Heteroplasmy of Mitochondrial DNA in the Ophiuroid *Astrobrachion constrictum*

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We demonstrate the presence of mitochondrial heteroplasmy for the cytochrome oxidase I (COI) gene of the brittle star (*Astrobrachion constrictum*). One of the 117 individuals analyzed contained two distinct single-strand conformation polymorphism (SSCP) haplotypes differing by two substitutions; another showed sequence evidence for heteroplasmy. We used polymerase chain reaction (PCR) cloning, SSCP, and sequencing of a 480 bp region of the 5′ end of COI to isolate and characterize these haplotypes. This is the first properly substantiated case of heteroplasmy in an echinoderm species and may have arisen from paternal leakage.

Mitochondrial DNA (mtDNA) has become a powerful tool for assessing relationships among individuals, populations, and species of animals (Avise 1994). As the number of studies using this genome increases, knowledge of the genetics of the genome itself is also increasing. Two of the more surprising discoveries have been the extent of heteroplasmy in animal populations (Lunt et al. 1998) and cases of biparental inheritance of the genome (Gyllensten et al. 1991; Hoeh et al. 1991). Heteroplasmy, the occurrence of more than one type of mtDNA in the same organism, can arise either from mutation of the genome within the individual, heteroplasmy of the original oocyte, or from biparental inheritance. Most published examples of heteroplasmy involve a variation in the number of repeats within the control region of the genome (Lunt et al. 1998). Although the control region is noncoding, it probably contains sequences that initiate replication and transcription (Clayton 1982). In echinoids and vertebrates, the displacement loop (d-loop) structure evidences replication (Matsumoto et al. 1974). The length of repeats found in this region ranges from small microsatellite-like repeats (Wenink et al. 1994) to large repeats of 1100 bp (Wallis 1987). Length heteroplasmy is generally explained by slipped-strand mispairing during replication (Densmore et al. 1985), and high frequencies of length heteroplasmic individuals can occur in some species (Lunt et al. 1998). In a few cases observed heteroplasmy has been attributed to biparental inheritance (Kondo et al. 1990; Magoulas and Zouros 1993). Paternal leakage is still thought to be rare in animals, with mussels of the genus *Mytilus* being a prominent exception (Wenne and Skibinski 1995; Zouros et al. 1994).

De Giorgi (1988) reported heteroplasmy in the eggs of the sea urchin (*Arbacia lixula*) using restriction fragment length polymorphism (RFLP) analysis. However, in the absence of additional data, it is difficult to rule out partial digestion for the single restriction enzyme (*Bam*HI) site that was seen to vary both within and among females. To date, no cases of heteroplasmy have been reported in ophiuroids. While conducting a population genetic study of the brittle star (*Astrobrachion constrictum*) in Fiordland, New Zealand, we discovered that 2 of 117 sampled individuals appeared to be heteroplasmic. The first was identified from an SSCP gel where the banding pattern of the individual appeared to be a combination of two previously recognized cytochrome oxidase I (COI) haplotypes. The second was identified by sequencing and appeared to be a combination of two previously characterized COI haplotypes. Through a combination of SSCP, cloning, and sequencing, we confirm heteroplasmy in this echinoderm species.

Materials and Methods

Sample Collection

Specimens of *A. constrictum* were collected by divers from seven sites in Fiordland, southern New Zealand: four sites within Doubtful Sound (Espenosa Point, Tricky Cove, Crowded House, and Oz) and one site each from Nancy Sound, Preservation Inlet, and Chalky Inlet. All animals were kept alive in seawater for transport to the lab, where they were then stored at −80°C. Total genomic DNA was extracted from a small portion of gonad tissue by boiling for 10 min in 50 μl H_2O. Extracted DNA was precipitated in cold 100% ethanol, washed once with 70% ethanol, and resuspended in 30 μl of milliQ H_2O.

PCR

A 480 bp fragment of COI was amplified using C1-J-1718 and C1-N-2191 primers (Simon et al. 1994). The 25 μl PCR reactions contained 3.5 mM $MgCl_2$, 200 μM each dNTP, 0.25 μM each primer, 0.25 units of Qiagen© *Taq* polymerase, 10× Qiagen© buffer, and 2 μl of template DNA. Amplification was effected by initial denaturation at 94°C for 1 min, 40 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 3 min. PCR products and a 1 kb molecular weight marker (Gibco BRL) were electrophoresed in 2% agarose gel stained with ethidium bromide. Gel plugs containing DNA of expected length were excised and purified using Prep-A-Gene® (BioRad).

Cloning

Purified PCR product was cloned using the Blunt Ended PCR Cloning Kit-pMOS blue (Amersham Pharmacia Biotech). Transformed cells were plated on LB agar (Sambrook et al. 1989), treated with X-gal and

IPTG for blue/white screening, and grown overnight at 37°C. White colonies were picked and cultured overnight in LB broth (Sambrook et al. 1989). Plasmids were purified from these cultures as follows. Approximately 1.5 ml of culture were tipped into an Eppendorf tube and centrifuged at 13,000 rpm for 30 s. All but 50–100 μl of supernatant was removed and the pellet was resuspended in the remainder. TENS buffer (300 μl; 1× TE, 0.5% SDS, 0.1 M NaOH) was added and the mixture vortexed briefly. Quickly following this 150 μl of 3 M NaOAc (pH 4.8) were added, then vortexed and centrifuged for 2 min at 13,000 rpm. The supernatant was removed and placed in a new tube, ethanol precipitated, and the pellet resuspended in 30 μl of milliQ H_2O with 1 μl of RNase added (Werman et al. 1996). The purified plasmid DNA was screened using SSCP.

SSCP

Polymerase chain reactions (PCRs) (10 μl) were carried out under the same conditions as described above with the addition of 0.05 μl of ^{33}P-labeled dATP per reaction. Following PCR an equal volume of formamide buffer (95% formamide, 20 mM EDTA, pH 8, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each reaction and denatured at 94°C for 5 min. Reactions were placed on wet ice and loaded immediately. Aliquots (4 μl) of each sample were applied to a 6% bis-acrylamide (37.5:1 ratio, BioRad premix) gel containing 5% glycerol in 0.5 × TBE. Electrophoresis was carried out at 4°C for 200 W-h running at approximately 13 W. Completed gels were lifted from the plates with blotting paper and dried using a Bio-Rad 583 Gel Dryer. Kodak BioMax™ film was exposed to the dried gels for 2–3 days. Haplotypes were scored by comparison of renatured single-stranded DNA migration patterns and this information was used to select cloned DNA for sequencing.

Sequencing

Minipreped DNA was quantified by electrophoresis with 1 kb Plus DNA ladder (Gibco BRL) on a 2% agarose gel stained with ethidium bromide. Cycle sequencing was carried out with BigDye™ chemistry according to manufacturer's recommendations (PE Applied Biosystems). The product was precipitated with EtOH/NaAc (PE Applied Biosystems) and sequenced on an ABI377. Sequences were aligned by eye using SeqEd version 1.03.

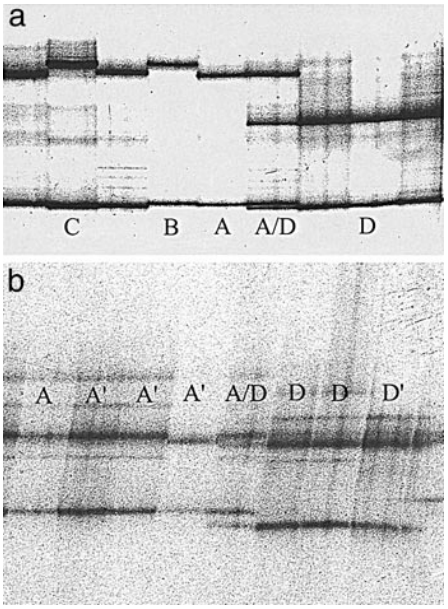

Figure 1. **(A)** SSCP gel of COI PCR products from heteroplasmic individual, ER11 (haplotypes A/D), with homoplasmic individuals either side (haplotypes A and D). Other haplotypes found in the Fiordland population (B and C) are also shown. **(B)** The same heteroplasmic ER11 sample (A/D) flanked by clones (A′ and D′) and homoplasmic individuals (A and D).

Results

A total of 117 individuals were screened for 473 bp at the 5′ end of COI using SSCP and sequencing. Of these, 113 showed clear, single SSCP products, and three failed to amplify. Eight haplotypes [A (84), B (1), C (2), D (14), E (3), F (2), G (4), H (3)] were recognized from SSCP and DNA sequencing. SSCP of one individual, ER11, showed a combination of two haplotypes, A and D. Repeated DNA isolations and SSCP analysis of ER11 showed the pattern to be real. Another individual, CR6, showed a single SSCP product, but sequence results indicated a combination of haplotypes A and G.

To determine whether these individuals were heteroplasmic, COI PCR products for both individuals and for a control individual of haplotype A (NR7) were cloned. Seventeen ER11 colonies, 20 CR6 colonies, and 5 control colonies were picked and cultured overnight. Plasmid preparations were made from all overnight cultures and subjected to SSCP analysis. On each gel, representatives of haplotypes A, D, and G were run as positive controls. Eleven clones from ER11 produced a banding pattern identical to haplotype A, while a single clone produced a banding pattern identical to haplotype D (Figure 1). All clones from CR6, and the NR7 control, produced haplotype A banding patterns. This suggests a higher proportion of haplotype A in both of the heteroplasmic individuals (see also Figure 1).

