between-males comparison within a female population.

* $P < .01$, ** $P < .001$.

A = Athens; T = Tel Aviv; N = New Caledonia; S = Seychelles; P = Spain.

Materials and Methods

Female interspecific mating can be estimated by the appearance of hybrid offspring or by the presence of foreign sperm in the female. To test for possible differences between these methods, 10 *D. simulans* virgin females and 10 *D. melanogaster* males were placed in a glass cylinder vial (12×3 cm) with 10 cc of food (baker's yeast 100 g, sugar 100 g, agar 12 g, salt 2 g, propionic acid 5 ml, water 1000 ml). Five days later, half of the females were individually placed in vials and reared in order to examine whether or not they left hybrid progeny; the other half were dissected in Ringer's solution and their ventral receptacle and spermathecas examined under the microscope for sperm presence. For each *D. simulans* population (A, T, N, S, and P), 140 females were examined, 35 for each combination of male type (MC or ML) and technique (hybrid offspring or female dissection).

Comparisons between the two techniques and between the two male strains were carried out by 2×2 contingency chi squares with 1 df (Table 1). Differences between techniques were not significant, but between male types, differences were significant in the sense that MC hybridized more than ML. Hereafter we used the appearance of offspring as a measure of hybridization, because it was easier to work with. We used MC as tester males because of their higher hybridization frequency.

Female interspecific mating propensity was examined in the 5 *D. simulans* populations and in the 20 F_1 crosses between them. For each cross, a total of 120 females were examined. All the interspecific mating tests were conducted at 21°C, under 12 h light:12 h darkness cycles.

Results

The hybridization percentages shown by the five parental populations appear at the top of Table 2. Comparisons between populations were carried out by homogeneity tests in the binomial distribution (Snedecor and Cochran 1967). Because differences were significant ($\chi^2 = 262.34$, df = 4, $P < .001$), we ascertained the population order by comparing the percentages by a posteriori unplanned STP method (Sokal and Rohlf 1995). The result of this test showed differences in hybridization frequency between all the populations, in the order S > A > T > N > P. This variation suggests an important genetic component for this trait in *D. simulans.*

The percentages of hybridization frequency in the 20 F_1 crosses are also shown in Table 2. To test for cytoplasmic effects on female mating propensity, we compared the values of the reciprocal crosses by contingency chi squares with 1 df (Table 2). In general, no significant differences were detected, indicating the absence of maternal effects.

To examine the inheritance mode of female propensity, we compared the mean value of each pair of reciprocal crosses

Table 2. Hybridization percentages of *D. simulans* females with *D. melanogaster* MC males in 5 populations and in 20 F_1 interpopulational crosses

Populations	Hybridization (%)	
A	65.83	
T	51.66	
N	28.33	
S	92.50	
P	4.17	
Interpopulational crosses Females × males		χ^2
A × T	17.50	0.08
T × A	16.66	
A × N	18.33	0.24
N × A	20.83	
A × S	55.83	1.10
S × A	62.50	
A × P	2.50	0.15
P × A	3.33	
T × N	25.00	0.02
N × T	25.83	
T × S	34.17	11.38*
S × T	55.83	
T × P	0.80	3.67
P × T	5.00	
N × S	65.83	0.87
S × N	60.00	
N × P	10.00	3.10
P × N	4.17	
S × P	8.33	0.06
P × S	7.50	

Contingency chi squares with 1 df compare reciprocal crosses. Each percentage is based on 120 females.

* $P < .001$.

Table 3. Average percentages of *D. simulans* female hybridization with *D. melanogaster* MC males in parental *D. simulans* populations and in their corresponding F_1 reciprocal crosses

Parental populations	Average (%)	F_1 crosses	Average (%)	χ^2
A and T	58.75	A × T and T × A	17.18	88.50*
A and N	47.08	A × N and N × A	19.58	40.84*
A and S	79.17	A × S and S × A	59.17	22.51*
A and P	35.00	A × P and P × A	2.91	80.36*
T and N	40.00	T × N and N × T	25.42	11.59*
T and S	72.08	T × S and S × T	45.00	36.27*
T and P	27.92	T × P and P × T	2.92	57.52*
N and S	60.42	N × S and S × N	58.75	0.32
N and P	16.25	N × P and P × N	7.08	12.20*
S and P	48.33	S × P and P × S	7.92	96.97*

Each percentage is based on 240 females. Comparisons between parental and F_1 crosses were done by 2 × 2 contingency chi squares with 1 df.

