Low Microsatellite Variation in Laboratory Gerbils

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The Mongolian gerbil has become a model organism of increasing importance for the understanding of aging, epilepsy, the process of domestication or sociobiological questions. We report the development and characterization of the first nine polymorphic dinucleotide repeat loci in this species. Average observed heterozygosity and allele number of laboratory animals measured 0.136 (SE = ±0.065) and 1.78 (SE = ±0.278) compared to 0.761 (SE = ±0.025) and 9.2 (SE = ±0.57) found for a reference group of wild gerbils. The extreme low genetic variation observed in laboratory animals is caused by several severe population size bottlenecks due to the initial founder event and the later establishment of subpopulations. Reduced levels of allelic polymorphism in experimental animals hamper genetic mapping or parental studies. Therefore experiments relying on kinship analyses have to be carried out on wild animals. Estimates of genetic identity and parental exclusion were calculated as $P_{id} = 2.8 \times 10^{-12}$ and $P_{ex} > 0.999$ in wild gerbils. Laboratory gerbil strains show the expected high degree of genetic similarity. However, significant allele frequency differences ($P < .001$) between American and European gerbils at some microsatellite loci may still allow discrimination between breeding lines.

The Mongolian gerbil (*Meriones unguiculatus* Milne Edwards, 1867) is a common rodent inhabiting the open steppe and semidesert regions of Mongolia and China. Its history as a laboratory animal started in 1935 when 20 pairs were captured in Mandschuria and brought to Japan for breeding. From there, 11 pairs of gerbils were imported into the United States in 1954, of which five females and four males at Tumblebrook Farm Ltd. formed the breeding stock for laboratories in the United States and Europe (Marston 1972; Schwentker 1963). Since then the Mongolian gerbil has become an extensively used experimental animal, for example, in neuroscience, auditory research, and physiology. The species serves as an important genetic model for aging (Spangler et al. 1997) and limbic epilipsy (Scotti et al. 1998). However, despite being an established model organism, the genetics of the Mongolian gerbil has hardly been explored (Gray and Wong 1990).

A number of behavioral and reproduction studies focus on the social organization and the cooperative breeding system of *M. unguiculatus* (e.g., Elwood 1979; Weinandy and Gattermann 1999), but most data were obtained from captive populations of laboratory animals and only a few field studies have been carried out (e.g., Ågren et al. 1989). Studies under laboratory and seminatural conditions (Ågren 1976, 1984) have shown that gerbils live in family groups and defend their territories. Within families there is usually a single reproducing pair suppressing reproduction of subordinated group members. Despite the formation of stable pair bonds, females often seek extrapair mating opportunities outside their territories to prevent inbreeding (Ågren 1990; Ågren et al. 1989).

The ongoing reproductive isolation of experimental gerbil strains has led to a number of anatomical, physiological, and behavioral pecularities not found in wild animals. Brain size reduction, seizures, and different learning performance are considered as evidence for a new case of domestication in a rodent species (Stuermer et al. 1997).

The low number of founder individuals in the Tumblebrook population has led to a substantial degree of inbreeding among captive gerbils. Previous data describing the genetic variability in laboratory bred animals are restricted to isozyme studies. Electrophoretic analysis of lactate dehydrogenase and alkaline phosphatase revealed no variation between different color morphs (Shimizu et al. 1996). A comparative survey of four gerbil strains kept in Japan found them indistinguishable at 23 protein loci except for liver acid phosphatase Acp2 (Okumura et al. 1995). These data support the view of a low inter- and intrastrain diversity among experimental animals.

Because of the obvious lack of genetic data and the need to evaluate the prognostics of genetic mapping experiments or parental studies, we have developed and characterized a set of microsatellite loci. Based on the data derived from nine loci we have estimated the genetic diversity of laboratory and wild gerbils.

Materials and Methods

The isolation procedure for microsatellites followed standard protocols with modifications (e.g., Neumann and Wetton 1996). DNA was extracted from six male gerbils (three wild and three laboratory) and doubly digested with restriction enzymes *Alu*I and *Hae*III. A genomic library was established in XL-1 Blue MRF′ (Stratagene) transformed with size fractionated (0.3–0.8 kb) DNA fragments ligated into the *Sma*I site of pUC18 (Pharmacia). Approximately 1500 recombinant clones were transferred to microtiter plates, replica plated onto nylon membranes, and screened with a cocktail of DIG-labeled oligonucleotides $(CA)_{15}$, $(TC)_{15}$, $(AGG)_5$, and $(GGAA)_4$. Hybridized colonies were visualized using the CSPD system (Roche). Thirty positive clones were sequenced containing 18*x* d(CA), 5*x* d(CT), 2*x* d(GGAA), 1*x* d(GGA), and 4*x* mixed d(CA/CT) motifs. Primers were designed for 13 loci using OLIGO 5.0 (MedProbe, Norway).

Table 1. Microsatellite loci in *Meriones unguiculatus*

Locus	GenBank accession no.	Primer sequence (5′-3′)	Repeat type	Annealing temperature
*Mung*μ1	AF200939	F:TGTGGCTGGCATCCTA R:AAGCAATTCTGTCTCTGTCTG	$d(GT)_{12}$ $d(GA)_{21}$	54–56°C
*Mung*μ2	AF200940	F:AGCCTTTATAGATGAGCAAGT R:GCCTACTAATGGTGAACTGA	$d(TC)_{23}$	54°C
*Mung*μ3	AF200941	F:CAGGCACCCCCAGTTT R:GTCTACACAGGCTGAGGATGT	$d(AC)_{17}$	55–57°C
*Mung*μ4	AF200942	F:GGCTCCTGATTCTACATTTCT R:CAACCATTGGCAACTCTC	$d(TG)_{22}$	54–56°C
*Mung*μ5	AF200943	F:GCTGGGCTTTAATGTTTATTT R:GGTGGCTCACACTTTCTGT	$d(GT)_{19}$	54°C
*Mung*μ6	AF200944	F:TTTCTGGGGTCTCTTTCTCTC R:CCATTCTGCAAGACTCCTCT	$d(TC)_{19}$	54°C
*Mung*μ7	AF200945	F:AGTCCCTATTACATCCACAAG R:TTATCCTGCAAAGCCTAAG	$d(GA)_{16}$	54–56°C
*Mung*μ8	AF200946	F:TGGGTCCTTTGGAAGA R:TGGCTTAAAATGAATCACTTA	$d(GT)_{21}$	54–56°C
*Mung*μ9	AF200947	F:GACAGAGTGGGAGGGGTATGT R:TGGCAAGTTTGGTTTGTTTGA	$d(CA)_{21}$	54–56°C

Repeat type refers to the motif obtained from the cloned allele. Only the longest repeat arrays (>10) are presented.

Polymerase chain reaction (PCR) was performed using the Ready-To-Go-system (Pharmacia). Thirty picomoles of each primer (one was labeled with Cy5 Amidite as fluorescent dye) and 0.01–0.1 μg of genomic DNA were added to a total volume of 25 μl. After an initial denaturation step of 180 s at 94°C the amplification proceeded for 35 cycles as follows; 60 s at 94°C, 60 s annealing at primer specific temperature (Table 1) and 120 s at 72°C (Thermocycler UNO II, Biometra). PCR products were electrophoresed through 6% denaturing polyacrylamide gels on an automated sequencer A.L.F. express II (Pharmacia). The amplification of nine dinucleotide repeat loci *Mung*μ1–*Mung*μ9 produced unambiguous allele patterns.

Inheritance and linkage of microsatellites were examined in seven gerbil families consisting of laboratory and second generation wild animals (2 laboratory × laboratory, 3 laboratory × wild, and 2 wild × wild). Linkage analysis was carried out using the sequential LOD score method (Morton 1955) compiled over all informative families.

To estimate the level of genetic polymorphism, 45 laboratory and 40 wild gerbils were analyzed. DNA was prepared from ethanol fixed liver tissues and frozen ear samples using a commercial kit system (E.Z.N.A. Tissue DNA Kit II, peqlab Biotechnologie GmbH). The laboratory animals comprise five different strains. Sixteen gerbils came directly from Tumblebrook Farm Inc./USA in 1995. A further 17 animals were supplied by the Leibniz Institute of Neurobiology in Magdeburg/Germany and are crossbred animals from the Charles River corporation (strain: CRW/Mon) and the Technical University of Darmstadt/Germany. Five animals belonged to a second strain kept in Magdeburg which originated from Molegart-breed/France. A single individual came from a laboratory at the University of Cologne/Germany and six animals represent the laboratory strain Zoh: CRW bred at the Institute of Zoology Halle/Germany. The latter population derived from three pairs supplied by Charles River Wiga (Sulzbach/Germany) stock label CRW/(Mon)BR in 1992.

Wild gerbils were captured during a joint expedition of the Leibniz Insitute of Neurobiology Magdeburg/Germany and the State University of Mongolia to Central Mongolia in 1995. Animals were trapped at six different locations about 130–140 km southwest and 100 km west of Ulaanbaatar.

All laboratory and wild animals were pooled into two separate groups defined as "laboratory" and "wild" because of the small number of individuals representing a particular strain or population. Expected heterozygosity (HET_{exp}) was calculated from Hardy–Weinberg assumptions for each locus $HET_{exp} = 1 - \Sigma q_i^2$. Deviations from Hardy–Weinberg equilibrium (HWE) were tested by the chi-squared test to detect potential genetic heterogeneity within the pooled samples. A test of genic differentiation combined over all polymorphic loci (Fisher exact test) was performed between American (Tumblebrook) and European laboratory gerbils using the computer program GENPOP, version 3.1d (Raymond and Rousset 1995). Based on the observed allele frequencies (f) we calculated the probabilities of profile identity (two random individuals share the same alleles = P_{id}) and parental exclusion (the probability of detecting an incorrectly assigned parent = P_{ex}) combined over all loci, according to Gundel and Reetz (1981) and Bruford et al. (1992).

Results

Primer sequences and characteristics of nine microsatellites in the Mongolian gerbil are presented in Table 1. Sequential LOD score analyses produced no evidence of close linkage ($z < -2$ for all $\theta < 0.05$ and $z < 0$ for all $\theta < 0.3$) between these markers. The two pure laboratory pedigrees provided no information because all parents proved homozygous at each locus. Allele segregation among all offspring followed Mendelian inheritance. No measurable frequencies of null alleles were detected.

A genetic variability comparison of laboratory and wild gerbils documented a dramatic reduction of allele numbers and observed heterozygosity in the domestic animals (Table 2). Four loci (*Mung*μ1, 2, 3, 9) were monomorphic and allele numbers at the five remaining loci ranged from 2 (*Mung*μ4, 5, 8) to 3 (*Mung*μ6, 7) in our sample, giving a mean allele number of 1.78 (SE = ±0.278). This differed significantly ($P < .001$, Mann–Whitney) from the high diversity of these loci in wild gerbils exhibiting an average allele number of 9.2 (SE = ±0.57). The mean observed heterozygosity in laboratory animals was 0.136 (SE = ±0.065) compared with 0.761 (SE = ±0.025) in wild gerbils ($P < .001$, Mann–Whitney). Significant differences in allele distribution were detected between animals from Tumblebrook Farm (USA) and European laboratories ($P < .001$). Private alleles restricted to the American or European strains were found at loci *Mung*μ6 and 7 in rather low frequencies. Alleles C ($f = 0.011$) and F ($f = 0.011$) at locus *Mung*μ6 were confined to Europe and allele E ($f = 0.022$) at locus *Mung*μ7 to Tumblebrook Farm (USA).

Two loci *Mung*μ8 and 9 showed deviation from HWE in wild gerbils ($P < .05$) due to heterozygosity deficiencies. An excess of observed homozygotes was also responsible for the violation of the HWE in laboratory animals at locus *Mung*μ8 ($P < .05$). Probability estimates of genetic identity were calculated as $P_{id} = 0.056$ and $P_{ex} = 0.467$ in laboratory and $P_{id} = 2.8 \times 10^{-12}$ and $P_{ex} > 0.999$ in wild gerbils.

Discussion

To evaluate the genetic variation in laboratory bred and wild Mongolian gerbils we have isolated a set of nine dinucleotide re-

Table 2. Allele frequencies, heterozygosity values, tests of Hardy–Weinberg equilibrium (HWE) for nine dinucleotide repeat loci in wild and laboratory gerbils

Locus	Allele no. wild/lab	Frequency wild/lab	HET_{obs} (HET_{exp}) wild/lab	HWE test
*Mungμ*1	10/1	A (0.013/–), B (0.100/–), C (0.125/–), D (0.163/–), E (0.013/–), F (0.125/1), G (0.288/–), H (0.138/–), I (0.025/–), J (0.013/–)	0.775/– (0.828/–)	ns/–
*Mungμ*2	10/1	A (0.013/–), B (0.100/–), C (0.175/–), D (0.138/–), E (0.150/–), F (0.300/1), G (0.013/–), H (0.050/–), I (0.013/–), J (0.050/–)	0.800/– (0.823/–)	ns/–
*Mungμ*3	8/1	A (0.163/–), B (0.050/1), C (0.013/–), D (0.175/–), E (0.325/–), F (0.150/–), G (0.050/–), H (0.075/–)	0.825/– (0.804/–)	ns/–
*Mungμ*4	7/2	A (0.113/–), B (0.063/–), C (0.163/0.433), D (0.200/0.567), E (0.113/–), F (0.238/–), G (0.113/–)	0.800/0.422 (0.835/0.491)	ns/ns
*Mungμ*5	12/2	A (0.025/–), B (0.050/–), C (0.025/–), D (0.100/–), E (0.075/0.011), F (0.125/0.989),G (0.188/–), H (0.063/–), I (0.163/–), J (0.025/–), K (0.088/–), L (0.075/–)	0.875/0.022 (0.886/0.022)	ns/ns
*Mungμ*6	9/3	A (0.038/–), B (0.100/–), C (0.013/0.011), D (0.363/–), E (0.188/0.978), F (0.050/0.011), G (0.138/–), H (0.100/–), I (0.013/–)	0.750/0.044 (0.790/0.043)	ns/ns
*Mungμ*7	9/3	A (0.025/–), B (0.063/–), C (0.350/0.278), D (0.050/0.700), E (0.200/0.022), F (0.100/–), G (0.063/–), H (0.100/–), I (0.050/–)	0.700/0.467 (0.804/0.432)	ns/ns
*Mungμ*8	11/2	A (0.375/0.311), B (0.013/–), C (0.075/–), D (0.088/–), E (0.050/–), F (0.038/–), G (0.050/–), H (0.125/0.689), I (0.063/–), J (0.113/–), K (0.013/–)	0.650/0.267 (0.807/0.429)	s/s
*Mungμ*9	7/1	A (0.013/–), B (0.200/–), C (0.300/–), D (0.125/–), E (0.138/–), F (0.088/1), G (0.138/–)	0.675/– (0.809/–)	s/–

ns = not significant; s = significant for $P < .05$.

peat loci. Their polymorphic nature and Mendelian inheritance was confirmed. All markers may be considered as genetically independent because no evidence for linkage was obtained. Pronounced differences in microsatellite variation between two pooled groups of laboratory and wild gerbils correspond with previous biochemical data and reflect the genetic history of the captive gerbil population. The low degree of polymorphism is a consequence of the initial founder event and following bottlenecks due to the establishment of subpopulations at different breeding locations. The genetic diversity of the investigated laboratory gerbil strains is below those observed in inbred mouse or rat strains (Love et al. 1990; Serikawa et al. 1992). In contrast, the data of wild gerbils fall in the range of variation found in other outbred wild *Cricetidae* populations (e.g., *Cricetus cricetus*; Neumann K, unpublished data).