One clone representative of each haplotype observed in ER11 and two clones representative of the haplotype observed in CR6 were sequenced and compared to reference sequences. Sequences from ER11 clones were identical to haplotypes A and D, as expected. Of the two clones sequenced for CR6, one gave a sequence identical to haplotype A but the other gave a novel sequence haplotype (I). Of the 480 bp cloned and sequenced from the 5′ end of COI, haplotypes present in ER11 differed by two bases, while haplotypes present in CR6 differed by only one (Figure 2). All three base substitutions are synonymous, third-codon position transitions.

Discussion

The majority of reported heteroplasmy is due to differences in repeat number within the noncoding control region of mtDNA. This length variation is easily identified through RFLP analysis, or more recently through PCR. In this study we have demonstrated the presence of heteroplasmy for base substitution variation in COI of the mitochondrial genome in two individuals of the brittle star (*A. constrictum*). As we have examined only gonad material, our observations reflect germline heteroplasmy. Sampling of other tissues could reveal higher levels of organismal heteroplasmy. Sequence information for these haplotypes shows that they differ by 2 bp in one individual and by a single base in the other. Base differences on this scale can be detected conveniently with methods such as SSCP which can separate short segments of DNA as a result of conformational differences resulting from minimal sequence variation (Lessa and Applebaum 1993). Cloning provides a relatively quick and easy way of separating different mitochondrial PCR products for SSCP screening. Direct sequencing of PCR product alone would not usually be sufficient to demonstrate heteroplasmy of this type, as results may often be misinterpreted as an unreliable sequence.

The haplotypes present in ER11 are two of the more divergent pairs of haplotypes found within the Fiordland population of *A. constrictum.* It is not likely that one of these variants arose de novo within this animal, since this scenario would require two mutations very early in development. It is more likely that either heteroplasmy has persisted through several germline

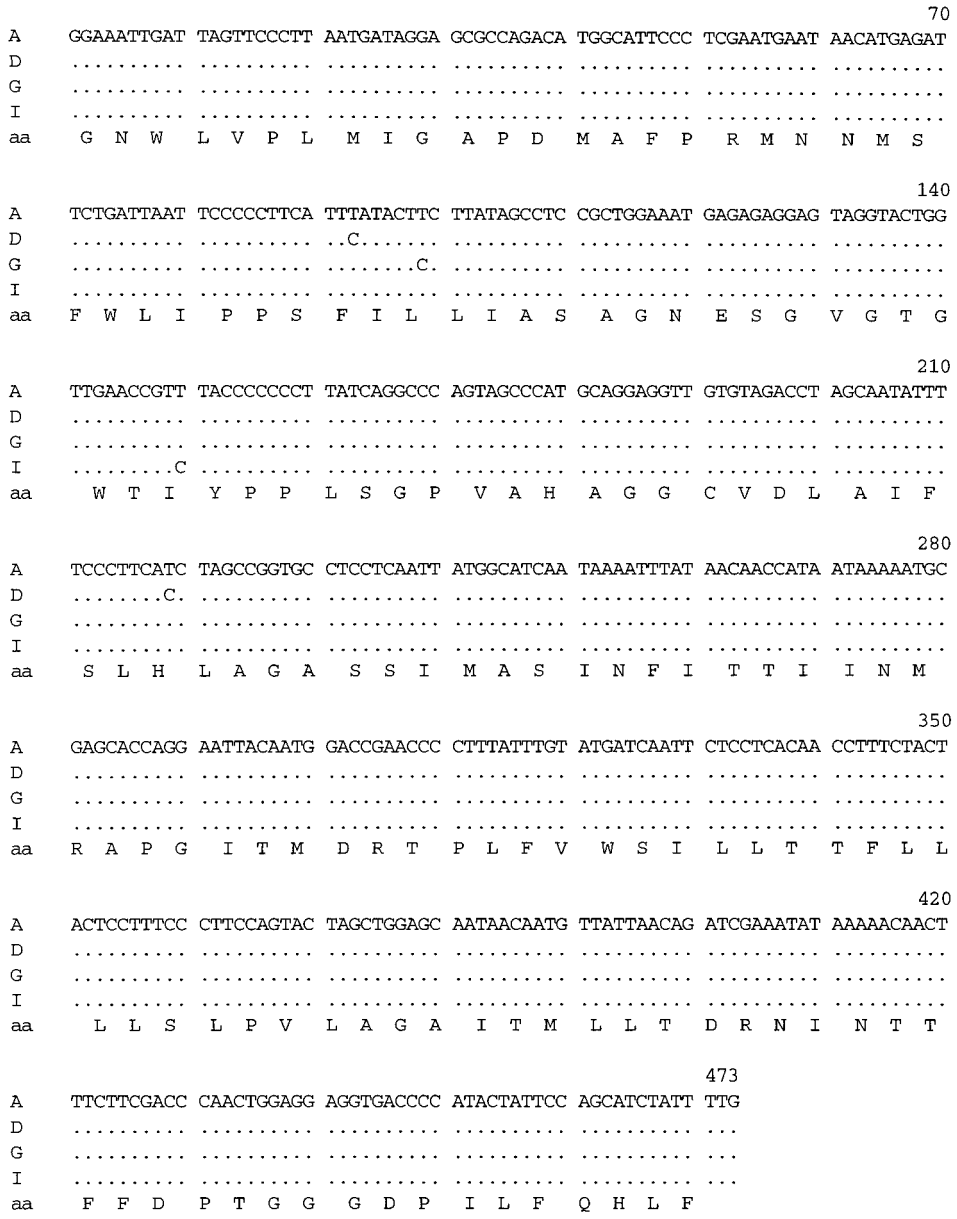

Figure 2. COI nucleotide and amino acid sequences for the haplotypes observed in the two heteroplasmic individuals.

replications, or there has been paternal leakage. For the first to occur, heteroplasmy would have to have persisted long enough in the same lineage to evolve two independent mutations.

From studies on *Drosophila* (Solignac et al. 1983) and crickets (Rand and Harrison 1986) it has been suggested that fixation is complete within a few hundred generations. This is a short time for two mutations to have evolved before sorting out into homoplasmic lineages, but without exact knowledge of the sorting out rates and mutation rates within COI of echinoderms we cannot discount this possibility. The second explanation, paternal leakage, seems more plausible. Experiments designed to detect low levels of paternal leakage through repeated backcrossing have shown partial paternal mitochondrial inheritance in *Drosophila* (Kondo et al. 1990) and mice (Gyllensten et al. 1991). These studies suggested that the observed heteroplasmy may be a result of reduced compatibility between egg and sperm due to the use of hybrid strains. However, heteroplasmy attributed to paternal input has been observed in natural populations of anchovy (*Engraulis encrasicolus*; Magoulas and Zouros 1993) and mussels of the genus *Mytilus* (Wenne and Skibinski 1995; Zouros et al. 1994). The extent and type of biparental mitochondrial inheritance in mussels appears to be unique to this genus. Magoulas and Zouros (1993) suggested that a lack of mitochondrial diversity within a population may give a false impression of the extent of paternal inheritance due to inheritance of the same molecule from both parents. This could be the case for *A. constrictum* as the recent study of the Fiordland population showed low levels of mitochondrial diversity (Steel 1999).

Low levels of heteroplasmy resulting from a small number of base substitutions are difficult to detect without methods designed to differentiate DNA molecules differing by only 1 bp. In this study we have used PCR and SSCP to identify heteroplasmy and cloning and SSCP to isolate and identify the haplotypes. We suggest that this approach, if employed widely, could reveal even higher levels of heteroplasmy in natural populations than are currently believed to exist.

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Received March 4, 1999
Accepted August 18, 1999

Corresponding Editor: Stephen J. O'Brien

X Trisomy in an Infertile Bitch: Cytogenetic, Anatomic, and Histologic Studies

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Three copies of the X chromosome were identified in a 5-year-old mixed breed infertile bitch. One year after the cytogenetic examination, the bitch died due to gastritis hemorrhagica, an inflammation of the mucus coat of the stomach. Dental studies showed congenital lack of some premolar and molar teeth. Ovaries were of normal shape and size. Also, histologic sections of the ovaries revealed their normal structure, with two corpora lutea and primary follicles. Phenotypic effects of X trisomy are discussed.

Precise identification of chromosome aberrations in the dog is rather a difficult task due to a large number of chromosomes ($2n = 78$), similar morphology of all autosomes which are acrocentric, and low resolution of banding patterns on small autosomes. On the other hand, the recognition of biarmed sex chromosomes is straightforward. The X chromosome is a large submetacentric and the Y chromosome is the smallest element in the karyotype. In addition, a large C-band block in the proximal half of the long arm is present on the X chromosome (Pathak and Wurster-Hill 1977).

Few cases of aneuploidy have been reported in dogs. Cytogenetic examinations of intersex dogs have revealed some cases of XXY trisomy and X monosomy (Mellink and Bosma 1989; Meyers-Wallen 1993), but until now only one case of X trisomy was diagnosed (Johnston et al. 1985).