* $P < .001$.

with the mean of the two corresponding parental populations by contingency chi-square tests with 1 df. If the effect of genes involved in this trait are essentially additive, both values should not differ. In 8 out of 10 comparisons, the means of reciprocal crosses were significantly smaller than the means of the corresponding parental populations (Table 3), showing dominance for low interspecific mating. The A-T crosses had lower hybridization than both parental populations suggesting overdominance for low expression of the trait. In contrast, the N-S crosses were not different from the mean values of the N and S populations. These results suggest different genetic systems for interspecific female mating propensity in some populations.

Discussion

Our results show that, for the trait receptivity of *D. simulans* females to *D. melanogaster* males, there is dominance for low receptivity in most *D. simulans* populations. This finding contrasts with the additive effects found in the same cross by Uenoyama and Inoue (1995), and in the reciprocal *D. melanogaster* females to *D. simulans* males cross by Carracedo et al. (1989), Eoff (1975), and Izquierdo et al. (1992). The differences detected in the mode of inheritance of female sexual isolation in some of our populations and the results of other authors are consistent with the hypothesis that the genes involved in speciation depend on different evolutionary processes in each population and species (Gould 1980; Lande 1982; Stanley 1979; Templeton 1981; Wright 1982).

Interpopulational genetic variation for hybridization frequency between *D. simulans* females and *D. melanogaster* males has been demonstrated (Das et al. 1995; Uenoyama and Inoue 1995; Welbergen et al. 1992, and the present article). However, the finding of interpopulation variation does not necessarily indicate the existence of intrapopulation variation. Thus, Welbergen et al. (1992) did not find variability in the *D. simulans* female mating propensity with *D. melanogaster* males, although their results could be due to the small number of lines analyzed and/or the lack of genetic variation for this trait in their base population. In this respect, our finding of dominance for low interspecific mating propensity in our *D. simulans* populations suggests the existence of natural selection against females that hybridize with foreign *D. melanogaster* males, selection that would deplete genetic variation for this trait. This selection, although it remains to be proved, could account for the low frequency of hybridization of *D. simulans* females with *D. melanogaster* males found in most populations.

D. simulans from the Seychelles has a remarkably high mating propensity with *D. melanogaster* males. One explanation of this fact could be the lack of selection against hybridization, since *D. melanogaster* is not present in these Islands (Lachaise et al. 1988). If this is correct, one should accept that reproductive character displacement for low hybridization has been acting in females of sympatric *D. simulans* populations. This conjecture might be erroneous however, since in the reciprocal cross, the frequency of hybridization is mainly determined by female receptivity (Carracedo et al. 1987, 1991, 1995), and is independent of the sympatric or allopatric origin of the examined populations (Carracedo et al. 1987; Das et al. 1995).

Coyne (1996) and Coyne et al. (1994) showed the importance of pheromones in sexual isolation among related species of the *D. melanogaster* group. While the populations in our study may differ in their cuticular hydrocarbon composition, our results suggest that several genetic systems, with different inheritance modes, are involved in female interspecific mating propensity. We agree with Paterson (1985) and Templeton (1989) that genes for sexual behavior may be very important in female intraspecific mating discrimination, leading to barriers against interspecific hybridization.