All alleles present in the studied laboratory strains could be traced back to alleles in wild gerbils and have probably originated from early founder animals. Indications for this are also the high frequencies of most of these alleles in the wild gerbil sample. However, the increase in single low-frequency alleles because of mutations cannot be excluded. Small length differences by one repeat, for example, at locus *Mungμ*7, are in agreement with common stepwise mutations in microsatellites.

Observed deviations from HWE at two loci *Mungμ*8, 9 ($P < .05$) in wild and one locus *Mungμ*8 ($P < .05$) in laboratory gerbils are most likely explained by genetic differences between sampled populations or strains, since a general trend for increased proportions of homozygotes is observed at almost all loci.

Laboratory gerbil strains show the expected high degree of genetic similarity. Four microsatellite loci were homozygous for the same alleles in all tested domestic animals. This is in concordance with the breeding and distribution history of gerbils in North America and Europe and proves their common origin. Significant allele frequency differences ($P < .001$) at the remaining five loci between animals from Tumblebrook Farm (USA) and European gerbils may still allow the discrimination between breeding stocks. The detection of private alleles in the two laboratory gerbil pools could be a result of the small sample sizes but may equally account for true genetic strain diversity. Unfortunately our material did not contain laboratory gerbils from Japan. Their genetic composition could be more heterogeneous due to the larger number of unrelated founders (but see Okumura et al. 1995).

Mapping and linkage studies of genetically important loci in Mongolian gerbils are possible but require a larger set of informative markers than in most other experimental animals. However, the low level of genetic variation among strains and the existence of obvious phenotypic differences, for example, seizure-sensitive and seizure-resistant strains may ease the identification of affected genetic loci.

High combined probability estimates of genetic identity and low parental exclusion strongly interfere with kinship analyses in laboratory gerbils. Therefore genetic studies concerning mating system and kinship have to be carried out on wild animals. The microsatellite markers reported here provide a powerful system to accomplish this aim.

Laboratory Mongolian gerbils are an example for a thriving captive rodent population despite its apparent lack of substantial genetic variation. The existence of some distinguishing features such as an increased susceptibility to cerebral infarctions in domestic versus wild animals may be caused by genetic alterations, for example, the fixation of rare alleles and could therefore present inbreeding effects.

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A Second Acromelanistic Allelomorph at the Albino Locus of the Mongolian Gerbil (*Meriones unguiculatus*)

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A new autosomal recessive coat color mutant in the Mongolian gerbil (*Meriones unguiculatus*) is described: chinchilla medium (symbol c^{chm}). The mutant has typical acromelanistic features similar to those of several acromelanistic *c* locus mutants of other species of mammals. Previously a more severe form of acromelanism ($c^h c^h$) has been described in the Mongolian gerbil. The new allele shows to be allelic with this form. On a nonagouti background compound heterozygotes ($aac^{chm}c^h$) show an intermediate phenotype that is very similar to that of the Siamese mouse (*Mus musculus*) and rat (*Rattus norvegicus*). Homozygotes ($aac^{chm}c^{chm}$) display a very dark acromelanistic phenotype reminiscent of that of the sable rabbit (*Oryctolagus cuniculus*). The gray phenotype (*gg*) in the Mongolian gerbil resembles the albino locus phenotype chinchilla ($c^{ch}c^{ch}$) in mice. We show that the new mutant is not allelic with gray. Fertility and viability of the new mutant are within normal range.

In 1866 the French Father Armand David sent some specimens of an unknown rodent species to the Musée d'Histoire Naturelle in Paris. At that time "père Armand" was staying in "la Mongolie chinoise," perhaps what is now known as northwestern Shansi, China (Allen 1940). In 1867 Milne Edwards described these specimens as *Gerbillus unguiculatus* (Milne Edwards 1867). Later, in 1908, they were reassigned by Thomas to *Meriones* and denominated *Meriones unguiculatus* (Thomas 1908). Popular names are Mongolian gerbil and clawed jird (Gulotta 1971). Observations of these animals in their natural environment are described by Ågren et al. (1989).

Mongolian gerbils have been bred in laboratories since 1935, when Dr. C. Kasuga caught 20 pairs in the basin of the Amur River in eastern Mongolia. They were sent to the Kitasato Institute with the intention of using them for rickettsial studies. Miss Michiko Nomura (Central Laboratories for Experimental Animals) obtained some animals from the Kitasato Institute in 1949, and in 1954 she sent four pairs of their offspring to Dr. Victor Schwentker at Brant Lake, New York. He established the first commercial colony in America at the Tumblebrook Farm. At that time many if not all investigators obtained their stock from Dr. Schwentker's colony (Rich 1968). Since then Mongolian gerbils are used in scientific research all over the world.

Mongolian gerbils can be managed quite easily and thrive on a diet of commercial rodent pellets. They are curious, gentle, easy to handle, and stress resistant. They can be transported without problems, reproduce throughout the year and are economical with water consumption, consequently their urine production is low. Their cages remain relatively dry and odorless unlike those of mice (*Mus musculus*) and rats (*Rattus norvegicus*). Mongolian gerbils can tolerate high population densities in captivity. All these features, combined with the fact that Mongolian gerbils are active both day and night, have made them very popular as pets and for fancy breeding mainly in Europe and the United States.

Gerbils have been used in pharmacological, parasitological, endocrinological, and cancer research. Their susceptibility to different types of bacteria and viruses has also been studied (Gulotta 1971; Rich 1968). They seem to have an unexpectedly high resistance to radiation (Chang et al. 1964). Because of their naturally high incidence (1 in 5 animals) of seizures (Thiessen et al. 1968) they have been used in many types of neurological research (Ellard et al. 1990; Gray-Allan and Wong 1990; Loskota et al. 1974a,b). Probably because of their distinct social behavior, they were used in many types of behavioral studies as well (Kaplan and Hyland 1972; Thiessen and Yahr 1977; Walter et al. 1963).

Surprisingly enough, Mongolian gerbils have rarely been used in genetic research. Their diploid number of chromosomes is 44, containing 22 metacentric, 10 submetacentric, and 10 acrocentric autosomes, a large submetacentric X and a smaller submetacentric Y chromosome (Nadler and Lay 1967). Between 1970 and 1985 seven mutants arose in scientific laboratory and

Table 1. Occurrence of Mongolian gerbil mutants

Phenotype	Occurred	Allele	Reference
Acromelanistic albino	1970	c^h	Robinson (1973) Cramlet et al. (1974)
Nonagouti	1971	*a*	Waring and Poole (1980) Silverstein and Silverstein (1976)
White spot	1968	*Sp*	Waring et al. (1978)
Gray	1976	*g*	Leiper and Robinson (1985)
Pink-eyed dilution	1977	*p*	Henley and Robinson (1981)
Hairless	1978	—	Swanson (1980)
Albino	1985	*c*	Matsuzaki et al. (1989)
Chinchilla medium	1994	c^{chm}	This article

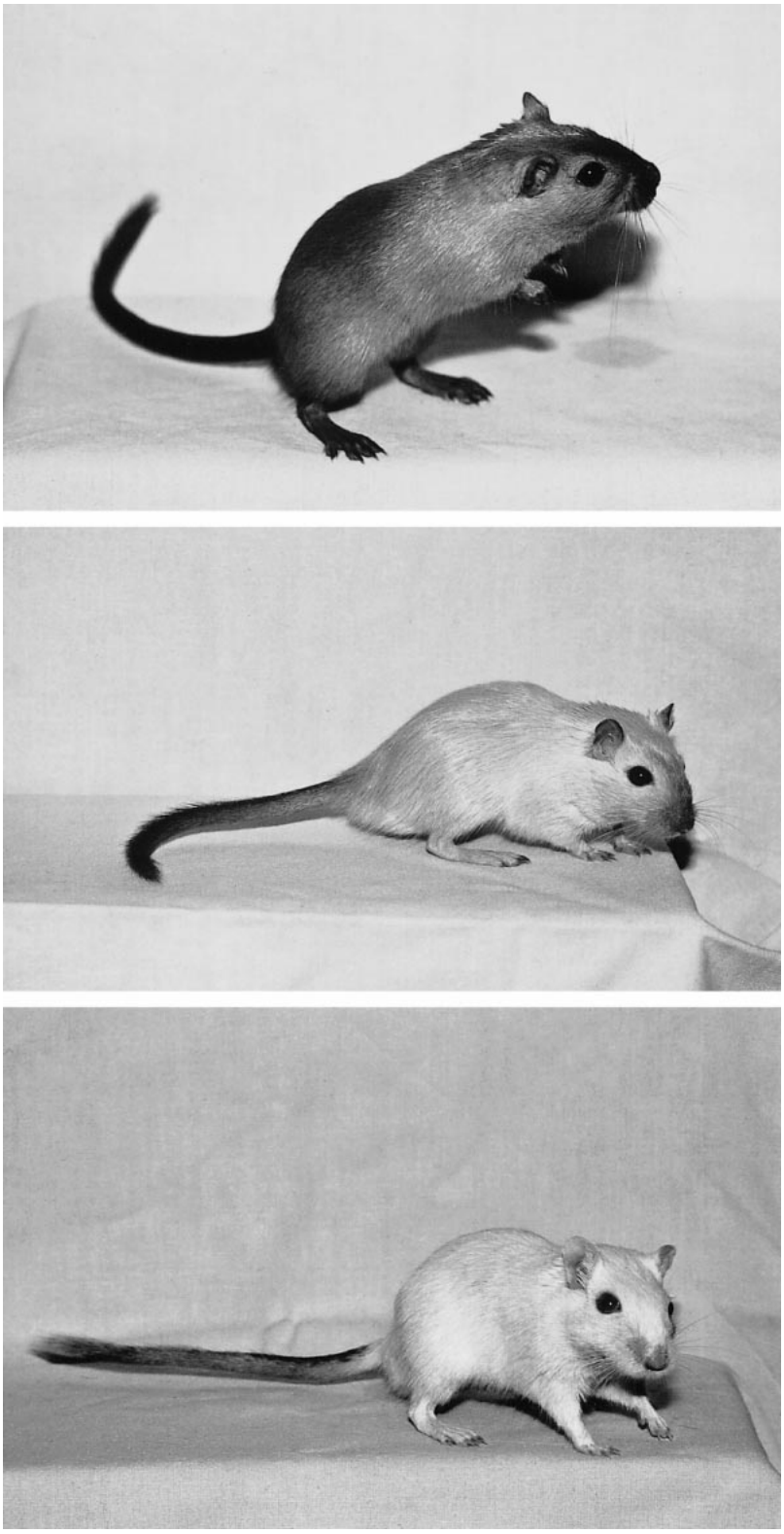

Figure 1. Phenotypic effects of gerbil *c* locus mutations on a nonagouti background shown in three adult animals: **(A)** black chinchilla medium ($aac^{chm}c^{chm}$), **(B)** Siamese ($aac^{chm}c^{h}$), **(C)** dark-tailed white ($aac^{h}c^{h}$).

pet populations (see Table 1). Recently we discovered four new coat color mutants in the Mongolian gerbil, three in pet populations and one in a laboratory population. Two of them are allelic and possibly related to the extension locus (Petrij F, unpublished data). The third one seems to be the dilution mutation (Pund T and Petrij F, unpublished data), a well-known coat color mutation, which is described in many other mammalian species (Searle 1968). The fourth one has proven to be allelic to the albino locus and will be discussed here.

Description of the Mutant

In November 1994 a British fancier discovered animals with two new phenotypes in a pet shop in the Republic of Ireland. Both phenotypes were of an acromelanistic type, although one was lighter than the other. He brought one dark- and three light-colored animals to the United Kingdom (Barker J, personal communication). About 1 year later offspring of these animals were imported to The Netherlands and Germany by fanciers. These animals formed the nucleus of our breeding experiments.

The darker version has a light brown body (see Figure 1A). The ventral side is slightly lighter in color in comparison to the dorsum. No clear demarcation line can be determined. The color is clearly lighter and more creamy than that of the dark sepia (Leiper and Robinson 1985) Mongolian gerbil (*aagg*). Compared with the typical brown coat color (*aabb*) of other rodents, such as mice (Searle 1968; Silvers 1979), the coat color of the new mutant is a lighter shade of brown. Nose, ears, and feet are covered with dark sepia hairs and the tail hairs are almost black. The scrotum is dark colored and covered with hairs that resemble the color of the ventral side. The color slowly fades out toward proximal. The nails are dark. The eyes are almost black but under bright illumination they show a dark red glow. The typical white patches on the upper lip, under the chin, and across the front feet of nonagouti animals are also recognizable in this new mutant. This, together with the nonagouti tail and the absence of the typical white belly produced by the agouti allele in this species, makes it likely that this phenotype has a nonagouti background.

The lighter version (see Figure 1B) can be described as a diluted form of the darker one and is reminiscent of the phenotype of the Siamese mouse (Green 1961) and rat (Moutier et al. 1973). The body color is a very creamy type of brown and the extremities are light brown. The tail, however, is quite dark and of a dark sepia coloration. The eyes are dark ruby and are clearly darker than those of pink-eyed dilution (*pp*) and pink-eyed white ($c^{h}c^{h}$) animals.