The objective of this study was a cytogenetic, anatomic and histologic examination of a new case of a trisomy X in an infertile bitch.

Materials and Methods

A 5-year-old mixed breed bitch was presented for cytogenetic examination due to infertility. The bitch had a normal phenotype, including external reproductive organs. It was reported by the owners that she had never been pregnant, in spite of frequent matings with different males. The behavior of this bitch was rather unusual. She did not bark and was fearful. The bitch died suddenly 1 year after the cytogenetic evaluation due to gastritis hemorrhagica, an inflammation of the mucus coat of the stomach.

Cytogenetic investigation was carried out with the use of QFQ and CBG banding and Giemsa staining on chromosome slides derived from a blood lymphocyte culture. International canine chromosome nomenclature was applied (Świtoński et al. 1996).

The ovaries were frozen for histologic study, due to the sudden death of the bitch, and later the samples were fixed in 10% neutral formaldehyde, routinely dehydrated, and embedded in paraplast. The sections were stained according to the Masson-Goldner technique.

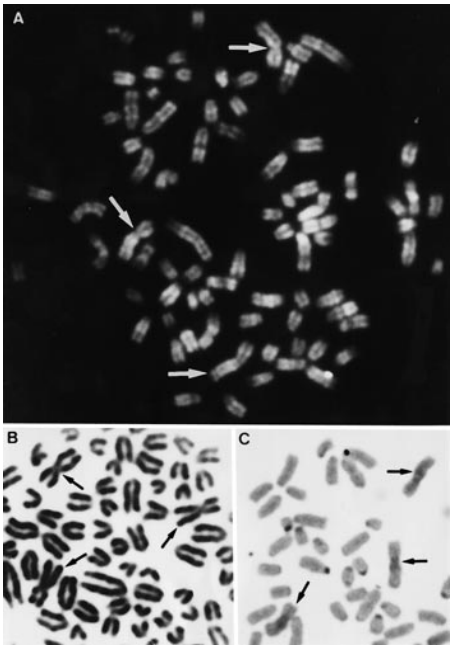

Figure 1. Triple X chromosome (arrows) in metaphase spreads from the infertile bitch: **(A)** QFQ-banded whole metaphase spread. **(B)** Giemsa-stained partial metaphase. **(C)** CBG-banded partial metaphase.

Results and Discussion

The analysis of Giemsa-stained chromosome slides revealed 79 chromosomes in all 40 analyzed spreads, including an extra biarmed chromosome indistinguishable from X chromosomes (Figure 1b). The application of QFQ- and CBG-banding techniques clearly showed that the extra chromosome was the X (Figure 1a,c). The CBG-banded chromosomes demonstrated dark staining in the pericentromeric region that extended to the proximal half of the long arm. The karyotype was designated 79,XXX.

Aneuploidy is rather rarely diagnosed in domestic animals. The exception concerns X monosomy, which is frequently found in infertile mares (Power 1991). Sex chromosome trisomies have been found in different species. In cattle, at least 13 cases of the XXY trisomy were described (Sysa and Słota 1984). This type of aneuploidy was only incidentally found in pigs (Makinen et al. 1998), but seven cases have been diagnosed in sheep (Long 1997). Trisomy X was found in 11 infertile mares (Power 1991), and 5 such cases were identified in cattle (Schmutz et al. 1994).

Anatomic studies revealed that the appearance, location, and size of the internal organs found in the thoracic cavity and in the abdominopelvic cavity were normal. It was found that the uterine horns were

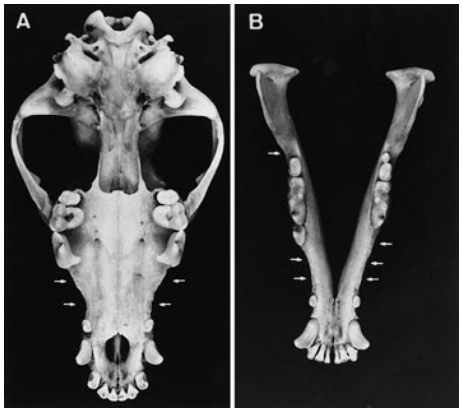

Figure 2. Dental arches of the X trisomy bitch: **(A)** upper dental arch, **(B)** lower dental arch. The missing premolar teeth are indicated by arrows.

curved on both sides instead of being straight. The uterine cavity was filled with a small amount of gray mucus. Ovaries were of normal shape and size.

In the bitch's mouth it was found that some premolar and molar teeth were missing in the upper and lower dental arches (Figure 2). The upper dental arch was symmetrical compared to the norm; on both sides two premolar teeth were missing—P_2 and P_3. The lower dental arch was not symmetrical. On the left side three premolar teeth were missing—P_2, P_3, and P_4—and on the right side two premolars—P_2 and P_3 were absent. Moreover, one molar tooth (M_3) was also missing. The formula for the dentition was as follows:

M2 P2(P_1, P_4) C1 I3	I3 C1 P2(P_1, P_4) M2
M3 P1(P_1) C1 I3	I3 C1 P2(P_1, P_4) M2(M_1, M_2)

All the teeth had normal structure and no dislocation in dental cavities was observed.

The incidence of trisomy X in newborn humans is estimated at approximately 0.1%, but among patients referred for karyotyping, underrepresentation of such cases is noticed. Thus it is assumed that the majority of trisomy X girls and women demonstrate normal phenotype. On the other hand, among identified trisomy X carriers reproductive failures, mental retardation, and facial dismorphism were noticed (Guichet et al. 1996). There are also reports pointing to dental anomalies in the carriers. In one of them, congenital absence of teeth in a girl with X trisomy was described (Miura et al. 1993). In the present case of an X trisomy bitch, abnormal behavior (lack of barking and fearfulness) and partial hypodontia were found. Partial hypodontia is quite often observed in dogs, especially in brachycephalic breeds (Evans and Christensen 1979). One can classify the present case of mixed breed dog, with a distinct share of shepherd dog breed, as a dolichocephalic dog. It suggests that the observed partial hypodontia was caused by trisomy X.

Ovaries were examined histologically and appeared to be normal. The cortex and medulla could be distinguished. The cortex contained multiple primordial and some more advanced multilaminar primary follicles. There were also some atretic follicles and two large corpora lutea, one in each ovary. In the hilum of one of the ovaries, a retential small cyst was observed. The cyst was covered by one layer of flat cells, and most probably it was of mesothelial origin. Domestic animals carrying monosomy X or trisomy XXY are usually infertile (Long 1997; Power 1991; Sysa and Słota 1984). The first trisomic X bitch ever described suffered from infertility and from a lack of both follicles and corpora lutea (Johnston et al. 1985). The present case of trisomy X was also attributed to infertility, but histologic findings revealed primary follicles and two corpora lutea in the ovaries. This finding is not surprising since it is well known that a high proportion of women who are trisomic for the X chromosome are fertile (Guichet et al. 1996). Also a case of a fertile X trisomy cow which gave birth to an XXY trisomic bull was reported (Schmutz et al. 1994).

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Received May 11, 1999
Accepted September 14, 1999

Corresponding Editor: William S. Modi

Trait Association of a Genetic Marker Near the IGF-I Gene in Egg-Laying Chickens

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The insulin-like growth factor I (IGF-I) gene was screened for genetic variants associated with trait means and trait correlations. Analysis of an unselected randomly mated White Leghorn population revealed a *Pst*I restriction fragment length polymorphism (RFLP) in the 5′ region of the gene which segregated at a frequency of 0.83 for the *Pst*I(+) allele (presence of a *Pst*I restriction site). A comparison of the three genotypic classes revealed that the *Pst*I(−/−) genotype was associated with a significantly lower egg weight measured in three different time periods, while the *Pst*I(+/−) genotype was significantly associated with a higher eggshell weight estimated from the egg weight and egg specific gravity. For eggshell weight, the effect was age dependent and significant only for the last two periods of egg laying. No genotype associations were found for body weight, feed consumption, and egg laying rates. Significant dominance effects of the IGF-I genotype were observed for two of the egg weight measurements and

three of the eggshell weight estimates. Partial correlation analyses in the two most frequent genotypic classes, *Pst*I(+/+) and *Pst*I(+/−), revealed the presence of a regulatory loop between feed consumption, body weight, egg weight, and the rate of egg laying. Several aspects of this regulatory loop were different between the two genotypic classes. In particular, for the *Pst*I(+/+) genotype, feed consumption was positively associated with egg weight, while there was no significant association for the *Pst*I(+/−) genotype. Further, the degree of association of body weight with egg weight decreased with age in the genotypic class *Pst*I(+/−), while it was constant for the *Pst*I(+/+) genotype. The results indicated that the marker in the IGF-I gene was not only associated with changes in some trait means, but also with changes in the stability of the coordination between feed intake, body weight, and egg production traits.