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Tobiano Spotting Pattern in Horses: Linkage of *To* With Al^A and Linkage Disequilibrium

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In a study of 2,786 tobiano and non-tobiano horses involved in paint horse breeding programs throughout the United States, the inheritance of the tobiano color pattern gene was tracked in pedigrees using the tightly linked polymorphic albumin gene. The dominant tobiano allele (*To*), which produces the tobiano spotting pattern in horses, was in coupling with both Al^A and Al^B alleles at the albumin locus. The frequency of the *To*:Al^A linkage phase, among all the homozygous tobiano horses in this study including offspring and parents ($N = 127$), was 0.08. The *To*:Al^B linkage phase was the most frequent (0.92). Linkage disequilibrium exists between the tobiano and albumin loci. The linkage disequilibrium parameter (D) was calculated as $D = 0.056$ at 79% of maximum linkage disequilibrium.

The tobiano spotting pattern is one of two types of white spotting patterns recognized by the American Paint Horse Association (1995) and is inherited as a simple Mendelian autosomal dominant allele (Castle 1954; Jones and Bogart 1971; Trommershausen-Smith 1978). This spotting pattern was originally referred to as a dominant piebald pattern (Klemola 1933). The white occurs on the neck, shoulders, back, or croup in what appears to be a dorsal to ventral distribution, but there is considerable phenotypic variation in the amount of white and the anterior-posterior positioning of the color pattern on the horse. The tobiano locus has been shown to be closely linked to the serum protein albumin locus (Trommershausen-Smith 1978) and is in equine linkage group II with six additional loci (Andersson and Sandberg 1982; Bowling 1987). Within this overall linkage group, the tobiano locus is part of a tight linkage cluster with both the albumin locus and the vitamin D binding protein locus (Bowling 1987; Sandberg and Juneja 1978; Weitkamp and Allen 1979). No recombination has been reported within this gene cluster and the gene order has not yet been determined. Bowling (1987) reported finding a conserved phase association between the dominant tobiano allele (*To*), the vitamin D binding *S* allele and the albumin *B* allele (*To*:Gc^S:Al^B).

For the past 10 years we have been using the tobiano-albumin linkage to follow the inheritance of the dominant tobiano allele and to distinguish between heterozygous and homozygous tobiano horses for horse breeders in North America who are interested in setting up homozygous (true-breeding) tobiano lines. In the course of this work we have documented tobiano lineages with a phase association between the dominant tobiano allele and the albumin *A* allele (*To*:Al^A), in addition to the more common association with the albumin *B* allele (*To*:Al^B). This communication reports our findings from these studies and estimates the linkage disequilibrium between the tobiano, non-tobiano locus alleles and the *A* and *B* alleles at the albumin locus.

Materials and Methods

Tobiano marker analysis was performed on samples from more than 4000 horses sent in by paint horse breeders throughout the United States and Canada. The horses were predominantly registered with the American Paint Horse Associa-

Table 1. Number of horses in each combination of genotypes for the tobiano, non-tobiano, and albumin *A* and *B* alleles

Tobiano genotypes	Albumin genotypes				Allele frequency	
	AA	AB	BB	N	Albumin *A,*	*B*
t t (non-tobiano)	148	603	601	1,352	$Al^A = 0.33$	$Al^B = 0.67$
To t (tobiano)	30	470	807	1,307	$Al^A = 0.20$	$Al^B = 0.80$
To To (tobiano)	1	17	109	127	$Al^A = 0.08$	$Al^B = 0.92$
Total	179	1,090	1,517	2,786	$Al^A = 0.26$	$Al^B = 0.74$

To = tobiano allele, *t* = non-tobiano allele; *A* and *B* are the albumin alleles. The albumin allele frequencies are given on the right for the three tobiano-related subsets (the non-tobiano horses, the heterozygous tobiano, and the homozygous tobiano horses), as well as for the study as a whole.

Table 2. Observed versus expected tobiano-albumin haplotypes among 2,786 tobiano and non-tobiano horses

Haplotype	Calculation[a]	Expected numbers	Observed numbers
$t:Al^A$	$5{,}572 \times 0.72 \times 0.26$	1,043	1,353
$t:Al^B$	$5{,}572 \times 0.72 \times 0.74$	2,969	2,658
$To:Al^A$	$5{,}572 \times 0.28 \times 0.26$	406	95
$To:Al^B$	$5{,}572 \times 0.28 \times 0.74$	1,154	1,466

To = dominant tobiano allele (frequency, 0.28), *t* = non-tobiano allele (0.72); albumin alleles = *A* (0.26) or *B* (0.74).