Juveniles don't show the full acromelanistic features like the adults do (see Figure

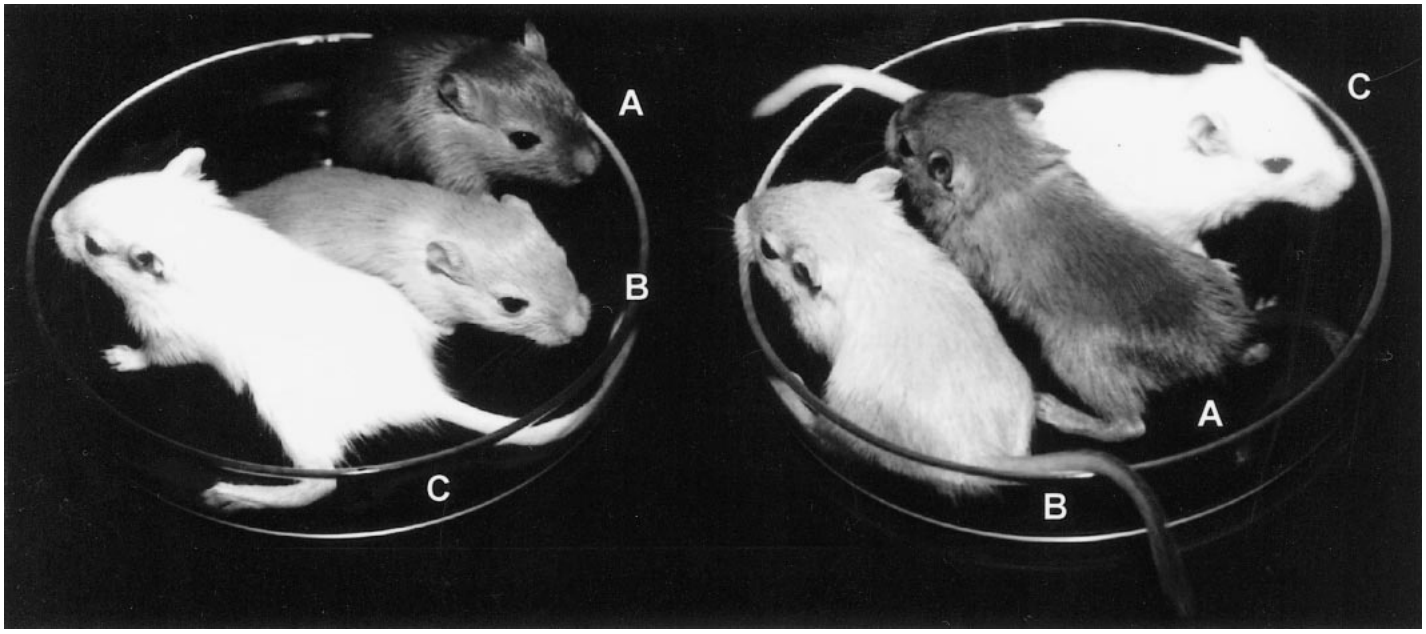

Figure 2. Phenotypic effects of gerbil *c* locus mutations on a nonagouti background shown in three juvenile animals (age 3 weeks): **(A)** black chinchilla medium ($aac^{chm}c^{chm}$), **(B)** Siamese ($aac^{chm}c^{h}$), **(C)** dark-tailed white ($aac^{h}c^{h}$).

2). Until their first molt the dark phenotypes have a more grayish-brown color with only a darker-colored tail. Nose, ears, and feet are the same color as the body. Similarly the juvenile of the lighter version also shows no dark coloration except for the tail. Both phenotypes develop dark extremities, ears, and nose as they reach maturity. Under the influence of low temperatures (<15°C) the mid-dorsal area tends to become darker than the rest of the body. Altogether both phenotypes are clearly acromelanistic.

Another acromelanistic phenotype of the Mongolian gerbil has previously been described (Leiper and Robinson 1984; Robinson 1973). In that case the pigmentation is more severely affected. The animals of this phenotype are pink-eyed whites with some light brown hairs on the tail ($AAc^{h}c^{h}$). On a pink-eyed dilution background ($c^{h}c^{h}pp$) these animals are pseudo-albinos. On a nonagouti background ($aac^{h}c^{h}$) the tail can become quite dark, therefore we would like to call this latter phenotype "dark-tailed white" (see Figure 1C).

Complete albinism (*cc*) was reported by Matsuzaki et al. (1989). However, no subsequent reports on this phenotype have been published. The question arises whether this mutation has been lost. It cannot be excluded that the pink-eyed dilution mutation (*pp*) or other modifying genes were involved in this specific stock.

Breeding Data

At first the darker animals were checked for their assumed nonagouti background. Crossings with nonagouti produced only nonagouti offspring (51 of 51), showing that the dark version is actually a nonagouti variant of the new mutant. In order to show that the dark mutant breeds true, dark individuals were mated with each other producing only dark variant F_1 animals (104 of 104). A similar experiment with animals of the light phenotype, subsequently called Siamese, resulted in three different phenotypes: dark variants, Siamese, and dark-tailed whites. This indicates that the Siamese were in fact compound heterozygotes of the new mutation and the earlier described more severe form of acromelanism.

To investigate the phenotypic effects of the new mutation on a wild-type agouti background, dark variants were mated to agouti animals and produced only agouti animals (51 of 51), and F_1 agouti animals were inbred or backcrossed to dark variant animals. $F_1 \times F_1$ matings resulted in 22 agoutis, 6 blacks, 2 dark variants, and 4 animals of a new phenotype. The new phenotype can be described as a light gray agouti with a somewhat diluted wild-type agouti tail (further referred to as chinchilla medium). F_1 agouti $\times$ dark variant animals resulted in 14 agoutis, 5 blacks, 12 dark variants, and 6 chinchilla medium animals. Both results are within the expected ratios of 9:3:3:1 and 1:1:1:1, respectively (see Table 2).

In the chinchilla medium animals it seems that the pheomelanin, which is almost completely washed out on the body, is less affected on the tail. The nose and ears of some individuals are slightly darkened. On close examination the mid-dorsal banded hairs (mainly awls) have a light sepia base, a creamy white subapical band, and light sepia tips. When compared to wild-type agouti it is clear that the new mutation dilutes pheomelanin more than eumelanin. Because the different phenotypes are more pronounced on a nonagouti than on an agouti background, all further experiments were performed on a nonagouti background.

Dark-variant animals were mated with pink-eyed white and gray agouti (*gg*) animals. Since acromelanism is a typical feature of the albino locus, pink-eyed white was chosen. For the same reason gray agouti was chosen: it mimics the chinchilla mutation at the albino locus of other rodent species (Leiper and Robinson 1985). Crossings between dark variants and pink-eyed whites only produced animals with the Siamese phenotype (118 of 118). Crossings between dark variants and gray agoutis only produced agoutis (39 of 39). Siamese F_1 animals were inbred to produce an F_2 of 40 dark variants, 59 Siamese, and 42 pink-eyed whites. Siamese backcrossed to dark animals produced 56 dark and 48 Siamese animals, and Siamese backcrossed

Table 2. Segregation ratios of crosses

Cross	Total	Progeny phenotype						Expected ratio	χ^2
		Agouti	Black	Chinchilla medium	Black chinchilla medium	Siamese	Pink-eyed white		
Black chinchilla medium × black chinchilla medium	104				104				
Black chinchilla medium × black	51		51						
Black chinchilla medium × pink-eyed white	118					118			
Black chinchilla medium × gray agouti	39	39							
Black chinchilla medium × agouti	51	51							
F_1 agouti × F_1 agouti	34	22	6	4	2			9:3:3:1	1.35 ($P > .50$)
F_1 agouti × black chinchilla medium	37	14	5	6	12			1:1:1:1	6.35 ($P > .05$)
Siamese × Siamese	141				40	59	42	1:2:1	3.80 ($P > .10$)
Siamese × black chinchilla medium	104				56	48		1:1	0.62 ($P > .25$)
Siamese × pink-eyed white	24					12	12	1:1	0.00 ($P = 1.00$)

to pink-eyed whites produced 12 pink-eyed whites and 12 Siamese. Segregation ratios and statistical analyses of all crosses are summarized in Table 2.

Our breeding data indicate an autosomal inheritance: no significant differences between distribution of phenotypes over the sexes could be observed. Fertility and viability seem to be within normal range.

In view of the fact that the (darker) homozygous phenotype has an acromelanistic expression, which is a typical feature of mutations at the *c* locus, and also because it is allelic with pink-eyed white (c^h), this new mutation can be located at the *c* locus with confidence. In comparison to the acromelanistic mouse and rat the new gerbil mutation differs in phenotype. Although similar acromelanistic phenotypes in rabbits (sable rabbits) show a darker coloration, their color characteristics and inheritance pattern have more resemblance with the here described mutation in the gerbil (see Table 3). Therefore we would like to propose to designate the gene symbol c^{chm} (chinchilla medium).

Discussion

The breeding data indicate that the mutation we describe is inherited as an autosomal recessive trait since all F_1 animals of mutant × agouti matings are of an agouti phenotype. In other words c^{chm} is fully recessive to *C.* Since all F_1 animals from black chinchilla medium × pink-eyed white matings are of an intermediate phenotype, c^{chm} is shown to be codominant to c^h.

Because in the Mongolian gerbil the *c* locus is linked to the *p* locus (Leiper and Robinson 1986) this will have consequences for the distribution of phenotypes in the F_2 progeny of, for instance, a black chinchilla medium ($aac^{chm}c^{chm}PP$) × argente golden (*AACCpp*) mating. These types of experiments were not performed by us, but it is surely worth testing in order to confirm the linkage. Another interesting question to investigate would be whether *pp* has a dominance modifying effect on the dominance of *C* over c^{chm}, like it has on the dominance of *C* over c^h (Leiper and Robinson 1984). Preliminary data indicate that this is indeed the case. Both the *A-*$Cc^{chm}pp$ and the $aaCc^{chm}pp$ animals are of a lighter color than the corresponding *CCpp* animals. In comparison to the argente creme (*A-*Cc^hpp) the *A-*$Cc^{chm}pp$ animal has a richer cast of yellow. In comparison to the silver ($aaCc^hpp$), the $aaCc^{chm}pp$ animal is of a more bluish cast. Both varieties have darker red eyes. We propose to call these phenotypes argente fawn and sapphire, respectively.

Furthermore, it would be interesting to see whether $c^{chm}c^{chm}$ has a dominance modifying effect on the dominance of *P* over *p.* In the mouse it has been reported that albinism (*cc*) and pink-eyed dilution (*pp*) have a reciprocal dominance modifying effect (Silvers 1979). In case of the pink-eyed whites (c^hc^h), this would be difficult to test because of the severe dilution of body color, only on the tail of dark-tailed white animals the difference between aac^hc^hPP and aac^hc^hPp might be visible. In chinchilla medium animals, however, the amount of remaining pigmentation will be easier to assess.

Mutations at the *c* locus affect the synthesis of tyrosinase (monophenol oxygenase). This copper-containing enzyme plays a major role in the synthesis of melanin by the catalyzation of the oxidation of tyrosine to L-dopa (3,4 dihydroxyphenylalanine) and the dehydrogenation of L-dopa to dopaquinone. These two catalytic reactions form the first two steps in the melanin biosynthetic pathway. Dopaquinone is a common precursor for eumelanin as well as pheomelanin. Certain tyrosinase mutations produce an extreme unstable and thermosensitive form of the enzyme (Halaban et al. 1988). In these cases melanin production is greater in colder areas of the body, leading to the typical acromelanistic phenotype seen in several mammalian species (Searle 1990), including humans (Giebel et al. 1991).

Because of the above described similar phenotype, inheritance pattern, and comparable interaction with the earlier described severe form of acromelanism assigned to the *c* locus, it is likely that the new mutation occurred at the albino locus of the Mongolian gerbil. Ultimate proof has to come from molecular studies, which will become possible as soon as the tyrosinase gene of the Mongolian gerbil has been cloned.

Table 3. Comparison of acromelanistic pheno- and genotypes in rabbits, gerbils, mice, and rats

Species	Full color	Dark acromelanistic	Medium acromelanistic	Light acromelanistic	Faint acromelanistic	Albino	Reference
Rabbit	*CC*	$c^{chm}c^{chm}$	$c^{chm}c^{chl}$ $c^{chm}c^h$	c^hc^h $c^{chm}c$	$c^{chl}c^{chl}$ $c^{chl}c^h$ $c^{chl}c$ c^hc	*cc*	Robinson (1958)
Gerbil	*CC*	$c^{chm}c^{chm}$	$c^{chm}c^h$	—	c^hc^h	*cc*[a]	Robinson (1973), Matsuzaki et al. (1989), this article
Mouse	*CC*	—	c^hc^h	—	c^hc	*cc*	Green (1961), Silvers (1979)
Rat	*CC*	—	c^hc^h	—	c^hc	*cc*	Moutier et al. (1973)

All described phenotypes are on a nonagouti (*aa*) background.

[a] Mutation possibly lost (see text).

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A Linkage Map for *CRINKLED PETAL*: A Homeotic Gene of *Clarkia tembloriensis* (Onagraceae)

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Homeotic mutations in flowers lead to the development of floral organs in abnormal locations. In most laboratory-induced examples of this type of mutation, two adjacent whorls of organs are affected, resulting in two whorls of abnormal organ formation. However, the *crinkled petal* mutant of *Clarkia tembloriensis* is interesting because it is a naturally occurring mutation and it affects only the second whorl of organs, producing sepaloid petals. In this study one wild-type population (Cantua Creek-2) and one crinkled petal mutant population (Red Rocks) were compared using 181 different primers in random amplified polymorphic DNA (RAPD) analysis. Bulk DNA from each parent population and their subsequent crosses were used to compare the genetic differences between the two populations and to search for molecular markers linked with the *CRINKLED PETAL* locus. A linkage map was developed for the *CRINKLED PETAL* gene, and markers were discovered which flanked both sides of the locus.

Clarkia tembloriensis (Onagraceae) is a wildflower found growing in discrete populations in the Temblor region of southern California. Within these populations, flowers with two distinct petal phenotypes have been observed (Vasek 1964). The typical wild-type flowers of *C. tembloriensis* possess four petals composed of a smooth-textured deltoid limb with a slender claw. These petals are bright pink in color, with a maroon spot at the base of the limb. All four petals are expanded and very prominently displayed. In striking contrast are the flowers from the homeotic mutant called *crinkled petal* (*cp*) (Smith-Huerta 1992, 1996; Vasek 1964). The *cp* mutant flowers also possess four "petals," but these organs are small and linear-lanceolate in shape, with abundant trichomes. They have a greenish-pink, wrinkled appearance, lack the maroon spot at the base, and are reflexed backward. In previous developmental studies, Smith-Huerta (1992) showed that the second whorl organs of the *cp* mutant are initiated in the proper time and location for wild-type petals, but in terms of color, morphology, and growth rate, they closely resemble wild-type sepals.

The *crinkled petal* phenotype occurs in at least 10 natural populations in California (Vasek 1964) and is controlled by a single recessive gene (Vasek 1966). In some populations both the wild-type and *crinkled petal* phenotypes occur together; in others, only one of the phenotypes is present (Vasek 1964). In this study, plants from the wholly wild-type population at Cantua Creek-2 (CC-2) were compared with plants from the *cp* mutant population at Red Rocks (RR). These two populations show minor differences in germination time, growth rate, and branching pattern (Leong L, personal observation), as well as the obvious major difference in floral morphology.

In this study we attempted to locate the *CRINKLED PETAL* gene using random amplified polymorphic DNA analysis (Welsh and McClelland 1990; Williams et al. 1990) to examine the genetic differences between two populations of *C. tembloriensis* that display different petal phenotypes.

Table 1. Linkage groups for *C. tembloriensis*

Group 1	Group 2
UBC-163_{1040}	UBC-211_{360}
UBC-207_{425}	UBC-211_{405}
UBC-251_{410}	UBC-281_{450}
cp+ (wild-type petal)	

Band names indicate the primer number along with the approximate size of the band in base pairs. All other markers were determined to be unlinked.

We used RAPDs with template DNA from parental wild-type and mutant plants, and DNA from subsequent crosses [F_3 for mutants; F_3 and selfed backcross (BC_2) for wild type] to examine the genetic variation between the CC-2 wild-type petal and RR *crinkled petal* populations. We then used the observed differences to search for markers linked with the gene responsible for the petal phenotype. Furthermore, by comparing the linkage of polymorphic bands with the segregation of the wild-type and *cp* mutant petal phenotypes in homozygous individuals (*cp*+/*cp*+ or *cp*/*cp*) of the F_3 generation, a linkage map was developed for the *CRINKLED PETAL* gene of *C. tembloriensis*.