The components which constitute the growth hormone (GH) axis affect a wide range of biological processes, ranging from growth and differentiation to reproduction (Chase et al. 1998; Feng et al. 1997, 1998; Kocamis et al.1988), immune responsiveness (Aggrey et al.1996; Johnson et al. 1997), and aging (Copras et al.1993; Feng et al. 1997). GH released from the pituitary gland may act directly on target tissues or indirectly by releasing IGF-I from the liver (Isaksson et al.1985). In addition to this major endocrine pathway mediated by the hypothalamus, pituitary gland, and liver, other tissues that produce GH and IGF-I have been identified, indicating that these hormones, together with their receptors and binding proteins, provide a complex regulatory network that coordinates a multitude of traits (Harvey and Hull 1997). Since IGF-I exerts a negative feedback control over GH expression, it is difficult to assign biological effects to either one of the two hormones. Nevertheless, IGF-I is thought to have a direct effect on the interface between nutrient intake and growth (Monaco and Donovan 1997; Thissen et al.1991), bone metabolism (Coxam et al. 1995; Ohlsson et al. 1998; Schoenle et al.1982), and ovarian function (Armstrong and Benoit 1996; Davoren et al. 1985).

In chickens, the IGF-I gene has been mapped to the short arm of chromosome 1 near the centromere and has been shown to be part of a syntenic group which is conserved in several vertebrate species (Klein et al. 1996). The association of IGF-I levels with traits has been studied mainly in broilers (Goddard et al.1988; Scanes et al.1989). It has been shown that circulating levels of IGF-I increase with age to reach a maximum at 6 weeks and then gradually decline (McCann-Levorse et al. 1993). Levels of circulating IGF-I decrease in proportion to the magnitude of feed restriction and may balance growth and feed intake (Clemmons and Underwood 1991). No studies have so far been reported in adult egg-laying chickens, with the exception of measuring the effects of exogenous administration of biosynthetic chicken GH on egg production (Donoghue et al. 1990). The latter experiment showed no effect. Nevertheless, chickens lay close to one egg per day and turn over about 10% of their total body calcium per day (Soares 1984), a biological extreme among vertebrates with respect to ovulation rate as well as calcium metabolism. From the known biological function of the GH axis, it is therefore to be expected that these traits are responsive to variations in these genes.

Some understanding of the genetic architecture of quantitative traits may be gained by systematically analyzing genetic markers in major metabolic pathways. We have previously identified markers in the GH and GH-receptor genes, which are still segregating in many noninbred strains of White Leghorn chickens, and have shown that they are associated with changes in body weight (Feng et al. 1998) and egg production rates (Kuhnlein et al. 1997), respectively. In this article we report the identification of a genetic marker in the IGF-I gene in a strain of White Leghorn chickens and show that it is associated with differences in egg weight and eggshell thickness and that it alters the coordination between feed intake, body weight, laying rate, and egg weight.

Materials and Methods

Experimental Chickens and Traits

Chickens were from a White Leghorn strain (strain 7) established in 1958 from four commercial stocks and maintained at Agriculture Canada (Ottawa) as a pedigreed randombred control strain reproduced with 100 sires and 200 dams (Gowe and Fairfull 1990; Gowe et al. 1993). The population analyzed was from a single hatch. Chickens were vaccinated against Marek's disease, avian encephalomyelitis, Newcastle disease, and avian infectious bronchitis following standard procedures. Rearing and lighting regimes were as described by Gavora et al. (1991). Birds were fed ad libitum. A flow chart of the traits measured is given in Figure 1. Hens were weighed at 140 days of age (HBWT), 265 days of age (MBWT), and at 365 days of age (FBWT). Egg weight (EGWT) and specific gravity (SPG) were measured on eggs laid during 5 consecutive days over three time periods starting from 240 days, 350 days, and 450 days of age and averaged for each period. Assuming that the density of the egg yolk and albumin is one, the increment of the SPG over one is approximately equal to the ratio between eggshell weight (mostly calcium carbonate) and total egg weight and can be used to estimate the amount of calcium carbonate deposited per egg. Feed consumption (FC) was measured between 247 days and 268 days of age.

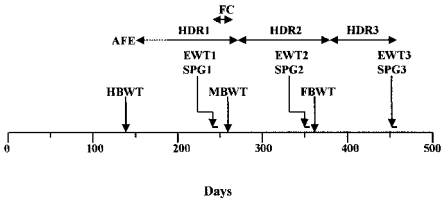

Figure 1. Flow chart of trait measurements. AFE, age at first egg; HDR, rate of egg laying; EWT, egg weight; SPG, specific gravity; HBWT, housing body weight; MBWT, mature body weight; FBWT, final body weight; FC, feed consumption.

Search for DNA Polymorphisms

The nucleotide sequence of the promoter region of the IGF-I gene (nucleotide position −650 to +312; Kajimoto and Rotwein 1991) was analyzed by SSCP analysis. Three sets of oligonucleotide primers were designed to amplify three adjacent fragments which encompassed the 940 bp promoter region. The primer sequence pairs were 5′-CAAGAGAAGCCCTTCGGCCTG-3′(forward) and 5′AGAGACAGGCAGGCAGTTTAC-3′(reverse), 5′GTAAACTGCCTGCCTGTCTCT-3′(forward) and 5′-AAGCTTACGCTGCCACGGAAA-3′(reverse), and 5′-TTTCCGTGGCAGCGTAAGCTT-3′(forward) and 5′-TCCATTGCGCAGGCTCTATCT3′(reverse) for the amplification of the 286 bp, 246 bp, and 325 bp fragments, respectively. For SSCP analysis, 1 μl of the PCR product was mixed with 15 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), denatured for 5 min at 100°C and subjected to electrophoresis on a 15% and 20% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide, 49:1) for 18 h or 12 h at 50 V. The gels were run in 1× Tris-borate (pH 8.3) EDTA buffer using a ver-

tical mini-gel apparatus (Bio-Rad, Hercules, CA) and silver stained. SSCP analysis of 15 chickens of strain 7 did not reveal any polymorphisms.

The search for DNA polymorphisms was subsequently extended to other parts of the IGF-I gene using RFLP analysis. The DNA of 20 individuals digested with either *Msp*I, *Sac*I, *Hin*dIII, *Pst*I, or *Taq*I was analyzed by Southern blotting according to the method of Westneat et al. (1988). The probes used for hybridization were prepared by subcloning *Eco*RI or *Hin*dIII digests of the lambda clones 15-4λ, 2-1λ, 5-2λ, and 12-3λ, of Kajimoto and Rotwein (1991) into PUC18. Hence they covered the first and last 30 kb of the 83 kb region harboring the IGF-I gene. The only polymorphism detected was a *Pst*I RFLP located about 7 kb upstream of the IGF-I promoter sequence in the 3′ region of clone 15–4λ.

Establishment of a RFLP-PCR Assay and Sequence Comparison Between Alleles

The 3′ end of a fragment of the 15-4λ clone which revealed the RFLP was sequenced, the polymorphic *Pst*I restriction site identified, and PCR primers spanning 621 nucleotides flanking this site were designed. The forward primer was 5′-GACTATACAGAAAGAACCCAC-3′ and the reverse primer was 5′-TATCACTCAAGTGGCTCAAGT-3′. PCR was performed in a reaction volume of 25 μl containing 100 ng of DNA, 0.5 μM of each primer, 1× PCR buffer (10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, and 50 mM KCl), 200 μM dNTP, and 0.625 units of *Thermus thermophilus* (*Tth*) DNA polymerase (Pharmacia). Amplification was carried out for 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 120 s, and extension at 72°C for 90 s using a DNA thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT). The PCR products were digested overnight at 37°C with *Pst*I and analyzed by electrophoresis on a 2% agarose gel. The *Pst*I(−) allele revealed a single band of 621 bp, whereas the *Pst*I(+) allele revealed two bands of 364 bp and 257 bp. Sequence comparison of two alleles revealed that the loss of the *Pst*I site was due to a C→T transition (Figure 2). No other base differences were observed within the 474 bp that were sequenced.

Statistical Analysis

Statistical analyses were carried out using the NCSS 97 program (Hintze JL, Kaysville, UT). Analysis for association of the IGF-I genotypes with traits was by single-factor analysis of variance (GLM procedure) and by the Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks. Egg weights and body weights were log transformed in order to obtain a normal trait distribution. The rate of laying (HDR) was transformed as $\arcsin\sqrt{x}$, but there was still deviation from normality. However, both the parametric and nonparametric tests gave the same result. Transformed values were also used in the factor analysis (Tabachnick and Fidell 1989), together with rowwise deletion of incomplete datasets. Multiple regressions, partial Pearson correlations, and partial Spearman rank correlations were carried out using untransformed trait values, except for HDR. Partial correlation coefficients were compared using Fisher's *Z* transformation (Zar 1984).