[a] Calculation for expected numbers = number of alleles × allele frequency.

tion or with the American Quarter Horse Association. Color pattern was verified on each horse by photograph or drawing. Heterozygosity versus homozygosity for the dominant tobiano allele was deduced based on establishing inheritance of the albumin alleles from sire and dam or by confirmation of inheritance of color pattern and albumin alleles in subsequent offspring. Linkage phase between specific tobiano and albumin alleles could be established in 2,768 horses. The *Gc* locus was not examined in these determinations.

Blood for the analysis of albumin was collected in both serum clot tubes and in EDTA or heparin blood tubes and shipped to the lab by 1- to 2-day delivery. Both serum and plasma albumins were analyzed by starch gel electrophoresis using a sodium acetate, EDTA buffer system at pH 5.0 (Harris and Hopkinson 1976). The electrophoretically "fast" allozyme is referred to here as the Al^A allele and the "slow" allozyme as the Al^B allele following Bowling (1987), Stormont and Suzuki (1963), and Trommershausen-Smith (1978). A third electrophoretic variant, Al^I (Sandberg 1972) was found in two related horses and was not in coupling phase with the dominant (*To*) allele of the tobiano gene.

Hardy–Weinberg equilibrium was tested by chi-square analysis, with a significance of $P < .05$. Linkage disequilibrium between the tobiano and albumin genes was calculated following Hartl (1980). The disequilibrium parameter ($D = P_{11}P_{22} - P_{12}P_{21}$) was compared to its hypothetical maximum by $D/D_{max} \times 100$.

Results

The distribution of tobiano and albumin genotypes for the 2,786 horses for which linkage phase could be determined is given in Table 1. We looked at the distribution of the albumin *A* and *B* alleles among the sample as a whole and among each of the tobiano and non-tobiano classes as subsets of the whole (allele frequencies given in Table 1). Although this sample of horses is not known to be from a randomly mating population, the albumin *A* and *B* alleles were distributed at Hardy–Weinberg equilibrium in the overall sample ($N = 2{,}786$; $\chi^2 = 0.78$, $P = .75$–$.5$; Zar 1974). The frequencies of the albumin alleles among the non-tobiano horses ($N = 1{,}352$) were also distributed at equilibrium among this subgroup ($\chi^2 = 0.11$, $P = .95$–$.9$), and although the allele frequencies among the homozygous tobiano horses compared with the non-tobiano subset were much different, the alleles were distributed at random ($N = 127$; $\chi^2 = 0.06$, $P > .95$). However, the frequencies of the albumin alleles among the heterozygous tobiano horses ($N = 1{,}307$) were not only different from those among the non-tobiano horses, but were not distributed at equilibrium ($\chi^2 = 16.79$, $P < .001$). This lack of equilibrium distribution in one of the tobiano subsets led us to quantify the linkage disequilibrium between the two loci.

Of the 1,434 tobiano horses tested, 1,307 (91%) were heterozygous and 127 (9%) were homozygous. The predominant linkage phase among the tobiano horses of this study group was $To{:}Al^B$. However, we found the opposite phase association of $To{:}Al^A$ in 94 horses, and 18 of these were homozygous tobianos (one with only *A* albumin alleles and 17 with both an *A* and a *B* allele). The rest were heterozygous tobianos with a *To*:*Al*A linkage phase and a non-tobiano allele linked with either an albumin *A* or *B* allele. We have followed the inheritance of this linkage phase through three generations. For one $To{:}Al^A/To{:}Al^B$ stallion, established by breeding record to be homozygous for the tobiano gene, we have verified both $To{:}Al^A$ and $To{:}Al^B$ linkage phases in his tobiano foals.