Materials and Methods

Clarkia tembloriensis seeds from the wild-type CC-2 population and the RR mutant population were sown on vermiculite and germinated in a growth chamber under a 12-h light/12-h dark cycle with a constant temperature of 13°C (Smith-Huerta 1992). Seedlings were transplanted into Metro Mix (Scott's) and grown in a growth chamber under a 16-h light/8-h dark cycle at a constant temperature of 23°C. Plants were watered as necessary with a dilute fertilizer solution (Liquid Miracle Gro, Scott's). To produce F_1, F_2, F_3, and backcross generations, flowers were emasculated 1 day prior to anthesis and hand pollinations were performed. F_3 populations were used to determine the genotype of the wild-type F_2 parents relative to the *cp* locus.

Healthy bud, stem, and shoot tissues were collected from plants of each type and generation. DNA extraction was performed using a CTAB method based on Doyle and Doyle (1987) with a 4× CTAB extraction buffer plus 5% PVP and 1% sodium bisulfite (dissolved in the buffer before tissue was added). DNA was resuspended in sterile deionized water. Salt concentrations were adjusted to 0.25 M, and then the protocol according to Michaels et al. (1994) was used to remove RNA and clean up polysaccharide contaminants. DNA was stored in sterile water at −20°C.

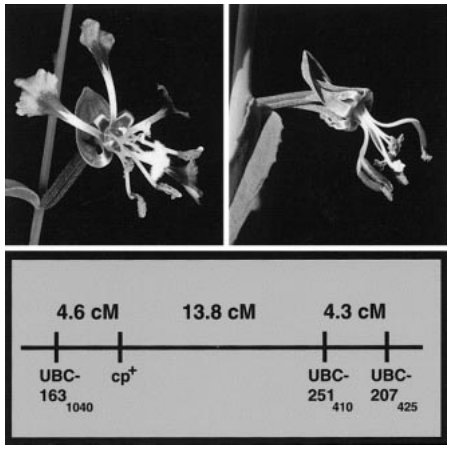

Figure 1. Mature flowers of *C. tembloriensis*: WT on left, *cp* mutant on right. Map of markers linked with the *cp*+ allele.

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR; Welsh and McClelland 1990; Williams et al. 1990) was performed in a thermocycler (Perkin-Elmer GeneAmp PCR System 2400) using the following components for each 12.5 μl reaction: 0.2 μM primer, 0.2 mM of each dNTP, 4 mM $MgCl_2$, 10× Stoffel Fragment PCR Buffer (final concentration 10 mM KCl, 10 mM Tris-HCl pH 8.3), 2% DMSO, 2× BSA, and 2 units of AmpliTaq DNA Polymerase Stoffel Fragment (Perkin-Elmer, Foster City, CA). The following program was used: 94°C for 3 min, followed by 50 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The reactions were then terminated by an extension of 72°C for 7 min and maintained at 4°C until loading for gel electrophoresis. PCR products were run on 1.3% agarose in 1× TAE buffer, and gels were then stained with ethidium bromide and photographed under ultraviolet (UV) light. Photography and analyses were performed using the AlphaImager camera system (Alpha Innotech, San Leandro, CA). Gels were compared visually for presence or absence of polymorphic bands. A total of 181 different random 10-mer primers (UBC-120–UBC-300; Nucleic Acid Protein Service, University of British Columbia, Vancouver, BC, Canada) were used in RAPD-PCR to compare the banding patterns of the two populations.

For initial population comparisons, DNAs from nine plants of the parental CC-2 population were combined together, as were DNAs from nine plants from the RR population (called CC-2 bulk and RR bulk, respectively). The purpose of bulking the DNAs from each population was to provide an estimate of the variation between the two populations and to disregard variation between individuals within each population (based on the idea of "bulked segregant analysis" from Michelmore et al. 1991). An ethanol precipitation from Michaels et al. (1994) was used to clean the bulked DNAs again. Bulk CC-2 and bulk RR DNAs were used in RAPD-PCR with each of the 181 primers. Primers that resulted in polymorphisms between the two population bulks were checked at least twice to confirm the polymorphism. Those primers producing a confirmed polymorphism were tested for linkage with the petal phenotype by running PCRs with DNA from various F_3 individuals bearing the mutant phenotype (genotype *cp*/*cp*). Primers resulting in a majority of mutant F_3 individuals showing the same band phenotype as one of the parental types were used again on F_3 and BC_2 (selfed backcross) individuals known to be homozygous for the wild-type *cp*+ allele (genotype *cp*+/*cp*+).

Segregation data for RAPD markers associated with the petal phenotype were analyzed using the genetic analysis program MapMaker/Exp version 3.0 (Lander et al. 1987; Lincoln et al. 1992).

Results

One hundred eighty-one 10-mer primers were screened through PCR with bulk DNAs from the CC-2 and RR populations, resulting in a total of 1060 scorable bands; 1018 bands were present in both populations (96.0%) and 42 were present in only one population (4.0%). To check further for linkage with the petal phenotype, PCR was performed on template DNA from individuals of the F_3 generation using those primers that produced polymorphic bands. Of the bands that were found to be polymorphic between the two populations, 30 showed segregation of the band in the F_3 population and were used to create a linkage map for the *cp* gene. Those bands that did not segregate at all in the F_3 were considered uninformative for mapping purposes.

Two linkage groups were determined using MapMaker/Exp version 3.0 (Table 1). Group 1 is significant because it demonstrates linkage between the *cp* locus and three different molecular markers. The linkage map for this group is shown in Figure 1. Primer sequences for the markers used in mapping were as follows: 5′-CCC CCC AGA T-3′ (primer UBC-163); 5′-CAT ATC AGG G-3′ (primer UBC-207); 5′-CTT

GAC GGG G-3′ (primer UBC-251). The marker UBC-163_{1040} correlated especially strongly with the wild-type allele, mapping only 4.6 cM apart. This band occurs in the wild-type CC-2 bulk and most of the F_3 and BC_2 individuals homozygous for the wild-type allele. It does not occur in the mutant RR bulk nor in any of the F_3 mutant individuals except F_3-25-08, indicating a tight linkage with the wild-type allele.

Discussion

When no sequence information is available for an organism, one way to determine the location of a gene is to identify markers that are physically close to it on a chromosome. The closer the proximity of two markers on a chromosome, the more likely they will segregate together, and the less likely that they will cross over separately during meiosis. Three bands were determined to be linked with the wild-type petal allele: UBC-163_{1040}, UBC-207_{425}, and UBC-251_{410}. In fact, the marker UBC-163_{1040} occurs in all scorable instances of the wild-type phenotype in the F_3 and BC_2 generations, with a very low frequency of occurrence in the F_3 mutant phenotype plants. Preliminary attempts to clone this marker were unsuccessful, but this may be a very useful marker for future attempts to locate the position of the *cp* locus. The likelihood of finding the *cp* locus in the future is enhanced by the fact that linked markers have been located on both sides of this locus: UBC-163_{1040} on one side and UBC-251_{410} on the other. As a demonstration of the potential usefulness of this finding, Thorlby et al. (1997) were able to determine a fine-scale molecular map position for the *Arabidopsis* male sterility gene *ms1* by measuring the recombination frequency of two molecular markers which flank both sides of the *ms1* gene. A similar mapping study could be performed on the *cp* gene with its flanking markers. Furthermore, by creating and searching a genomic DNA library for *C. tembloriensis,* it might be possible to find contiguous DNA fragments that span the region between the two flanking markers. A chromosome walk across this region could then lead to a more accurate mapping of the *cp* locus and would make sequencing the *cp* gene a viable option.

To explain the mechanism of floral organ development, Bowman et al. (1991) have suggested a series of three overlapping floral factors or functions called A, B, and C which, when expressed in different combinations, specify the development of the four different floral whorls. The expression of factor A alone results in sepals, factors A and B together produce petals, B and C make stamens, and C alone stimulates the production of carpels. According to this 'ABC model,' a mutation in one of these factors should have an impact on two adjacent whorls of organs since each factor is required for the development of two whorls. Indeed, this prediction is consistent with many studies describing homeotic mutations that disrupt the development of two adjacent floral whorls in a variety of plant systems (Coen and Meyerowitz 1991). However, not all homeotic mutants behave exactly as this model predicts. Exceptions such as the *superman* mutation of *Arabidopsis,* which affects the carpel whorl only (Bowman et al. 1992; Sakai et al. 1995), and the *green petals* mutation of *Petunia* which affects the petals only (Angenent et al. 1992; Halfter et al. 1994; van der Krol and Chua 1993; van Tunen and Angenent 1991), do not follow the pattern of the basic ABC model. In the same way, the *cp* mutant does not fit the normal model, as it produces a mutant petal whorl, but otherwise appears to be unaffected anywhere else. However, this deviance from the model does not necessarily imply that the ABC model is not relevant for *C. tembloriensis* or other one-whorl mutants. Although the *cp* mutant is considered a class B mutant (Smith-Huerta 1992), it may be that the *CRINKLED PETAL* gene is not responsible for the production of the "B" factor of *C. tembloriensis,* but is involved instead in the response of the plant to the presence of the A + B factor product.

The *crinkled petal* mutant of *C. tembloriensis* is significant not only because it represents a deviation from the more common two-whorl mutant effect, but also because it is not a laboratory-induced mutation as is the case of most of the commonly studied floral mutants. The *cp* mutation has been observed in at least 10 populations in the wild (Vasek 1964). Other studies have reported apparently homologous sepaloid petal phenotypes in other species of *Clarkia* (*crinkled petal* form in *C. exilis,* Vasek 1966; *bicalyx* mutant in *C. concinna,* Ford and Gottlieb 1992) and in other genera of the Onagraceae including *Epilobium* and *Oenothera* (*cruciata* mutants; Renner 1959). All of these mutants are fully fertile and appear to be unaffected in the other three floral whorls. The existence of these mutations suggests that the single-whorl mutant condition, while by no means a common occurrence, is not as unusual in nature as might be anticipated, at least in the Onagraceae. Further pursuit of the *cp* locus in *C. tembloriensis* could thus prove very useful to other studies. Although it was shown by Vasek (1966) that the *CRINKLED PETAL* genes of *C. tembloriensis* and *C. exilis* are homologous, it is not known whether the same is true for the rest of the sepaloid petal mutations in the Onagraceae, or for other sepaloid petal mutants in other plant families. Continued investigation of the *crinkled petal* mutant of *C. tembloriensis* and other similar mutants will help us to compare and contrast the relationships between the different floral whorls in different plant systems and may help to provide insight into the evolution of the flower itself.

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Production of Wheat Doubled Haploids by Pollination With Job's Tears (*Coix lachryma-jobi* L.)

K. Mochida and H. Tsujimoto

Wheat (*Triticum aestivum* L.) haploids were produced by crossing with Job's tears (*Coix lachryma-jobi* L.) as the pollen parent. Pollination was followed by 2,4-D treatment, detached tiller culture, and embryo culture, as described for maize pollination. The frequency of embryo formation was similar to that obtained by crossing wheat with maize pollen. Job's tears is a perennial plant which forms several stalks and its pollen can be collected throughout the year when the plant is maintained in a controlled environment. Our results indicate that Job's tears can be used as the pollen parent for wheat crosses for haploid production without requiring synchronization of flowering dates.

Haploid production followed by chromosome doubling results in the creation of genetically pure lines within a short period of time. This is an important procedure to shorten the time for crop improvement. In addition, a set of double haploid lines is useful for analyses of quantitative trait loci (QTLs) (Cadalen et al. 1998; Yan et al. 1999).

In wheat, haploids can be produced by interspecific crosses. In this procedure, haploid embryos appear as a result of selective elimination of the alien chromosomes during embryogenesis. *Hordeum bulbosum* was first used as the pollen parent for this purpose (Barcley 1975). However, subsequent studies indicated that this species was inappropriate for wheat cultivars due to the presence of dominant *Kr* genes, which reduce crossability (Snape et al. 1979). When maize pollen is crossed with wheat stigma, the sperm nuclei fertilize the wheat egg (Laurie and Bennett 1986; Zenkteler and Nitzsche 1984). However, the maize chromosomes are eliminated at early embryonic stages (Laurie and Bennett 1989). When hexaploid wheat or durum wheat is crossed with maize pollen, the efficiency of haploid production is not affected by *Kr* genes (Almouslem et al. 1998; Inagaki 1997; Laurie and Bennett 1987, 1988b). Other species related to maize, such as Teosinte (*Zea mays* L. spp. *mexicana*) and eastern gamagrass (*Tripsacum dactyloides* (L.) L.), are alternative pollen donors for wheat haploid production (Li et al. 1996; Riera-Lizarazu and Mujieeb-Kazi 1993; Suenaga et al. 1998; Ushiyama et al. 1991).

Cytological studies have provided evidence for the successful fertilization and elimination of paternal chromosomes from hybrid zygote in sorghum (*Sorghum bicolor* (L.) Moench) and pearl millet (*Pennisetum glaucum* (L.) R. Br.) crosses, and thus these species are also possible pollinators for wheat haploid production (Ahmad and Comeau 1990; Inagaki and Mujeeb-Kazi 1995; Laurie 1989; Laurie and Bennett 1988a). Here we report a new potential pollinator, Job's tears (*Coix lachryma-jobi* L.). Job's tears is a vigorous perennial grass of the tribe Maydeae, subfamily Panicoideae of Poaceae. It includes both cultivated and wild plants of Southeast Asian origin. The plant has thick rosetting stems. The top of the stem divides into several branches, and the plant is about 60–100 cm high. The cultivated type (ssp. *mayuen*) is used as food and medicine in eastern and southern Asian countries. The wild species inhabits wet ground such as riverbanks and the flowers bloom in summer to autumn. One male inflorescence contains several florets, and thus collection of the pollen is easy.

In this study we describe the potential use of Job's tears as a novel pollinator for wheat double-haploid production.

Materials and Methods

Plant Material

Common wheat (*Triticum aestivum* L.) cv. Chinese Spring was used as the female parent. Wheat plants were grown in potting soil in a temperature-controlled greenhouse [10°C/23°C (min/max)]. Job's tears plants were collected from the Maioka River close to our institution and were transferred to pots. As a control, the maize line 919J, which was kindly provided by the Maize Stock Center, was used as the pollen parent. Both pollen parents were grown in a temperature-controlled greenhouse [18°C/27°C (min/max)] with a light:dark cycle of 16:8 h. Flowering of Job's tears was maintained by growing lateral buds.

Crossing

The size of the anthers of Job's tears is similar to that of maize anthers (5–7 mm) (Figure 1). Several anthers could be obtained from the flowering florets in the male inflorescence. Fresh anthers of Job's tears collected by forceps and placed on the palm of the hand readily dehisced and were used for fertilization of the emasculated wheat spikes. Maize pollen was used as a control to pollinate wheat, using the method described previously by Inagaki (1997).