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AACAAAATTTACACAAATAAAAGCTACATTGATTGTTATTGATGTGTGCTTCACTGTAAT
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AAAAAGATAACCGATGTCATTTAAGGTCCAGCCTCCATCTATATATCCCTAGGATATAGC
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AACTATTTCTGCAACTGTAGTCCTAAGAAGTATTGGCTTGTCAGATGACCAAATCACATT
**********************************************************

                                    ↓
TTCTTTTCTTCTTTCCAAATAATTTCTAAAATAAATAGTCCCTTGGACTGCAGCAGAATT
**********************************************CTGTAG*******

AGGCCCTGCATTTAGAATACTAGGTGCAGATCTACCCTAACTCATCCATGGATTCTATAG
**********************************************************

TTCTGAACAATAACTGTTTATTAAGTGCAATGTATTTTTCACCTTTTATTTTCATCAGCTT
**********************************************************

TTTTATTTTCATAGCATATGTTTTTAAGTTTTCCAAACAGTGATGGTAGCTTAGGTCATTA
**********************************************************

AGACTGTTTTTACAGTTTCAGATTTTGATGCATCCTGTATTTGAATGCATTGAA
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Figure 2. Sequence comparison of the IGF-I alleles. The upper and lower lines represent the *Pst*I(+) and *Pst*I(−) alleles, respectively. The sequence in bold represents the polymorphic *Pst*I site and the arrow indicates the C to T transition that gives rise to the RFLP.

Results

Association of the IGF-I Marker Genotype with Trait Means

A total of 359 hens of strain 7 were genotyped. The frequency of the *Pst*I(+) allele was 0.83. The distribution of individuals among the genotypic classes did not deviate significantly from a Hardy–Weinberg equilibrium ($\chi^2 = 1.29$, $P > .2$).The association of the *Pst*I genotypes with the means of phenotypic measurements in the hen population was examined by the Kruskal–Wallis one-way ANOVA by rank and by single-factor ANOVA (Table 1). Both the nonparametric and parametric analyses gave similar results. There was no significant association with the age at first egg (AFE), rate of egg production measured during three consecutive time intervals, as well as with the average feed consumption. There was a trend for association with body weight before the beginning of egg laying, but not with mature body weight. Egg weight and specific gravity (a measure of the eggshell weight) were determined in three time periods starting from days 240, 350, and 450. For egg weight, the association with the IGF-I genotype was significant for all three periods, and the average weight was higher for the *Pst*I(+/+) and *Pst*I(+/−) genotypes than for the *Pst*I(−/−) genotype. For specific gravity and eggshell weight (mostly calcium carbonate) the association was significant for periods 2 and 3, and the average was higher for the *Pst*I(+/−) genotype than the other two genotypes.

Analysis of Marker-Trait Associations by Multivariate ANOVA

A factor analysis followed by varimax rotation was carried out with 14 variables consisting of age at first egg, feed consumption and the three measurements of body weight, the rate of egg laying, egg weight, and specific gravity. The first five factors, which accounted for 100% of the variance of the dataset were maintained for further analysis. The factor loadings indicated that F1 represented the three body weight measurements and feed consumption, F2 the rates of egg laying, F3 the egg specific gravity measurements, F4 the egg weights, and F5 a combination of age at first egg and body weight prior to the

Table 1. Association between IGF-I genotypes and traits means

Traits[a]	Significance		IGF-I genotype			Orthogonal contrasts[b] (*P* values)	
	Kruskal–Wallis	ANOVA	*Pst*I (−/−) (*N* = 12–14)[c]	*Pst*I (+/−) (*N* = 83–97)	*Pst*I (+/+) (*N* = 208–248)	Linear (additive)	Quadratic (dominant)
AFE (d)	0.17	0.43	167	167	166	0.808	0.351
HBWT (g)	0.145	0.083	1242	1267	1297	0.160	0.884
MBWT	0.192	0.192	1715	1729	1776	0.320	0.656
FBWT	0.247	0.216	1689	1706	1745	0.327	0.711
HDR1 (%)	0.269	0.140	85.8	85.7	83.7	0.415	0.624
HDR2	0.256	0.365	74.3	70.5	69.2	0.252	0.750
HDR3	0.439	0.576	55.1	56.7	54.3	0.926	0.460
EWT1 (g)	0.052	0.045	50.3[d]	52.8[e]	52.5[e]	0.026	0.023
EWT2	0.048	0.065	55.5[d]	58.1[e]	58.1[e]	0.020	0.077
EWT3	0.008	0.017	56.9[d]	60.7[e]	60.7[e]	0.004	0.030
SPG1	0.420	0.306	86.1	86.0	85.2	0.464	0.670
SPG2	0.082	0.045	82.2	82.9[d]	81.5[e]	0.544	0.177
SPG3	0.037	0.013	78.9	80.6[d]	78.5[e]	0.817	0.058
ESWT1 (g)	0.098	0.106	4.34	4.54	4.48	0.180	0.035
ESWT2	0.043	0.050	4.57[d]	4.83[e]	4.74	0.113	0.015
ESWT3	0.004	0.010	4.49[d]	4.90[e]	4.78[f]	0.037	0.002
FC (g/hen/day)	0.247	0.218	109.6	114	115.2	0.101	0.448

[a] Unless evident, the units were as follows (see Materials and Methods): SPG1, SPG2, and SPG3 are the actual specific gravities (g/cm^3) minus 1 and multiplied by 10^3. It approximates the ratio between eggshell weight and egg weight (in mg/g). ESWT1, ESWT2, and ESWT3 (eggshell weights) were computed from the egg weight and the specific gravity as indicated in Materials and Methods. FC is the average amount of feed consumed per hen per day measured between 247 and 268 days of age.

[b] The data were regressed on orthogonal polynomials and the significance of the linear and quadratic term (i.e., additive and dominance effects) are indicated.

[c] Number of hens. Variations in number are due to missing data.

[d,e,f] Different superscripts within a row indicate that the means are significantly different from each other ($P < .05$).

onset of egg laying (Table 2). Multivariate ANOVA indicated that the three genotypic classes differed significantly. ANOVA of the individual factors indicated significant differences for F3 (specific gravities) and F4 (egg weights), in agreement with the univariate analysis of the original variables (Table 3).

Association of the Marker Genotype with Differences in Trait Interactions

A partial correlation analysis (i.e., correlation between pairs of traits corrected for the remaining traits) between feed consumption, body weight, egg weight, and the rate of egg laying was carried out for the two most frequent genotypic classes, *Pst*I(+/+) and *Pst*I(+/−). It indicated that the IGF-I genotype affected trait interactions (Figure 3). For the *Pst*I(+/+) genotype, which was the most frequent, there were significant correlations among all traits, with the exception of the correlation between body weight and the rate of egg laying. A comparison between different time periods revealed no significant changes with age. The *Pst*I(+/−) genotype differed in several aspects from the *Pst*I(+/+) genotype. In all time periods the correlation between feed consumption and body weight was higher in the *Pst*I(+/−) class than in the *Pst*I(+/+) class, while the correlation between feed consumption and egg weight was reduced to nonsignificant levels. Further, in contrast to the *Pst*I(+/+) genotype, there were time-dependent changes for two of the partial regression coefficients. The correlation between body weight and egg weight decreased from 0.336 in period 1 to 0.091 in period 3, whereas the correlation between the egg weight and the rate of egg laying decreased from −0.165 to −0.389. The decrease in the correlation between the former two traits was significant ($P = .038$), while for the latter two it was not ($P = .30$). Near identical results were obtained by partial Spearman correlation analysis by rank or by successively regressing each variable on the remaining variables. The latter analysis showed that despite similar variances, there were systematic differences in the variance of a trait "explained" by the remaining traits (Table 4). In particular, the range of R^2 values with the egg weights as dependent variables were between 31% and 33% for the (+/+) genotype, but only between 12% and 18% for the (+/−) genotype. Similar systematic differences were observed with feed consumption or body weight as dependent variables. However, in these cases the direction of change was reversed; R^2 values were higher for the (+/−) genotype (>57%) than for the (+/+) genotype (<44%).

Trait correlations of specific gravity, an indicator of the ratio between eggshell weight and egg mass, were of a lesser magnitude and not significant in most cases. They were not further analyzed. The frequency of the (−/−) genotypes ($N = 10$) was too small for meaningful correlation analyses.

Discussion

In this study we investigated sequence variations in the chicken IGF-I gene and their association with traits of economic importance. SSCP analysis of the promoter region of the gene revealed no sequence variation under the conditions used, and RFLP analysis revealed a single *Pst*I poly-

Table 2. Factor analysis: factor loadings after varimax rotation

Traits	Factor loadings after varimax rotation					Commonality[b] (%)
	F1 (28%)[a]	F2 (20%)	F3 (21%)	F4 (24%)	F5 (7%)	
AFE	−0.012	−0.047	0.000	0.053	**0.578**	34
HBWT	**0.617**[c]	−0.001	0.021	0.183	**−0.455**	62
MBWT	**0.863**	0.051	−0.088	0.241	0.057	82
FBWT	**0.964**	0.015	−0.096	0.173	−0.016	97
EWT1	0.288	−0.112	0.003	**0.743**	0.018	65
EWT2	0.204	−0.255	−0.054	**0.882**	−0.003	87
EWT3	0.240	−0.193	−0.009	**0.821**	0.048	77
SPG1	−0.091	−0.098	**0.739**	0.001	0.122	58
SPG2	−0.028	0.103	**0.899**	−0.028	0.083	83
SPG3	−0.047	0.041	**0.717**	−0.027	−0.266	59
HDR1	0.074	**0.495**	−0.007	−0.122	−0.017	27
HDR2	0.103	**0.893**	0.002	−0.103	−0.045	82
HDR3	−0.014	**0.786**	0.122	−0.165	−0.028	66
FC	**0.557**	0.221	−0.054	0.281	0.009	44

[a] Percent of total variation explained by the particular factor. The total variation explained by the five factors was 100%.