Comparison of the observed versus expected haplotype frequencies for the tobiano and albumin genes (Table 2) suggests significant linkage disequilibrium (χ^2=447, $P < .0001$). The linkage disequilibrium parameter (D) was calculated as $D = 0.057$ and the percent of maximum disequilibrium ($D/D_{max} \times 100$) was 79%.

Discussion

The horses examined in this study were almost entirely of quarter horse origin. The frequencies of the Al^A and Al^B alleles among the non-tobiano horses ($N = 1{,}352$; $Al^A = 0.33$, $Al^B = 0.67$) was similar to that reported by Bowling and Clark (1985) for quarter horses ($A = 0.355$, $B = 0.645$). In contrast, the albumin allele frequency estimated from the homozygous tobiano horses ($N = 127$) was $Al^A = 0.08$, $Al^B = 0.92$. The two alleles were distributed at equilibrium among the non-tobiano horses and the homozygous tobiano horses, but not among the heterozygous tobiano horses. Presumably this is a reflection of the linkage disequilibrium resulting from different distributions of the albumin alleles among non-tobiano horses and the tobiano founders of this breed in the United States. Due to the tight linkage of the tobiano and albumin loci (Trommershausen-Smith 1978), the albumin alleles have not reached equilibrium in the heterozygous tobianos. Trommershausen-Smith (1978), following inheritance of tobiano and albumin genes from a doubly heterozygous tobiano stallion, reported complete linkage disequilibrium between the dominant tobiano allele and the *B* allele of the albumin locus ($To{:}Al^B$). We have not detected any crossovers between the tobiano and albumin genes in any of the lineages followed in our laboratory, supporting tight linkage, but we have found and followed the inheritance of the opposite phase ($To{:}Al^A$). The estimate of linkage disequilibrium based on our data is $D = 0.056$, which is 79% of the maximum linkage disequilibrium for these two loci.

The $To{:}Al^A$ linkage phase was found in

at least six distinct tobiano lineages, but these lineages could only be examined in detail three to four generations back. There is a very long history of the tobiano spotted pattern, as paintings from the 17th and 18th centuries in Europe show horses with this pattern. It would be interesting to investigate the American lineages further to see whether the Al^A and Al^B phase differences can be used to represent historic recombination between the tobiano and albumin loci from a common ancestor. On the other hand, there is also the unlikely possibility that the Al^A and Al^B phases have arisen as independent mutations of the tobiano or albumin genes.

Use of the albumin locus as a marker for tracking inheritance of the dominant tobiano allele is becoming more common among breeders in North America, with the result that the numbers of identified homozygous tobiano horses (especially mares) has increased and the frequency of the *To*:Al^A linkage phase in offspring of the last 5 years has also increased slightly (0.09 in the offspring alone, compared to 0.08 in the older horses). There were 127 homozygous tobianos present in our current study, double the number of the previous year. Breeders are continuing to work toward breeding for and identifying homozygous tobianos using the albumin gene as a marker. As they use both linkage phases in their breeding programs, the frequency of the tobiano-albumin linkage phases can be expected to continue to change.

Tobiano is considered to be a dominant trait; however, Bowling (1987) reported four examples of tobianos which had nontobiano parents, but a tobiano grandparent. Parentage was confirmed. The "nontobiano" parents were reported to exhibit characteristic white markings on all four legs, but to have no characteristic white crossing the dorsal midline. In the course of our study we had minimally spotted foals reported from homozygous tobiano matings. Two of the cases that were investigated proved to be nonpaternity; however, in the others, the midline white was expressed only around or on the tail, or on one side or the other of the dorsal line. The animal would not fit the definition for "tobiano" used by the American Paint Horse Association. This highlights an area for further investigation. The finding of minimal color and the possibility of developmental variance in the positioning of the tobiano spotting pattern, including the extent and position of the midline white, white on the face, and white on the legs, suggests that the tobiano pattern description used as the standard to identify tobiano horses may need to be refined.

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