Detached Tiller Culture

After pollination, wheat tillers were cut off and cultured for 14 days in a solution containing sucrose (4 %) and 2,4-D (100 mg/l). They were incubated in a growth chamber set at a constant temperature of 22.5°C, with a 16:8 h light:dark cycle and 80–90% relative humidity. The liquid medium was regularly changed with fresh medium throughout the cultivation period.

Double-Haploid Production

Culture embryos, plant rescue, and chromosome doubling were performed according to the procedures described by Inagaki (1997). The seeds were removed from the spikes and immersed in 70% ethanol for a few seconds then sterilized for 10 min in

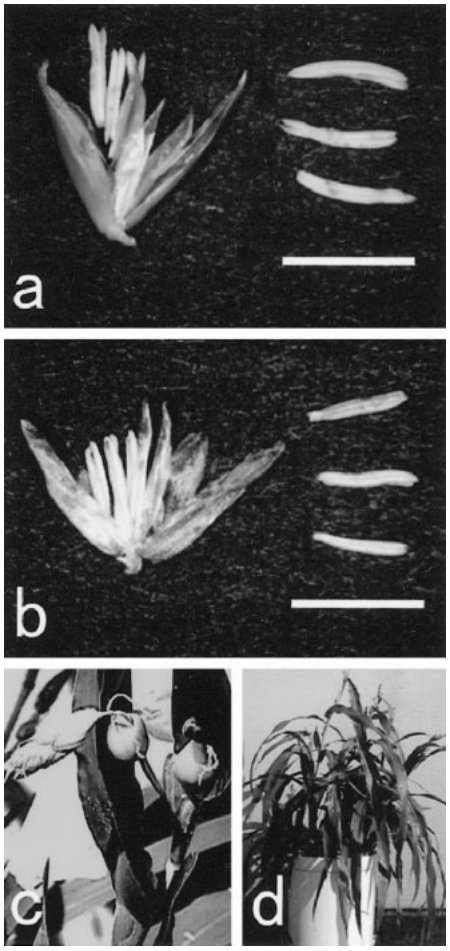

Figure 1. Male inflorescences of **(A)** Job's tears and **(B)** maize. Bar = 5 mm. **(C)** Job's tears inflorescences and **(D)** whole plant in a pot.

a solution containing 1–2% sodium hypochloride and a few drops per liter of Tween-20. After rinsing twice with sterile water, the seeds were dissected on sterilized filter papers, and the embryos were examined under the stereoscopic microscope. The embryos were transferred onto Murashige and Skoog medium (M-5519 one pack per liter; Sigma Chemical Co., St. Louis, MO) with 2% sucrose and 0.6% agarose in plastic dishes. The embryo rescue procedure was performed under aseptic conditions on a clean bench. The embryos were cultured at 22.5°C in a room set with a light:dark cycle of 16:8 h. After about 1 month the new plants grown from the embryos were transplanted to potting soil and grown in a greenhouse set at 22°C with relative humidity of 80% and 16:8 h light:dark cycle.

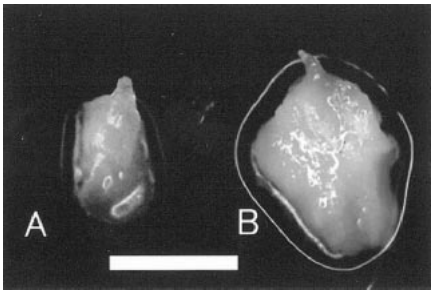

Figure 2. Comparison of wheat embryo formed **(A)** by crossing wheat and Job's tears and **(B)** by crossing with maize. Bar = 1 mm.

The plant somatic chromosomes were examined in squashed preparations of root-tip cells stained with acetocarmine. To induce chromosome doubling, the plants were treated with colchicine solution [0.05% colchicine, 2% dimethyl sulfoxide (DMSO), 15 drops/l Tween-20] for 12–15 h in darkness at room temperature, rinsed with water, and transferred to soil. Self-pollinated seeds were obtained from the plants. The likelihood of embryo and haploid formation was estimated using the following formulas:

Frequency of embryo formation
= Number of embryos carrying caryopses
÷ Total number of pollinated florets

Efficiency of haploid formation
= Total number of regenerated plants
÷ Total number of pollinated florets

These values were subjected to analysis of variance (ANOVA) after arc sine transformation.

Results and Discussion

Wheat florets crossed with the pollen of Job's tears bore caryopses at 14 days after pollination that were smaller than those obtained by self-pollination. The caryopses were filled with an aqueous solution instead of the solid endosperm normally found in the wheat, and some contained immature embryos (Figure 2). The obtained embryos grew well and 30% formed normal shoots and roots. We examined the somatic chromosomes of all six regenerated plants. The root-tip cells of these plants carried 21 chromosomes of wheat, indicating a complete elimination of the chromosomes of Job's tears (Figure 3). ANOVA results indicated that the frequencies of embryo and haploid formation were not significantly different from those obtained by crossing with maize pollen (Table 1). Plants were treated with colchicine and diploid plants were obtained. The seeds of the double plants showed 42 normal chromosomes of common wheat.

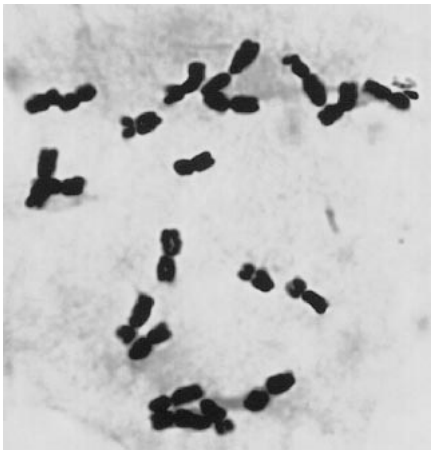

Figure 3. Somatic chromosomes ($2n = 3x = 21$) of a plant derived by crossing wheat and Job's tears.

Collection of pollen from Job's tears is not a straightforward process when compared to collection of maize pollen. However, Job's tears is a perennial plant that forms lateral buds, and thus pollen can be obtained throughout the year (Figure 1). In the present study, although only a few plants of Job's tears were grown in the greenhouse, we were able to cross the pollen whenever wheat flowered. Thus we were able to easily produce wheat haploids without synchronization of the pollen donor. Consequently Job's tears can be used as a suitable pollen donor for wheat haploid production using the wide-cross method.

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Table 1. Frequencies of haploid formation following crossing between wheat and Job's tears or maize

Cross	No. of crossed florets	No. of formed embryos (%)	No. of haploid plants (%)
Wheat × Job's tears	218	23 (10.6)	6 (2.8)
Wheat × maize	1196	125 (10.5)	33 (2.8)

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A Recessive Allele Inhibiting Saponin Synthesis in Two Lines of Bolivian Quinoa (*Chenopodium quinoa* Willd.)

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Quinoa cultivars currently grown in North America and Europe require removal of bitter-tasting saponins from the grain prior to human consumption. This need for postharvest processing is a barrier to expanding production of the crop outside its Andean area of origin. Grain saponin content in quinoa shows continuous variation and is considered to be a quantitative trait. However, segregation for the presence or absence of grain saponin in F2 generations derived from crosses between high- and low-saponin parents indicates a major gene effect, with plants homozygous for a recessive allele sp1 having no detectable grain saponin. Variation in saponin levels among F2 plants with detectable grain saponin was consistent with polygenic inheritance. It appears that grain saponin level in quinoa is both qualitatively and quantitatively controlled, with saponin production requiring at least one dominant allele at the *Sp* locus and the amount of grain saponin being determined by an unknown number of additional quantitative loci. Introgression of sp1 into day-neutral lines will facilitate the development of short-season "sweet" quinoa cultivars which do not require postharvest processing to remove grain saponin.

Quinoa (*Chenopodium quinoa* Willd.) is a traditional Andean grain with many useful attributes. The species is drought and frost tolerant, will grow in poor soils at high elevation, and the grain is rich in essential amino acids (Koziol 1992). For these reasons quinoa is attracting attention as an alternative crop outside its area of origin, especially in the western United States, Canada, and northern Europe (Jacobsen et al. 1996; Ortiz et al. 1998). A significant barrier to expanding quinoa production in these areas, however, is the grain saponin content of short-season day-neutral varieties which can be grown at higher latitudes. Plant saponins are triterpenoid glucoside compounds found in many genera; most plant saponins have an intensely bitter flavor and all are potentially toxic if ingested in large quantities (Koziol 1992). Mizui et al. (1988; 1990) identified 13 different saponins in quinoa bran, 10 of them previously unknown. Quinoa saponins are concentrated in the external layers of the grain, which is botanically a fruit with a tightly adhering pericarp covering two seed coat layers (Varriano-Marston and DeFrancisco 1984). The tissue containing saponins is therefore of maternal origin, and grain saponin content reflects the genotype of the plant from which the grain is harvested. Saponins are traditionally removed from quinoa by washing the grain before cooking it, although mechanical dehulling by abrasion is also effective (Chauhan et al. 1992; Reichert et al. 1986). This method is used by U.S. quinoa producers, but it increases costs and is a major deterrent to potential growers who do not have access to the appropriate equipment. The key to developing commercial quinoa cultivars for North America and Europe is to combine early maturity and high yield with a sufficiently reduced grain saponin content to eliminate the need for postharvest processing (Jacobsen et al. 1996; Johnson and Ward 1993).

Estimates of grain saponin content in different quinoa cultivars range from 0.00 to 11.2 mg/g (Koziol 1992; Wahli 1990). Some Ecuadorian and Bolivian cultivars have extremely low levels of saponin in the grain, which can be consumed directly without washing or milling; however, these low-saponin or "sweet" lines ("quinoas dulces") are late maturing and perform very poorly at higher latitudes. There has been little investigation of the genetic basis of quinoa saponin content. Gandarillas (1979) proposed that the trait was controlled by two alleles at a single locus, with "bitter" (high saponin) dominant to "sweet" (low saponin). More recently researchers have observed that grain saponin content in quinoa is a continuously distributed variable and is therefore more likely to be polygenically controlled and quantitatively inherited (Galwey et al. 1990; Jacobsen et al. 1996).

Gas chromatography and spectrophotometric techniques have been used to characterize and quantify some individual quinoa saponins (Ng et al. 1994). Such methods are unsuitable for screening total grain saponin content in large numbers of samples, however, as they are time consuming and expensive, and at least 13 different saponins must be quantified, not all of which have been fully characterized. Afrosimetric methods, based on the quantity of foam generated when saponins are shaken in water, cannot quantify the individual saponins present but do offer the advantage of providing a rapid and economical estimate of total grain saponin content. Koziol (1991) has described a standardized afrosimetric test for estimating grain saponin levels in quinoa. This method has some minor disadvantages: it underestimates very high levels of grain saponin, and results may be affected by the presence of other surfactants. However, the test readily identifies "sweet" quinoa lines that contain no detectable saponins, as these produce no measurable

Table 1. Grain saponin content (in mg/g) of parent plants and 20 F_1 progeny from each of four different quinoa crosses

Cross	Female parent	Male parent	F_1 Progeny mean	F_1 Standard error	F_1 Progeny range
Amachuma × Tango	6.37	1.65	9.33	0.36	7.16–11.03
Calcha × Baer	6.52	2.50	6.70	0.19	5.41–8.13
Isluga × Sajama	6.50	0.00	7.67	0.44	4.18–10.13
Amachuma × Sayana	6.25	0.00	10.26	0.05	10.13–10.64

foam. Coefficients of variation obtained for repeated tests using this method are within acceptable limits, and the standardized test has proved satisfactory when screening for saponin content of individual plants within quinoa populations (Jacobsen et al. 1996; Ward 1994).

Genetic analysis of economically important traits in quinoa has been hampered by the difficulty of making controlled crosses. Quinoa is a predominantly self-pollinating allotetraploid with large numbers of very small (3 mm diameter) perfect flowers clustered on an inflorescence. This floral structure makes artificial hybridization very difficult and hinders the production of segregating generations large enough for analysis. The research described here used cytoplasmic male sterile (CMS) quinoa lines as female parents in four different crosses, two of which used low saponin "sweet" quinoa cultivars as pollen parents. Subsequent F_1, F_2, and F_3 generations were screened for saponin content using Koziol's standardized afrosimetric test. The goal of this research was to characterize genetic control of grain saponin content in quinoa, especially in low-saponin lines.

Materials and Methods

All quinoa plants used in this study were grown in a greenhouse in Fort Collins, Colorado, in 1998 and 1999. Five plants were grown in each 25 cm diameter pot, using commercial potting compost supplemented by liquid fertilizer. Greenhouse temperature was maintained at 25°C and plants were grown under broad-spectrum halogen lamps with a 16 h photoperiod maintained throughout the experiment. Four individual crosses were made using CMS quinoa plants as female parents (Table 1). The CMS lines were derived from a Peruvian accession (PI 510536) from the USDA-ARS *Chenopodium* collection; this CMS system and the associated maintainer and restorer lines have been described previously (Ward 1998). The pollen parents were selected from these restorer lines to generate fully fertile F_1 generations. The four crosses were made by enclosing a male sterile inflorescence with exserted stigmas together with a male fertile inflorescence at anthesis within a single waxed paper pollination bag for 5 days; this also enabled simultaneous selfing of the pollen parent. Grain saponin content of all parent plants was determined by using Koziol's (1991) standardized afrosimetric test on the F_1 seed harvested from the female parents and on the S_1 seed harvested from the male parents. Twenty F_1 progeny from each of the four crosses were grown and self-pollinated by enclosing the inflorescence in a waxed paper pollination bag throughout anthesis, and the grain saponin content of the F_1 plants was determined by using Koziol's test on the F_2 seed harvested from each F_1 plant. Details of the four initial crosses are given in Table 1.

Seed from a single F_1 plant from each cross was planted to produce an F_2 of at least 160 plants. Three of the four F_2 generations segregated for presence or absence of the nuclear restorer allele, and in these cases all male sterile F_2 plants were discarded at flowering. Each F_2 plant was self-pollinated by enclosing the inflorescence in a waxed paper pollination bag throughout anthesis, and F_2 grain saponin contents were determined by testing the F_3 seed harvested from each F_2 plant. F_3 families of 40 plants were raised from each of 10 selected zero-saponin F_2 parents, 5 of which were derived from cross 3 and 5 from cross 4. These F_3 plants were self-pollinated and tested for grain saponin content as already described. Chi-square tests for goodness-of-fit were performed on the two F_2 generations segregating for presence or absence of detectable levels of grain saponin, using Yates' continuous correction factor (Gomez and Gomez 1984).