[b] Percent of the variance of a particular trait explained by the factors.

[c] Values above 0.4 are marked in bold.

Table 3. Analysis of variance of factor means[a]

Genotype	*N*	Factor means[b] F1 (BWT, FC)	F2 (HDR)	F3 (SPG)	F4 (EWT)	F5[c] (HBWT, AFE)
*Pst*I (−/−)	10	−0.296	−0.113	−0.051	−0.786[a]	0.357
*Pst*I (+/−)	79	−0.138	0.217	0.307[a]	0.102[b]	0.076
*Pst*I (+/+)	201	0.069	−0.080	−0.118[b]	−0.001[b]	−0.048
ANOVA (*P* value)		0.222	0.121	0.012*	0.047*	0.557

[a] Multivariate analysis of variance indicated that the differences between genotypes was significant at $P = .0084$ (Wilk's lambda, Hotelling–Lawley trace, Pillai's trace) and $P = .006$ (Roy's largest root).

[b] Within each column means which differ significantly are marked with different superscripts.

[c] A high score of F5 is diagnostic for low HBWT and/or late AFE.

Table 4. Variation explained by regression model[a] and coefficients of variation

Dependent variable	IGF-I genotype	Period[b]	Coefficient of variation	R^2 value of regression (%)
FC	+/+	1	0.088	43.8
		2		38.1
		3		34.9
	+/−	1	0.065	57.1
		2		58.4
		3		58.8
BWT	+/+	1	0.097	40.8
		2		41.1
		3		36.7
	+/−	1	0.079	59.4
		2		55.7
		3		57.9
HDR	+/+	1	0.097	18.0
		2	0.150	17.2
		3	0.217	11.4
	+/−	1	0.103	10.5
		2	0.147	21.0
		3	0.205	18.9
EWT	+/+	1	0.055	32.1
		2	0.067	31.0
		3	0.069	32.8
	+/−	1	0.064	15.8
		2	0.065	11.7
		3	0.070	17.7

[a] Each of the four traits (dependent variable) was successively regressed on the remaining three traits (independent variable). Body weight was the average of the weights measured at 275 days and 365 days. Measurements were transformed as indicated in Materials and Methods.

[b] See Figure 1.

morphism in the 5′ region of the gene. This is in contrast with other genes (ornithine decarboxylase, growth hormone, mitochondrial phosphoenolpyruvate carboxykinase, endogenous viral genes) which had been analyzed in the same strain (Li et al. 1998). In particular, an analysis of growth hormone with only two restriction enzymes revealed three RFLPs, and additional polymorphisms were present in regions flanking the gene (unpublished results). The apparent paucity of nucleotide polymorphism may reflect a relatively recent coalescence of the IGF-I alleles present in the White Leghorns (Chakravarti 1999).

GLM ANOVA revealed a significant influence of the IGF-I genotype on egg weight and specific gravity (Table 1). In the case of egg weight, the *Pst*I(+) allele was dominant and eggs of chickens carrying this allele were 4–6% heavier than eggs of chickens homozygous for the *Pst*I(−) genotype. The effect on specific gravity was age dependent and restricted to differences between the *Pst*I(+/−) heterozygote and the *Pst*I(+/+) homozygote. A comparison of the mean eggshell weights estimated from the specific gravity and the egg weight indicates overdominance (ratio d/a = 2) in all three periods of measurement. In the last period of measurement, where all means differed significantly from each other, the largest difference observed was between the *Pst*I(−/−) and *Pst*I(+/−) genotypes and amounted to 10% of the daily eggshell weight.

In order to account for the correlation and multiplicity of the traits analyzed, a factor analysis was conducted which partitioned the traits into five factors, representing feed consumption and body weights, the rates of laying, the egg weights, specific gravities, as well as the age at first egg and juvenile body weight (Table 2). Hence the grouping of the original variables into five factors was biologically meaningful. The significance of a multivariate test using these five factors exceeds the significance of any of the univariate tests which had not been corrected for multiplicity. Thus, as would be expected from the pleiotropic nature of IGF-I, other traits besides egg weight and specific gravity contribute to the phenotypic differences associated with the three IGF-I

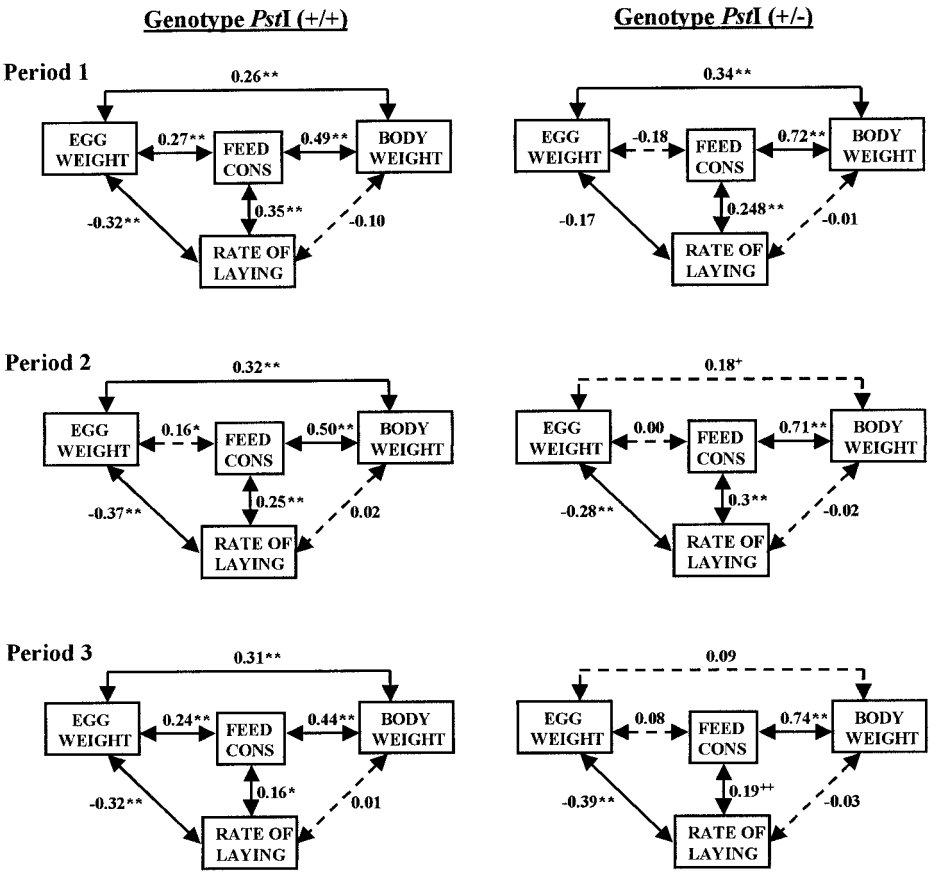

Figure 3. Regulatory loops for periods 1, 2, and 3 between feed consumption, adult body weight (average of MBWT and FBWT), egg weight, and the rate of egg laying. Dotted lines indicate partial correlations with $r < 0.2$ ($P > .05$ for $N = 90$). The significance of the partial correlation coefficients taking the actual sample size into account is indicated by † ($P < .1$), * ($P < .05$), and ** ($P < .01$). Abbreviations are as in Figure 1.

genotypes. Additional contributions in decreasing order are the factor which represented the rates of egg laying, and the factor which represented body weight plus feed consumption (Table 3).

Partial correlation analyses (i.e., correlation between two traits corrected for the influence of the remaining traits) indicated the presence of a regulatory loop which coordinates body weight, feed consumption, egg weight, and the rate of laying (Figure 3). Thus in the *Pst*I(+/+) genotypic class, heavy chickens tended to lay heavier eggs, presumably reflecting the larger size of their reproductive organs. As expected, heavier chickens will also consume more feed, required to maintain a larger body size. In addition, the increased feed consumption had a direct positive effect on egg weight as well as the rate of laying. The latter two traits were negatively correlated, providing a feedback control between egg weight and the rate of laying. Based on the regression of egg weight and laying rate on body weight, the net effect of an increased body weight (effect without corrections for the other traits) is an increased egg weight, but no changes in the rate of egg laying. This relationship is observed for all three time periods with standardized regression coefficients ranging from 0.443 and 0.470 ($P < 10^{-6}$) for egg weight and from −0.08 to −0.01 ($P > .80$) for the laying rate, respectively.

In the *Pst*I(+/−) genotypic class the correlations between several components of the regulatory pathway were altered and, in addition, were age dependent. In period 1, heavy chickens also tended to lay heavier eggs and consumed more feed. However, in contrast to the *Pst*I(+/+) genotype, the increased feed consumption did not have a direct effect on egg weight and the negative correlation between egg weight and the rate of egg laying was reduced. In periods 2 and 3, the dependency of the egg weight on body weight progressively decreased, while the magnitude of the negative correlation between egg weight and rate of laying increased. The positive correlation between feed consumption and the rate of egg laying was similar as for the *Pst*I(+/+) genotype and did not change with time. The net result (not correcting for other traits) was a decreased dependency of the egg weight on body weight as reflected by decreasing standardized regression coefficients (0.335, $P < .001$ in period 1; 0.190, $P = .069$ in period 2; 0.175, $P = .114$ in period 3). Hence, in contrast to the (+/+) genotype, the dependency of egg weight on body weight decreased with time. The standardized regression coefficients of the rate of laying on body weight showed a more complex age dependency. They were 0.175 ($P = .087$), 0.264 ($P = .009$), and 0.053 ($P = .63$) for the three time periods, while in the *Pst*I(+/+) genotype they were nonsignificant for all time periods.