Results

All of the CMS plants used as female parents had high grain saponin levels, while two of the male parent plants had medium saponin levels and two were effectively zero saponin, with grain saponin levels below the limits of detection using afrosimetric methods (Table 1). All F_1 plants were high saponin, although variation in saponin levels among plants within individual F_1 generations was observed in three of the four crosses. All F_2 offspring from cross 1 (Amachuma × Tango) and cross 2 (Calcha × Baer), where a medium-saponin male parent was used, had measurable levels of grain saponin, although considerable variation was observed between plants within F_2 families. F_2 progeny from cross 3 (Isluga × Sajama), where the male parent lacked saponin, segregated 148:41 for the presence or absence of saponin. This corresponds to a ratio of 3:1 ($\chi^2 = 1.02$, $P = .34$). F_2 progeny from cross 4 (Amachuma × Sayana), where the male parent also lacked saponin, segregated 139:27. This ratio falls between 3:1 ($\chi^2 = 6.53$, $P = .013$) and 15:1 ($\chi^2 = 29.97$, $P < .01$). In both cross 3 and cross 4, variation among F_2 plants that contained measurable levels of grain saponin was observed. Frequencies of plants containing different levels of grain saponin are summarized for all four crosses in Figure 1. None of the 40 F_3 progeny grown from each of the 10 selected zero-saponin F_2 plants had detectable grain saponin levels.

Discussion

Segregation for a continuous range of saponin levels was seen in the F_2 populations from crosses 1 and 2, where both parents had either high or medium saponin levels. This is consistent with the hypothesis that grain saponin content in quinoa is a quantitative trait controlled at multiple loci. The high saponin levels in the F_1 generations from all four crosses and the skewed frequency distributions for F_2 progeny from crosses 1 and 2 also indicate that dominance effects may be significant contributors to the observed phenotypic variance. F_1 saponin levels exceeded parental values in all four crosses, which could be a heterotic effect due to overdominance. This suggestion should be treated with caution, however: as a quantitative trait, grain saponin level may also be affected by the environment, and it is possible that even in a greenhouse growing the parent and F_1 plants at different times of the year (parents in late summer 1998, F_1 plants in winter 1998–1999) generational differences in grain saponin content may be observed. The nature and extent of environmental influences on grain saponin in

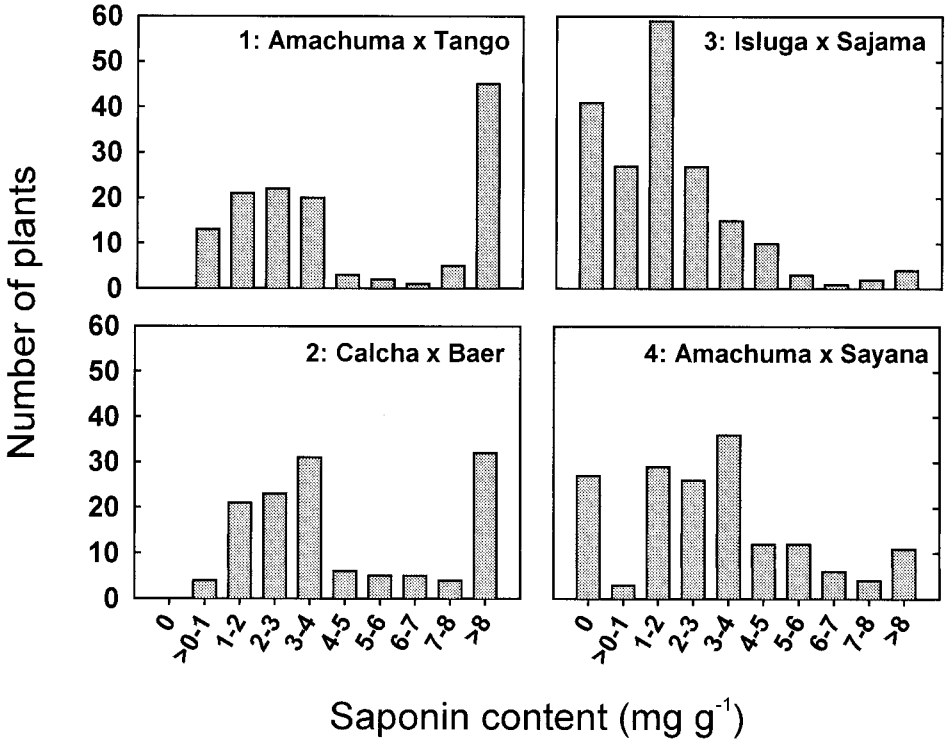

Figure 1. Frequency distributions of grain saponin content of F_2 progeny from four different quinoa crosses.

quinoa and the possibility of heterosis for the trait clearly need further investigation. The amount of between-plant variation observed in three of the F_1 generations indicates heterozygosity at the relevant multiple loci at one or both of the parents used in those crosses. This was not unexpected: although quinoa is largely self-pollinating, at least 1–2% outcrossing typically occurs between adjacent plants (Espindola G, personal communication). The male fertile restorer lines used in this study were maintained as individual bulk populations, isolated from other lines but without bagging individual plants, so complete homozygosity of all plants cannot be assumed. As an allotetraploid, quinoa may also be subject to fixed heterozygosity at some loci, even when self-pollinated.

The F_2 progeny in crosses 3 and 4, where zero-saponin pollen parents were used, show frequency distributions for those plants with measurable quantities of grain saponin consistent with quantitative inheritance and similar to those from crosses 1 and 2. A striking difference in the F_2 generations from crosses 3 and 4, however, is the clear segregation for presence or absence of grain saponin, with a ratio of 3:1 in cross 3 and a distorted segregation falling between 3:1 and 15:1 in cross 4. As quinoa is an allotetraploid, multiple genotypes are possible: the variation in F_2 progeny ratios between crosses 3 and 4 reflects different genotypes at the *Sp* locus in the CMS female parents, and consequent differences in genotype between the F_1 plants selfed to produce the segregating F_2 generations. The F_2 ratios observed here are typical of allotetraploid segregation at a single locus present in both genomes, where partial pairing between homologous chromosomes produces a range of erratic segregations. Similar segregation ratios have been observed for other single-gene traits in quinoa (Ward 2000). This segregation for presence or absence of grain saponin is consistent with the observation made by Gandarillas (1979) that a cross between "bitter" and "sweet" Bolivian quinoas produced a high-saponin or "bitter" F_1 and an F_2 generation that segregated for presence or absence of grain saponin ("bitter" or "sweet") in a 3:1 ratio. Gandarillas did not report any F3 data; however, the 400 F_3 plants derived from 10 zero-saponin F_2 individuals in this experiment were all uniformly zero-saponin, indicating that both they and their F_2 parents were homozygous at the relevant locus.

These results suggest that Sayana and Sajama, the Bolivian cultivars used as pollen parents in crosses 3 and 4, possess a recessive allele that inhibits saponin production when homozygous. This allele may be a mutated or null version of a gene encoding either an enzyme critical for saponin synthesis or a regulator of the saponin synthetic pathway. Crossing these zero-saponin cultivars with saponin-containing lines produces F_1 plants in which saponin synthesis is restored, possibly because at least one functional copy of the key gene is now present. The between-plant variation in grain saponin content in three of the four F_1 generations, and the more extensive variation seen among F_2 plants containing saponin, indicate that in plants with at least one functional copy of the key gene, grain saponin levels will depend on alleles present at an unknown number of additional loci. Variation for this trait as seen in the F_2 distributions (Figure 1) does not indicate simple additive effects, which is not surprising given the number of different saponins known to be present in quinoa. More extensive biochemical analysis is needed to determine the relative contributions of the different triterpenoid glucosides in quinoa to the total grain saponin content.

Similar combinations of qualitative and quantitative variation for a single trait have been reported elsewhere: in maize silks, for example, maysin concentration is controlled at a major locus *p1* coding for a transcription activator, with additional minor loci only active in the presence of a functional *p1* allele (Byrne et al. 1996). Another possibility is that zero-saponin quinoa plants result from the accumulation and fixation of null alleles at the relevant quantitative loci, regardless of any additional major gene effect. However, no zero-saponin plants were identified in a total of 263 segregating F_2 plants from crosses 1 and 2, suggesting that if this does occur it is a relatively rare event.

An unresolved question is whether the "sweet" quinoa plants identified in this experiment are totally lacking in grain saponin or are failing to produce only certain types of saponin compounds. As described earlier, Mizui et al. (1988; 1990) identified more than 13 different saponins in quinoa bran. Ng et al. (1994) reported that using thin-layer chromatography and mass spectrometry they were able to detect saponins containing phytolaccagenic acid and oleanolic acid in three quinoa lines identified as "sweet," while "bitter" lines also contained the sapogenol hederagenin. However, the total saponin contents reported by these researchers for the "sweet" lines tested ranged from 1.3 to 3 mg/g, levels which would actually taste bitter to most humans and which are above the 1.1 mg/g threshold for "sweet" as defined by Koziol (1991). These results

indicate that different types of saponins are found in varying proportions in different quinoa varieties, again suggesting that multiple loci are involved. As discussed earlier, Koziol's afrosimetric test cannot distinguish between different types of saponins, but it does distinguish effectively between quinoa plants containing measurable levels of some form of saponin and those which do not. Seed from all the plants classified as zero saponin in this experiment failed to generate any measurable foam when shaken with water, and such seed also had no detectable bitterness when tasted.

This recessive allele, provisionally designated *sp1,* offers a number of advantages to breeding programs developing commercial quinoa varieties for North America and Europe. Introduction of this allele into the early maturing day-neutral lines via conventional backcross techniques should be straightforward and less time consuming than attempting to manipulate grain saponin content as a quantitative trait. Plants that are homozygous for *sp1* can be identified easily and inexpensively using afrosimetric methods, will produce uniformly zero-saponin progeny, and will eliminate the need for postharvesting processing of the grain. A potential disadvantage is that zero-saponin quinoa lines homozygous for *sp1* must be grown in isolation if seed is to be saved, as even low levels of cross-pollination from saponin-containing lines will result in the loss of the "sweet" trait in subsequent generations. It is also likely that the presence of saponins in quinoa grain provides protection against bird and insect attacks, and such damage in the field may be a greater problem with zero-saponin quinoa lines. These disadvantages, however, are offset by eliminating the need for postharvest grain processing, which will increase the potential for quinoa as an alternative crop for non-Andean countries.

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Parental Effects in the Inheritance of Nonnodulation in Peanut

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A nonnodulating line (M4-2) and three normal nodulating lines (UF 487A, PI 262090, and Florunner) of peanut (*Arachis hypogaea* L.) were crossed in full diallel to investigate the inheritance of nodulation. Data from F_1, F_2, F_3, F_1BC_1, and F_2BC_1 generations indicated that three genes control nodulation at three independent loci, with nodulation being a product of two genes and inhibited by a third gene when it is dominant and the others are homozygous recessive. A genetic model has been proposed that describes the nonnodulated genotypes as $n_1n_1n_2n_2N_3N_3$ or $n_1n_1n_2n_2N_3n_3$ and all other genotypes as normally nodulated except $n_1n_1N_2n_2N_3$–, which has reduced nodulation when the $n_1n_2N_3$ male gamete unites with the n_1N_2– female gamete or when the $n_1n_2n_3$ male gamete unites with the $n_1N_2N_3$ female gamete.

Most legumes, when infected by the appropriate *Rhizobium* strains, form root nodules that are capable of N_2 fixation. However, nonnodulation mutants derived either spontaneously or through mutagenesis have been reported from eight species that normally nodulate. A single recessive gene, rj_1, controls nonnodulation in a spontaneously derived soybean (*Glycine max* L. Merr.) mutant (Williams and Lynch 1954) and two ethyl methanesulphonate (EMS)-induced soybean mutants (Mathews et al. 1989). However, a third EMS-derived soybean mutant is controlled by another single recessive gene different from rj_1 (Mathews et al. 1989). In wild pea (*Pisum* spp.), a homozygous recessive *sym-2* gene controls nonnodulation (Holl 1975), and in an EMS-induced pea mutant nonnodulation is conditioned by *sym-5* (Kneen and LaRue 1984). In common bean (*Phaseolus vulgaris* L.), a single recessive gene, *nnd-2,* controlled nonnodulation in an EMS-derived mutant (Park and Buttery 1994). In nonnodulating mutants of sweetclover (*Melilotus alba* Desr.), a single recessive gene is responsible for the trait; however, allelism tests demonstrated that at least five different genes, *sym-1, sym-2, sym-3, sym-4,* and *sym-5,* are involved in nodulation (Miller et al. 1991). In three chickpea (*Cicer* spp) mutants derived by γ radiation, nonnodulation is controlled by different single recessive genes, *rn1, rn2,* and *rn3* (Davis et al. 1986). Later, spontaneous nonnodulating chickpea mutants were identified that also were controlled by different single recessive genes (Singh et al. 1992; Singh and Ruplea 1998). Nonnodulation in red clover (*Trifolium pratense* L.) is controlled by a recessive gene, *r,* which is affected by a cytoplasmic factor and the presence of zygotic and postzygotic lethals (Nutman 1949). In a spontaneous mutant of alfalfa (*Medicago sativa* L.), nonnodulation is controlled by two te-

Table 1. Cross description, parental genotype, and nodulation classification of field-grown F_1 plants

Cross ♀	Cross ♂	Genotype	No. of plants Nodulated	Few	Nonnodulated
UF 487A	M4-2	$N_1N_1n_2n_2\ N_3N_3 \times n_1n_1n_2n_2N_3N_3$	32	0	1
M4-2	UF 487A	$n_1n_1n_2n_2\ N_3N_3 \times N_1N_1n_2n_2\ N_3N_3$	26	0	0
PI 262090	M4-2	$n_1n_1N_2N_2n_3n_3 \times n_1n_1n_2n_2\ N_3N_3$	1	24	8
M4-2	PI 262090	$n_1n_1n_2n_2\ N_3N_3 \times n_1n_1N_2N_2n_3n_3$	30	0	0
Florunner	M4-2	$N_1N_1N_2N_2n_3n_3 \times n_1n_1n_2n_2\ N_3N_3$	51	1	0
M4-2	Florunner	$n_1n_1n_2n_2\ N_3N_3 \times N_1N_1N_2N_2n_3n_3$	31	0	0
UF 487A	PI 262090	$N_1N_1n_2n_2\ N_3N_3 \times n_1n_1N_2N_2n_3n_3$	17	0	0
PI 262090	UF 487A	$n_1n_1N_2N_2n_3n_3 \times N_1N_1n_2n_2\ N_3N_3$	28	0	0
Florunner	PI 262090	$N_1N_1N_2N_2n_3n_3 \times n_1n_1N_2N_2n_3n_3$	25	0	0
PI 262090	Florunner	$n_1n_1N_2N_2n_3n_3 \times N_1N_1N_2N_2n_3n_3$	18	0	0
Florunner	UF 487A	$N_1N_1N_2N_2n_3n_3 \times N_1N_1n_2n_2\ N_3N_3$	29	0	0
UF 487A	Florunner	$N_1N_1n_2n_2N_3N_3 \times N_1N_1N_2N_2n_3n_3$	20	0	0

trasomically inherited recessive genes, nn_1 and nn_2 (Peterson and Barnes 1981).