An influence of the IGF-I genotype on the dependence of the "output" traits (egg weight and rate of egg laying) on the "input" traits (feed consumption and body weight) was also apparent from the R^2 values obtained when each one of the four traits were regressed on the remaining three traits (Table 4). Among the *Pst*I(+/−) genotypes, close to 60% of the variations in feed consumption and body weight was "explained" by variations in other traits. This reflects primarily the high coordination between body weight and feed consumption observed for this genotype. In the *Pst*I(+/+) genotypic class, the R^2 values were 10–20% lower. It was concomitant with an increase in the coefficient of variation in body weight and feed consumption, indicating a less stringent physiological control of these parameters. For egg weight the direction of change was reversed. In the *Pst*I(+/+) genotypic class, about 30% of the variation was explained by the other traits, but only half of that was explained in the *Pst*I(+/−) genotype. Despite these differences, the coefficients of variance were similar, indicating the presence of additional regulatory loops that stabilize the egg weight.

In summary, the results indicated that the marker in the IGF-I gene was associated with changes in the average egg weight and eggshell weight, but not the rate of egg laying, the body weight, or feed consumption. Such differences may reflect variations in IGF-I expression, since IGF-I is known to be involved in nutrient partitioning (Monaco and Donovan 1997; Thissen et al. 1991), ovulation (Armstrong and Benoit 1996; Davoren et al. 1985), and calcium metabolism (Coxam et al. 1995). However, linkage disequilibrium of the IGF-I marker with variants in neighboring genes cannot be excluded, despite the large effective size of the population analyzed. Based on the metabolic role of the IGF-I gene, one may speculate that the *Pst*I(+) allele is associated with a higher IGF-I expression. In addition, the age-dependent effect on eggshell weight may be consistent with the observation of declining serum levels of IGF-I with age (Burnside and Cogburn 1992; McMurty et al. 1994; Radecki and Scanes 1997). However, little is known about the age dependency of IGF-I expression in peripheral tissues where IGF-I may act in a paracrine and/or autocrine fashion.

Complex regulatory systems such as the GH axis have presumably evolved to coordinate traits in order to optimize fitness. It is therefore to be expected that most genetic variations affect trait coordinations rather than trait means. This is observed in the case of the *Pst*I(+/−) and *Pst*I(+/+) genotypes, which differ marginally in trait means, but have a large effect on trait correlations. Similar observations have been made for genetic markers in the growth hormone gene and the growth hormone receptor gene (Feng et al. 1997) as well as in the mitochondria and the mitochondrial phosphoenol pyruvate carboxykinase gene, a major regulatory gene of gluconeogenesis (unpublished results). Analyses of such trait dependencies may provide more insight into the genetic architecture of quantitative traits than the comparison of trait means.

From the Department of Animal Science, McGill University, Macdonald Campus, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9. This research was supported by grants from the Natural Sciences and Research Council of Canada and Shaver Poultry Breeding Farms Ltd. We are thankful to L. Volkov and the staff of the Center for Food and Animal Research of Agriculture Canada for technical support, and to Dr. Peter Rotwein (St. Louis, MO) for generously providing us with the chicken IGF-Iλ clones. Address correspondence to Urs Kuhnlein at the address above or e-mail: kuhnleinu@macdonald.mcgill.ca.

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Received March 10, 1999
Accepted September 14, 1999

Corresponding Editor: Lyman Crittenden

Neutrality Tests on mtDNA: Unusual Results from Nematodes

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McDonald–Kreitman tests of neutrality on mitochondrial DNA (mtDNA) of butterflies, *Drosophila,* and a variety of vertebrates usually show excess (over the neutral expectation) intraspecific polymorphism at nonsilent sites. These results are of great interest because they are the opposite of what is usually found for nuclear genes, in which the neutral pattern or evidence of adaptive divergence between species is usually observed. However, only vertebrates and insects have been tested so far, so it is not clear whether this intriguing pattern is typical for mtDNA in all taxa. Here I tested three pairs of nematode species and found that they all show a deficit of replacement polymorphism. Taken at face value, this result suggests that adaptive evolution proceeds more efficiently in nematode mtDNA than in mtDNA of vertebrates or insects. An alternate explanation is that the nematode pattern is an artifact of silent-site saturation that results from the rapid and composition-biased way in which nematode mtDNA evolves. Further studies are needed to distinguish between these two hypotheses.

McDonald and Kreitman (1991) showed that for closely related species evolving under neutrality, the ratio of the number of synonymous (silent) sites polymorphic within species to the number fixed between species should equal the ratio of nonsynonymous (replacement) sites polymorphic within species to the number fixed between species. One can symbolize the first ratio as $r_{\text{pd-silent}}$ (for ratio of polymorphism to divergence at silent sites), and the second ratio as $r_{\text{pd-replacement}}$ (for ratio of polymorphism to divergence at replacement sites). The neutrality index, NI ($= r_{\text{pd-replacement}}/r_{\text{pd-silent}}$), indicates the degree to which the ratios for a species pair deviate from the value of 1.0 expected under neutrality (Rand and Kann 1996). Here values greater than 1 represent an "excess" of replacement polymorphism within species and values between 0 and 1 represent "excess" replacement substitutions fixed between species. Usually a 2×2 contingency table is used to test the hypothesis of neutrality, in which one dimension is silent versus replacement sites, and the other dimension is polymorphism within species versus fixation between species.

Most applications of the McDonald–Kreitman (MK) test to nuclear gene sequences found an excess of replacement substitutions fixed between species (NI $<$ 1), a result usually interpreted as evidence of adaptive evolution (Brookfield and Sharp 1994; Eanes et al. 1993; McDonald and Kreitman 1991). In contrast, MK tests on mtDNA sequence data from many vertebrate and a few insect species pairs (*Heliconius* butterflies and *Drosophila*), found that almost all of the datasets show an excess of replacement polymorphisms (NI $>$ 1) and that the excess is statistically significant in about half of them [summarized in Nachman (1998) and in Rand and Kann (1998); also Wise et al. (1998)].

The most common explanation for the persistent pattern of excess replacement polymorphism is that the mtDNA as a whole evolves under a slightly deleterious model of evolution (Nachman 1998; Nachman et al. 1996; Ohta 1992; Rand and Kann 1996, 1998). Under this model the slightly deleterious variants are under just enough selection to prevent them from drifting to fixation, but under weak enough selection

Table 1. Species and DNA sequence used

Species pair	bp	p_b	Number unique	p_w
Genus *Haemonchus*	459	0.152		
H. contortus			37 (50)	0.025
H. placei			31 (40)	0.019
Genus *Teladorsagia*	390	0.134		
T. circumcincta			39 (40)	0.023
T. boreoarcticus			8 (11)	0.005
Genus *Heterorhabditis*	474	0.134		
H. marelatus			4 (58)	0.018
H. bacteriophera			1 (5)	0

bp = length of coding sequence used in that comparison (3′ end of the *ND4* gene); p_b = average sequence difference between the two species; number unique = the number of unique sequences used (out of the total number of individuals sequenced, in parentheses). For example, 37 *H. contortus* and 31 *H. placei* sequences were used in the MK tests. p_w = average sequence difference between individuals within each species.

Table 2. Tables for McDonald-Kreitman tests

	Haemonchus			*Teladorsagia*			*Heterorhabditis*		
	Fixed	Polymorphism	r_{pd}	Fixed	Polymorphism	r_{pd}	Fixed	Polymorphism	r_{pd}
R	16	27	1.7	11	14	1.3	13	1	0.08
S	15	94	6.3	18	65	3.6	45	14	0.31
S/R	1.0	3.6		1.6	4.8		3.5	14	
	NI = 0.30			NI = 0.36			NI = 0.26		
	P = .003			P = .038			P = .27		

R = replacement, S = silent, S/R = ratio of silent to replacement substitutions, NI = neutrality index. *P* values are for two-tailed Fisher's exact test.

that we observe multiple transient polymorphisms at any point in time. Nevertheless, the details of how mutation and selection might be interacting to generate this excess of replacement polymorphism are still not entirely clear. MK tests on a wider variety of taxa having different demographic and genetic properties (variation in effective sizes, in tempo and mode of mtDNA evolution, and so on) would be very useful in testing predictions of different models (Rand and Kann 1996, 1998). Therefore I performed MK tests on mitochondrial ND4 sequences in three pairs of nematode species.

Materials and Methods

Partial *ND4* gene sequences were obtained for three species pairs representing three genera and two families: *Heterorhabditis marelatus* versus *H. bacteriophera* (Blouin et al. 1999; Liu et al. 1999), *Haemonchus contortus* versus *H. placei* (Blouin et al. 1997), and *Teladorsagia circumcincta* versus *T. boreoarcticus* (Blouin et al. 1995; Hoberg et al. 1999). Table 1 lists the number of sequences analyzed, length of *ND4* region sequenced, and measures of intra- and interspecific diversity for each species pair. Note that the trichostrongylid species are much more variable than the *Heterorhabditis* species. Base compositions at the three codon sites are very similar in each species pair.