Gorbet and Burton (1979) were the first to describe a nonnodulating peanut (*Arachis hypogaea* L.), which originally was identified in the F_3 generation from the hybridization of UF 487A, a University of Florida breeding line, with PI 262090. Nigam et al. (1980) also identified nonnodulating peanut plants from the cross PI 259747 with NC 17 and NC Ac 2731. They reported that two independent duplicate genes control nodulation, with nonnodulated plants reported as homozygous recessive at both loci ($n_1n_1n_2n_2$) (Nigam et al. 1982). However, Dutta and Reddy (1988) proposed a three-gene model where two genes produce nodulation and a third gene inhibits nodulation when it is dominant and the other genes are homozygous recessive ($n_1n_1n_2n_2N_3$_). In addition, Essomba et al. (1991), in studies of the inheritance of stem color and nonnodulation in peanut, also reported that nonnodulation may be inherited by three independent genes. However, in that study the three genes showed additive effects, and nonnodulation was conditioned by any two of the three genes being in a homozygous recessive state. In their model, plants with an $n_1n_1n_2n_2n_3n_3$ genotype would be nonnodulating, whereas in the Dutta and Reddy (1988) model that genotype would be nodulated. Therefore the inheritance of nonnodulation in peanut remains unclear.

The objective of this study was to examine the inheritance of nonnodulation in peanut using the nonnodulating peanut line, M4-2, selected from the original nonnodulating germplasm described earlier (Gorbet and Burton 1979). Our results support the three locus model of Dutta and Reddy (1988). However, crosses with n_1N_2_ female gametes fertilized by $n_1n_2N_3$ pollen or $n_1N_2N_3$ female gametes fertilized by $n_1n_2n_3$ pollen resulted in reduced or nonnodulation, whereas the reciprocal crosses produced normal nodulation.

Materials and Methods

Three normal nodulating lines, UF 487A (University of Florida line), PI 262090 (Robore, Bolivia), and Florunner (cultivar unrelated to the above peanuts), and a nonnodulating line, M4-2, derived from the cross UF 487A × PI 262090, were used as parents in a diallel cross. F_1 plants were backcrossed to M4-2 or PI 262090. The F_1, F_2, F_3, F_1BC_1, and F_2BC_1 generations were field grown at the University of Florida Agricultural Research and Education Center at Marianna, Florida. Recommended agronomic practices were utilized, including inoculation of seed at planting with cowpea-type *Rhizobium* sp.

Leaf color ratings of individual plants were taken from a representative leaf from each plant just prior to digging. Each plant was tagged so that foliage color could be compared with nodule characteristics. Plants were dug using a conventional peanut digger-inverter, cutting roots 20–25 cm below the soil surface. Immediately after digging, nodulation of roots of individual plants were rated according to size and number as nodulated (equivalent to the number and size of nodules found in Florunner), few (less than 50 nodules whose diameters were almost twice the size of Florunner nodules), and nonnodulated. Pods were hand picked from individual plants that were to be progeny tested. In classifying the nodulation trait in crosses subsequent to the F_1 generation, the few class was considered as nodulated. This classification scheme is consistent with those used by others examining nodulation in peanut (e.g., Dutta and Reddy 1988). Data were analyzed by chi-square tests for goodness-of-fit to the two-locus (Nigam et al. 1982) and three-locus models for nodulation (Dutta and Reddy 1988; Essomba et al. 1991).

Results and Discussion

Field observation indicated three nodule categories: nonnodulated, few nodules, and normally nodulated. Leaf colors were yellow-green or dark green. Yellow-green plants either had few or no nodules, and dark green plants had normal nodulation. Most plants with few nodules also had larger nodules than a normally nodulated plant. Peanut plants with few, very large nodules also were observed by Nambiar and Dart (1980) in populations that were segregating for nodulating and nonnodulating plants.

Reciprocal crosses in the F_1 involving the nonnodulating mutant, M4-2, showed differences in nodulation (Table 1). The three crosses with M4-2 as the female parent resulted in all progeny being normally nodulated. However, the three crosses with M4-2 as the male parent exhibited reduced nodulation in the F_1. This effect was particularly pronounced in F_1 plants generated from PI 262090 × M4-2. Of the 33 F_1 plants rated, 8 were nonnodulated, 24 had few, and only 1 was normally nodulated.

F_2 data were analyzed by chi-square tests for goodness-of-fit to the expected ratios for the two-locus (Nigam et al. 1982) and three-locus models for nodulation (Dutta and Reddy 1988; Essomba et al. 1991). Only the three-locus model proposed by Dutta and Reddy (1988) fit the observed F_2 data (Table 2). The results indicate that three gene loci are operating in these crosses. The genotypes proposed for the parents are $n_1n_1n_2n_2N_3N_3$ for M4-2, $n_1n_1N_2N_2n_3n_3$ for PI 262090, $N_1N_1n_2n_2N_3N_3$ for UF 487A, and $N_1N_1N_2N_2n_3n_3$ for Florunner (Table 1). The cross UF 487A ($N_1N_1n_2n_2N_3N_3$) × M4-2 ($n_1n_1n_2n_2N_3N_3$) and the reciprocal cross segregated 3 nodulated:1 nonnodulated. This indicates that the N_1 allele is completely dominant to n_1 and results in nodulation. The cross PI 262090 ($n_1n_1N_2N_2n_3n_3$) × M4-2 ($n_1n_1n_2n_2N_3N_3$) segregated 10 nodulated: 6 nonnodulated because half of the plants that were heterozygous at the N_2 locus ($n_1n_1N_2n_2N_3$_) were formed as a result of either the union of an $n_1N_2N_3$ female gamete and an n_1n_2_ male gamete or an $n_1N_2n_3$ female gamete and an $n_1n_2N_3$ male gamete which would produce plants that were nonnodulated as a result of the parental effect described earlier.

In accordance with this model of inheritance, the crosses Florunner ($N_1N_1N_2N_2n_3n_3$)

Table 2. F_2 data analyzed by chi-square test for goodness-of-fit to the proposed model

Cross		Number of F_2 plants				
♀	♂	Nodulated[a]	Nonnodulated	df	χ^2	*P*
UF 487A × M4-2		1354	426	1	1.08 (3:1)	.30
M4-2 × UF 487A		1440	485	1	0.03 (3:1)	.86
PI 262090 × M4-2		1462	855	1	0.36 (10:6)	.55
M4-2 × PI 262090		1077	668	1	0.48 (10:6)	.49
Florunner × M4-2		2082	237	1	2.03 (58:6)	.15
M4-2 × Florunner		1698	198	1	2.48 (58:6)	.12
UF 487A × PI 262090		1094	124	1	0.97 (58:6)	.32
PI 262090 × UF 487A		943	83	1	1.94 (58:6)	.16
Florunner × PI 262090		913	1			
PI 262090 × Florunner		744	0			
Florunner × UF 487A		803	0			
UF 487A × Florunner		459	0			

[a] Normally nodulated plus few nodules.

× M4-2 ($n_1n_1n_2n_2N_3N_3$), UF 487A ($N_1N_1n_2n_2N_3N_3$) × PI 262090 ($n_1n_1N_2N_2n_3n_3$), and reciprocals produced the same F_2 segregation ratios since they had the same F_1 genotypes ($N_1n_1N_2n_2N_3n_3$), with an expected ratio of 58 nodulated:6 nonnodulated. All N_1_ _ _ _ _ and _ _N_2N_2 _ _ genotypes were normally nodulated. Half of the plants that were $n_1n_1N_2n_2N_3N_3$ and $n_1n_1N_2n_2N_3n_3$ would be phenotypically classified as nonnodulated and the other half as normally nodulated, for the same reason mentioned in the previous cross.

The crosses Florunner ($N_1N_1N_2N_2n_3n_3$) × PI 262090 ($n_1n_1N_2N_2n_3n_3$), Florunner ($N_1N_1N_2N_2n_3n_3$) × UF 487A ($N_1N_1n_2n_2N_3N_3$), and their reciprocals produced all normally nodulated plants since both parents were homozygous dominant at the N_1 and/or N_2 locus. All but one of 2920 segregates were nodulated. This one easily could have been nonnodulated due to a number of factors including environmental conditions or mutations. The chi-square values for all F_2 data had probabilities above the 10% level, strongly supporting the three-locus model (Table 2).

The number of F_2 families (classified F_3 plants) derived from nodulated F_2 plants was determined by calculating the ratio of each expected F_2 genotype that had normal nodulation and then determining the expected segregation ratio of the progeny for each genotype, and the results supported the three-locus model of nodulation. For example, from the cross UF 487A × M4-2 and the reciprocal, one would expect one-third of the nodulated plants to produce only nodulated progeny and two-thirds to segregate 3 nodulated:1 nonnodulated. These expected ratios were observed (P = .49 and .54).

The F_1BC_1 data also provided strong evidence to support the proposed model (Table 3). For example, in the last two entries involving PI 262090 and M4-2 (Table 3) the genotypes of the parents are $n_1n_1N_2N_2n_3n_3$ and $n_1n_1N_2n_2N_3n_3$, when the female gamete was $n_1N_2n_3$ and the male gamete was $n_1n_2N_3$, the resulting F_1BC_1 plant was nonnodulated with a genotype of $n_1n_1N_2n_2N_3n_3$. However, when the female gamete was $n_1n_2N_3$, and the male was $n_1N_2n_3$, the F_1BC_1 plant was nodulated and had the same genotype as the previous example, $n_1n_1N_2n_2N_3n_3$. So, the genotypes are the same, but their nodulation differs relative to inheritance from the maternal or paternal parent. Similar results were also obtained for F_2BC_1 data.

Our proposed model for inheritance of nonnodulation in peanut suggests that the phenotype of peanut plants can be different even though the genotypes are the same because of a parental effect. Mouli and Patil (1975) reported a similar mode of inheritance for an X-ray-induced foliaceous stipule mutant in peanut. They found that F_1 plants showed normal stipules when the mutant was used as the female parent, but when the mutant was used as the pollen parent all F_1 plants had foliaceous stipules. Similar to our results, parental influence on expression also was evident in their F_1BC_1. A parental effect in peanut has also been described in a spontaneously derived shriveled seed mutant (Jakkula et al. 1997b). Jakkula et al. (1997a) found that when the shriveled seed mutant was the male parent, the progeny segregated in the expected Mendelian fashion, 3 normal:1 shriveled in the F_2 derived from a cross with a wild-type peanut. However, when the mutant was used as the female parent, only normal seed was produced in F_1, F_2, and F_3 seed progenies.

In this study, results of our F_2 and backcross data make it appear unlikely that extranuclear inheritance plays a role in nonnodulation. One possible interpretation of our results may be that the inheritance of nodulation in peanut is controlled by parental or gametic imprinting (for a review see Matzke and Matzke 1993). In such genomic imprinting, either the maternally or paternally derived allele is actively expressed, while the other is silent. Imprinting involves an epigenetic mechanism such as DNA methylation. In the mammalian genome, it has been shown that there are allele-specific regions of differential methylation in imprinted genes, and a trend for imprinted genes to be clustered (for a review see Constância et al. 1998). In our study, it is possible that imprinting of the N_2 locus results in reduced expression when inherited through the egg. However, when inherited through the male gamete, imprinting of the N_2 locus is erased and this allele becomes fully active. Since a parental effect has been detected for several different characters in peanut, it supports the hypothesis that peanut may regulate expression of a number of genes through some form of genomic imprinting.

Table 3. F_1BC_1 data with each cross grouped according to the genotype of the parents and analyzed by chi-square test for goodness-of-fit to the proposed model

Cross		Nodulation		Chi-square test		
♀ × (♀ × ♂) and (♀ × ♂) × ♂		Nodulated[a]	Nonnodulated	df	χ^2	*P*
M4-2 × (UF 487A × M4-2)	ER[b]	1	1			
M4-2 × (M4-2 × UF 487A)	O	38	33	1	0.35	.55
(UF 487A × M4-2) × M4-2	ER	1	1			
(M4-2 × UF 487A) × M4-2	O	39	34	1	0.49	.48
M4-2 × (PI 262090 × M4-2)	ER	1	1			
M4-2 × (M4-2 × PI 262090)	O	16	12	1	0.57	.45
M4-2 × (Florunner × M4-2)	ER	3	1			
(M4-2 × (M4-2 × Florunner)	O	40	13	1	0.01	.92
PI 262090 × (M4-2 × PI 262090)	ER	3	1			
PI 262090 × (PI 262090 × M4-2)	O	8	3	1	0.03	.86
(M4-2 × PI 262090) × PI 262090	ER	1	0	0		
(PI 262090 × M4-2) × PI 262090	O	14	0			

[a] Normally nodulated plus few nodules.

[b] ER = expected ratio, O = observed frequency.

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Genetic and Linkage Analysis of Cleistogamy in Soybean

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Early maturing cultivars of soybean [*Glycine max* (L.) Merr.] native to the shores of the Sea of Okhotsk (Sakhalin and Kuril Islands) and eastern Hokkaido (northern Japan) have been used in breeding for chilling tolerance. These cultivars have a strong tendency to produce cleistogamous flowers throughout their blooming period. This study was conducted to determine the genetic basis of cleistogamy in an early maturing cultivar, Karafuto-1, introduced from Sakhalin. Genetic analysis was performed using F_1 plants, the F_2 population, and 50 F_3 families produced by crossing between Karafuto-1 and a chasmogamous cultivar, Toyosuzu. F_1 plants had chasmogamous flowers, indicating that chasmogamy was dominant to cleistogamy. Analysis of F_2 populations and F_3 families generated segregation data that was close to a two-gene model with epistatic interactions, although a portion of the pooled F_3 data on the frequency of chasmogamous segregants from cleistogamous families significantly deviated from the model. The results suggested that a minimum of two genes with epistatic effects were involved in the genetic control of cleistogamy. Furthermore, cleistogamy was associated with early flowering in the F_2 and F_3 populations. A gene for cleistogamy was linked to one of the recessive genes responsible for insensitivity to incandescent long daylength.

Cleistogamy, production of open (chasmogamous, CH) and closed (cleistogamous, CL) floral forms by one species, is widespread among the angiosperms. Lord (1981) classified cleistogamy into four categories: preanthesis cleistogamy in which pollination occurs followed by anthesis; pseudocleistogamy in which no morphological differences occur between CL and CH flowers other than a lack of expansion of petals and anthesis in CL flowers; complete cleistogamy in which species produce only CL flowers; and true cleistogamy in which floral dimorphism results from divergent developmental pathways in a single species or individual. Pseudocleistogamy is occasionally induced by environmental factors such as drought and low temperatures (Uphof 1938).

Cleistogamy has been described in members of the genus, *Glycine*. Newell and Hymowitz (1980) and Kenworthy et al. (1989) reported cleistogamy of *Glycine tabacina, G. tomentella,* and *G. arenaria*. Cleistogamy has also been observed in cultivated soybean [*Glycine max* (L.) Merr.] and its wild relative [*G. soja* Sieb. & Zucc.]. Soybean usually produces both CH and CL flowers on an individual plant; fertilization occurs without opening of petals in CL flowers. Thus, based on the classification of Lord (1981), soybean is pseudocleistogamous.

The production of CH and CL flowers on individual plants depends on developmental stages. Miyashita et al. (1999) studied the dynamics of CH and CL flowers in five *G. max* cultivars and two *G. soja* populations. The soybean cultivars produced primarily CH flowers at the early stage of flowering, whereas CL flowers were produced almost exclusively at the later stage. Among these cultivars, the proportion of CL flowers produced by an individual plant ranged from 22.6 to 66.5% of the total number of flowers.

In contrast, early maturing landraces (maturity group 000–00) native to the shores of the Sea of Okhotsk (Sakhalin and Kuril Islands) and eastern Hokkaido (northern Japan) usually produce only CL flowers when cultivated in Hokkaido. In these cultivars, fertilization occurs without opening of petals, or without any appearance of petals at anthesis. However, they have been observed to produce CH flowers at the early flowering stage during years with high temperatures.

The early maturing cleistogamous landraces have been used in breeding for chilling tolerance in Japan and Sweden (Holmberg 1973; Sanbuichi 1979). Soybeans are sensitive to low temperatures at various stages from germination to maturation (Raper and Kramer 1987). Low temperatures at the flowering stage, which is most sensitive to chilling stress, induce flower and pod abortion (Holmberg 1973; Hume and Jackson 1981, Takahashi and Asanuma 1996) or discolored and cracked seed coats (Sunada and Ito 1982; Takahashi and Abe 1994, 1999). Minimum temperatures

required for good flowering and pod set varied among cultivars (Holmberg 1973; Hume and Jackson 1981). The early maturing landraces and some of their descendants, such as the cultivar Fiskeby V, have somewhat lower critical temperatures, and flowering and seed formation were generally not interrupted by low temperatures (Holmberg 1973; Hume and Jackson 1981).

Cleistogamy may be advantageous under severe environmental conditions because the energetic investment in fertilization costs (i.e. sepals, petals, pollen, and nectar) of CL flowers appears to be considerably lower than CH flowers. Schemske (1978) evaluated the energetic costs of CH and CL flowers in pseudocleistogamous plant species, *Impatiens pallida,* and found that CH flowers had an energetic investment of more than 100 times higher than that of CL flowers. Further, CL flowers possibly shelter pollen from chilling temperatures at fertilization because chilling temperatures hindered pollen formation, anther dehiscence, and fertilization in soybean (Goto and Yamamoto 1972). Detailed physiological and genetic studies are necessary to evaluate possible roles of cleistogamy in relation to chilling tolerance. This study was conducted to investigate the genetic basis of cleistogamy in the early maturing soybean cultivars.

Materials and Methods

Plant Material

A CL cultivar, Karafuto-1, was pollinated by a CH cultivar, Toyosuzu, in 1997 and 1998. Toyosuzu is a cultivar (maturity group II) developed at the Tokachi Agricultural Experiment Station that has gray pubescence and a yellow hilum (*IIrrtt*). Toyosuzu has chasmogamous flowers irrespective of environmental conditions, except for the later stage of flowering. Karafuto-1 is a pure line selected from an early maturing landrace introduced from Sakhalin (maturity group 00); it has brown pubescence and brown hilum (*i-ii-irrTT*). Karafuto-1 has cleistogamous flowers throughout flowering time, except for years with high temperatures. The hybridization of F_1 plants was ascertained by brown pubescence color.

Genetic and Linkage Analysis

Twenty seeds from each parent and 120 F_2 seeds were sown in a field at Tokachi Agricultural Experiment Station, Memuro, Hokkaido, Japan (42°53′N, 143°05′E) on May 20, 1998. Twenty seeds from each parent, 4 F_1 seeds, and 20 seeds from each of 50 F_3 families were sown at the same location on May 19, 1999. Seeds of parents were sown in duplicate and thinned after emergence. Cleistogamy of each plant was determined by assessing flowers that fertilized at the first date of anthesis (R1; Fehr et al. 1971), because parental differences in floral forms were evident at an early period of flowering. The date of anthesis in CL plants was visually evaluated by the size of flower buds, and it was confirmed by comparing sizes of developing pods between CL and CH plants 5 days after anthesis. Floral forms were classified as follows; CH included open flowers with fully expanded petals, whereas CL included flowers that did not open fully, that is, those with slightly expanded petals that protruded out of calyxes, or those whose petals did not come out of calyxes.

Flowering response to incandescent long daylength (ILD) was also evaluated for parents and 98 F_3 families at Hokkaido University, Sapporo, Hokkaido, Japan (43°03′N, 141°20′E) in 1999. Toyosuzu is sensitive to ILD and flowering was retarded under ILD, whereas Karafuto-1 is insensitive to ILD and its flowering was not affected by daylength. Twenty seeds for each parent and 98 F_3 families were planted in a field where natural daylength was extended to 20 h using incandescent lamps. The response to ILD for individual plants was evaluated by growth stages at 60 days after planting. Classification of growth stage followed Fehr et al. (1971).

Results and Discussion

Inheritance of Cleistogamy

Table 1 shows temperatures from June 26 to July 25 in 1998 and 1999 at Memuro. All 10 plants of Karafuto-1 and Toyosuzu had CL and CH flowers at anthesis in both years. Date of anthesis of the F_2 plants in 1998 and that of the F_3 plants in 1999 ranged from July 15 to 25 and July 11 to 21, respectively. High temperatures from July 21, 1999, apparently had no affect on cleistogamy.

Due to virus infection, only two F_1 plants and 98 F_2 plants grew normally. The two F_1 plants had CH flowers, indicating that chasmogamy was dominant to cleistogamy (Table 2). Ninety-eight F_2 plants were classified into 77 CH and 21 CL plants. The result agreed with the dominance relationship observed in the F_1 plants. F_2 segregation fitted both a ratio of 3:1 under a single recessive gene model ($\chi^2 = 0.67$, $.4 < P < .5$) and a ratio of 13:3 under a single recessive gene (*a*) and a dominant gene (*B*) model, in which the former is epistatic to the latter ($\chi^2 = 0.46$, $.4 < P < .5$).

Segregation was further evaluated using 10 plants each from 50 F_3 families including 9 F_3 families derived from F_2 CL plants and 41 F_3 families derived from F_2 CH plants. Four of the nine CL families produced only CL plants, whereas the remaining five families segregated into CH and CL plants. This result evidently contradicts the single-gene control model, but it seemed to fit a segregation ratio of 0:2:1 for CH-fixed family:segregating family (*aaBb*):CL-fixed family (*aaBB*) under the two-gene model with epistatic interaction ($\chi^2 = 0.50$, $.7 < P < .8$). On the other hand, the 41 families derived from CH F_2 plants produced 28 CH-fixed families and 13 segregating families, with the segregation ratio in agreement with the expected ratio of 7:6:0 ratio for CH-fixed family (*AA–, Aabb, aabb*):segregating family (*AaBB, AaBb*):CL-fixed family ($\chi^2 = 3.44$, $.1 < P < .2$).

Taking into account a possible bias resulting from the small number of plants tested in each F_3 family, segregation of

Table 1. Maximum, minimum, and mean temperatures of June 26–July 25 in 1998 and 1999 and long-term average at Memuro

	Temperatures					
Year	Jun 26–30	Jul 1–5	Jul 6–10	Jul 11–15	Jul 16–20	Jul 21–25
1998						
Maximum	23.8	22.2	23.3	20.1	24.2	21.9
Minimum	12.4	14.0	13.7	10.2	12.2	16.6
Mean	17.8	17.5	18.0	14.1	17.6	18.8
1999						
Maximum	18.9	18.8	18.9	23.1	23.7	31.3
Minimum	11.3	12.5	12.1	16.5	15.8	18.7
Mean	14.9	15.1	14.7	19.3	19.3	25.0
Average						
Maximum	21.6	22.8	22.0	22.1	23.4	24.4
Minimum	11.7	12.1	12.7	13.8	14.4	16.0
Mean	16.3	17.0	16.9	17.5	18.4	19.6

cleistogamy in F_3 was evaluated on an individual-plant basis. A total of 495 plants could be evaluated due to lack of germination in some of the F_3 families. The 41 CH families produced 376 CH plants and 30 CL plants, closely fitting the expected number of 367 CH plants (47/52) and 39 CL plants (5/52) ($\chi^2 = 2.30$, $.1 < P < .2$). The nine CL families, however, produced a surplus of CH segregants that was significantly different from the expected number of 15 (1/6) based on the model ($\chi^2 = 29.9$, $P < .01$). These results suggested that cleistogamy in Karafuto-1 is not controlled by a single gene, and a minimum of two genes with epistatic effects may be involved in genetic control. However, the effects of different climatic conditions between the two years or the microclimatic differences due to the different flowering dates within the segregating populations were not factored in the present study. Neither penetrance nor expressivity were taken into account as factors influencing the expression of cleistogamy in the segregating populations, although parents exhibited constant and clear differences in our experiments. A further characterization of cleistogamy and/or experiments under controlled environments may therefore be necessary to exactly determine the contributions of genetic and environmental factors on the expression of cleistogamy in Karafuto-1.

Table 2. Cleistogamy of Toyosuzu (P_1), Karafuto-1 (P_2), and their F_1, F_2, and F_3 plants

Generation	Year	Number of plants		
		Total	CH	CL
P_1	1998	10	10	0
	1999	10	10	0
P_2	1998	10	0	10
	1999	10	0	10
F_1	1999	2	2	0
F_2	1998	98	77	21
F_3 (CL)[a]	1999	89	34	55
(CH)	1999	406	376	30

[a] F_3 (CL) and (CH) represent pooled F_3 plants derived from CL and CH F_2 plants, respectively.

Table 3. Association of cleistogamy with ILD response

ILD response of F_3 family	Number of F_2 plants	
	CL	CH
Karafuto-1 type	8	0
Karafuto-1 and intermediate type	1	4
Karafuto-1, intermediate, and Toyosuzu type	3	15
Karafuto-1 and Toyosuzu type	3	10
Intermediate type	0	4
Toyosuzu and intermediate type	2	28
Toyosuzu type	0	10

Association of Cleistogamy with ILD Insensitivity

Toyosuzu is sensitive to ILD and its flowering was retarded under ILD, whereas Karafuto-1 is insensitive to ILD and its flowering was not delayed. At 60 days after planting, Karafuto-1 reached R4, whereas Toyosuzu still remained vegetative. F_3 plants were classified into the following three types: Karafuto-1 type, Toyosuzu type, and intermediate type whose growth stage reached R2–R3. Segregation of intermediate type in F_3 families may indicate that dominance of genes for ILD sensitivity of Toyosuzu was not complete, unlike *E3* or *E4* (Buzzell 1971; Buzzell and Voldeng 1980). Of the 98 F_3 families tested, 88 families with a minimum of 15 plants each were classified into seven classes: (1) family fixed for Karafuto-1 type, (2) family segregating for Karafuto-1 and intermediate type, (3) family segregating for all of the three types, (4) family segregating for Karafuto-1 and Toyosuzu type, (5) family fixed for intermediate type, (6) family segregating for Toyosuzu and intermediate type, and (7) family fixed for Toyosuzu type (Table 3). When all segregating or intermediate phenotypes (2–6) are considered as one phenotypic class, the observed number of Karafuto-1 class, segregating or intermediate class, and Toyosuzu class was close to a two-gene (1:14:1) model ($\chi^2 = 5.45$, $.05 < P < .1$), suggesting the involvement of two recessive genes in ILD insensitivity of Karafuto-1. As shown in Table 3, all of the 8 F_3 families fixed for ILD insensitivity were derived from CL F_2 plants, while all of the 10 F_3 families fixed for ILD sensitivity were derived from CH F_2 plants. Segregation of cleistogamy and ILD insensitivity was thus significantly associated ($\chi^2 = 40.1$, $P < .005$). The results strongly suggested that one of the genes for ILD insensitivity was closely linked with a gene for cleistogamy. CL plants flowered 3 days earlier than CH plants in both F_2 and F_3 populations probably due to the close linkage (Figure 1).

Seven loci have so far been reported to control time to flowering and maturity in soybean: *E1* and *E2* (Bernard 1971), *E3* (Buzzell 1971), *E4* (Buzzell and Voldeng 1980), *E5* (McBlain and Bernard 1987), *J* (Ray et al. 1995), and an unnamed gene for ILD response (*e*(*t*)) (Abe et al. 1998). The *E3, E4,* and *e*(*t*) loci are known to be involved in the initiation of flowering under ILD. The recessive alleles *e3* and *e4* or *e*(*t*) jointly confer insensitivity to ILD (Abe et al. 1998; Buzzell 1971; Buzzell and Voldeng 1980). When combined with *e3* and *e4, E1* markedly retards flowering under ILD or natural daylength relative to *e1* (Cober et al. 1996). Of these genes, *E1* was linked to *T* with a recombination frequency of 3.9% (Weiss 1970). The association between the genotype at the *T* locus and ILD insensitivity in the F_2 population was not significant (data not shown), suggesting that *E1* may not be a gene responsible for the varietal

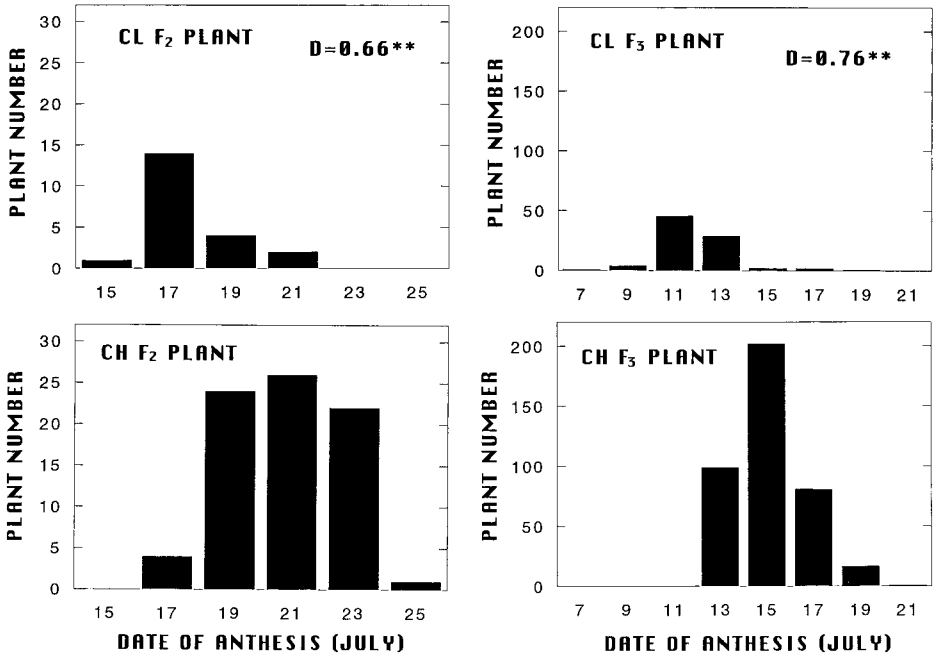

Figure 1. Date of anthesis in CL and CH plants of F_2 (left) and F_3 population (right). Differences in frequency distribution were significant at the 1% level by Kolmogorov–Smirnov test in the both populations.

differences in ILD insensitivity. Linkage analysis using DNA markers may be useful to identify the maturity gene associated with cleistogamy.

This study revealed that cleistogamy in an early maturing soybean cultivar was primarily genetic and a few major genes were involved. Near-isogenic lines regarding cleistogamy should be developed to help clarify the relationship between cleistogamy and chilling tolerance.

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