Silent and replacement polymorphisms and fixations in each dataset were scored by hand and by using DnaSP (Rozas and Rozas 1997). When more than one site was variable within a codon, a parsimony approach was used to decide whether each site in that codon was scored as silent (S) or replacement (R). All possible paths among the codons were evaluated and the shortest path was considered to be the correct one. For example, say three codons segregating at a site in a species were ATA (met), GTA (val), and ATG (met). The path GTA (val) ↔ ATA (met) ↔ ATG (met) would be considered the best one, and so one would score a replacement polymorphism at the first position and a silent polymorphism at the third position. GTA ↔ ATG is less parsimonious because it requires two steps, passing through the unobserved codon GTG. When two equally parsimonious paths disagreed over whether a nucleotide site is silent or replacement, that site was excluded from the analysis. Preferentially using paths that maximize the number of S's or R's has little effect on the final tables (not shown), so the above approach could not have caused any spurious results.

Results and Discussion

Table 2 shows the MK test tables for each pair of species. All three tables show NI ≈ 0.3, and the tests on the *Haemonchus* and *Teladorsagia* tables are significant. Taken at face value, these results suggest that nematode mtDNA (or at least the *ND4* gene) is unique in that it consistently gives a pattern opposite to that typically observed in vertebrates and insects. In other words, this may be evidence for adaptive mitochondrial differentiation between species, or if one prefers, stronger purifying selection within species. For the mildly deleterious model to work, the product of effective size (N_e) and selection (s) must be in a narrow range around 1. If N_es is very large, then deleterious mutations will contribute little to either divergence or polymorphism, and we will see only variation owing to neutral alleles and to advantageous alleles fixed between species. If mutation rates and N_es were both unusually large in nematodes, then the pattern observed here might result. Circumstantial evidence suggests that mutation rates are high in nematode mtDNA (Anderson et al. 1998; Blouin et al. 1995). The trichostrongylid species also appear to have unusually large effective sizes, although the same is not obvious for *Heterorhabditis* (Anderson et al. 1998; Blouin et al. 1995, 1999).

An alternate explanation for the nematode result is that it is simply an artifact of silent-site saturation. An assumption of the MK test is that there have been few multiple substitutions per site in the between-species comparison. Because silent sites saturate faster than replacement sites, substantial saturation can cause the silent fixations cell to be underestimated, which results in downward biased NI values (e.g., Maynard Smith 1994; Nachman 1998). The downward bias may be particularly high in nematode species for two reasons. First, nematodes appear to have a high mtDNA mutation rate (Anderson et al. 1998; Blouin et al. 1995), yet are very conservative in gross morphology. Thus by the time two populations are recognized as distinct species their mtDNAs are highly divergent. For example, for 12 pairs of congeneric nematode species (from five genera) that were originally defined on the basis of morphology, and for which mtDNA sequence data are also available, interspecific differences ranged from 8% to 20%, with a mean of 14% (Blouin et al. 1998). By the time this level of difference is observed, silent sites are probably substantially saturated. Indeed, the average transition:transversion ratio in the third position site is about eight times higher in pairwise intraspecific comparisons than in comparisons between sequences from congeneric species (Blouin et al., 1998). Second, nematode mtDNA is also highly A + T rich (Hugall et al. 1997; Hyman and Azevedo 1996; Okimoto et al. 1992; Thomas and Wilson 1991), apparently as a result of a strong mutational bias from C and G to A and T (Blouin et al. 1998). The multiple hits problem will be exacerbated in

DNA such as this because there are effectively fewer possible character states, so sites saturate quickly (e.g., Brower and DeSalle 1998; Wolfe and Sharp 1993). Unfortunately, how one would accurately correct these tables for multiple hits is not obvious. Any post hoc correction to the tables will need to incorporate a realistic model of the mode of substitution, which in nematode DNA is extremely biased and is not fit by any of the standard models (Blouin et al. 1998).

Further research is needed to determine if selection really acts differently in nematode mtDNA, or if the MK test results are purely an artifact of silent-site saturation. The simplest test would be to find pairs of nematode species that have not diverged as much as these species pairs. Although the search for such pairs might be difficult, it would be worth the effort because nematodes would be an excellent taxon in which to do a comparative study of selection on mtDNA. Nematodes appear to vary much more widely than vertebrates or *Drosophila* in specieswide effective population sizes (Anderson et al. 1998; Blouin et al. 1998, 1999), which makes them useful for testing certain predictions of nearly neutral models of mtDNA evolution (Nachman 1998; Rand and Kann 1998). Because nematodes have such biased mtDNA base composition and code for all their genes on the same strand of mtDNA (Okimoto et al. 1992), they should be useful for testing predictions about the interaction between mutation and selection in driving codon usage, and on the extent to which synonymous sites really are neutral (e.g., Akashi 1995; Rand and Kann 1998). This taxon thus represents an underutilized, but potentially very useful system for studying selection on metazoan mtDNA.

From the Department of Zoology, Cordley 3029, Oregon State University, Corvallis, OR 97331-2914. T. Anderson first suggested applying the MK test to the *Haemonchus* dataset. Thanks to J. McDonald, M. Kreitman, and J. Rozas for suggestions on the best way to perform the MK test given multiple polymorphic sites within a codon. Special thanks to M. Nachman and D. Rand for discussions that greatly clarified my thinking on this subject, in particular on the effect of variation in N_e on R:S ratios. Sequences used in this study are available under GenBank accession numbers AF070736-AF070825 for *H. contortus* and *H. placei,* AF070877-AF070916 for *Teladorsagia circumcincta,* and AF144551-AF144561 for *T. boreoarcticus.* The four *H. marelatus* sequences are published in Blouin et al. (1999), and the *H. bacteriophora* sequence is GenBank number AF066888. Address correspondence to Michael S. Blouin at the address above or e-mail: blouinm@bcc.orst.edu.

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Received July 1, 1999
Accepted October 18, 1999

Corresponding Editor: Sudhir Kumar

Possible Genetic Basis of Pederin Polymorphism in Rove Beetles (*Paederus riparius*)

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In *Paederus riparius,* (+) females able to biosynthesize the unique hemolymph toxin pederin and (−) females lacking this ability co-occur in natural populations. Larvae descended from both types of females were reared in the laboratory and the imagoes were crossed in order to get information about a possible genetic basis of this polymorphism. The daughters of (+) mothers become (+) females or (−) females, while the progeny of (−) mothers comprises only (−) females. This suggests a matrilineal trait because pederin biosynthesis cannot be inherited from the father. The rather stable proportion of nearly 90% (+) females in collected females is not maintained, however, when the beetles are reared in the laboratory. This observation is discussed with regard to artificial rearing conditions, where individuals are kept separate and cannot prey on conspecifics.

Rove beetles of the genus *Paederus* (Coleoptera: Staphylinidae) are notorious for their irritant hemolymph toxin which causes dermatitis linearis when the beetles are crushed and smeared on the skin. The affliction has been studied extensively, resulting in about 5% of the more than 600 *Paederus* species being known as causative (Frank and Kanamitsu 1987). Isolation of the toxic substance, which was named pederin by Pavan and Bo (1953), led to the discovery of an amide with complex structure (Cardani et al. 1965b; Matsumoto et al. 1968). The biosynthesis of this unusual compound, which immediately became a target of scientific interest (Cardani et al. 1965a), could not be re-

solved in detail but is regarded as a polyketide synthesis (Cardani et al. 1973). This pathway implies the presence of multifunctional proteins that biosynthesize (part of) the substance through metabolic channeling (Luckner 1990).

After experimental application of *Paederus* hemolymph to human skin, no reactions were observed by Ito (1934) using *P. poweri* and by de Leon (1952) using *P. fuscipes,* two species which were shown by other authors to cause dermatitis (Frank and Kanamitsu 1987). Such a negative result was attributed to immunization of the test person (Théodoridès 1952). Recent chemical analysis of *P. riparius* and *P. fuscipes* (Kellner and Dettner 1995), however, indicate that contradicting evidence using the same species is a real phenomenon due to pederin polymorphism. In both species studied, most of the females accumulate pederin and transfer it into their eggs, whereas some females are obviously unable to biosynthesize the substance and lay eggs without pederin. The former are concisely called (+) females, the latter (−) females. Like the (−) females, larvae and males do not increase their pederin content by themselves but sequester the substance received maternally or consumed if given access to conspecifics.

Polymorphism for a defensive compound is known in great detail from an example in plants: *Trifolium repens* has a cyanogenic and an acyanogenic morph which differ in mollusk acceptability (Dirzo and Harper 1982). Cyanogenesis, the production of HCN, has long been known to be dependent on the presence of cyanogenic glucosides and a specific β-glucosidase (Jones 1972). It is widely accepted that the cyanogenic polymorphism is controlled by alleles of two loci (Hughes 1991): Alleles at locus *Ac* determine the presence or absence of two cyanogenic glucosides, linamarin and lotaustralin, while alleles at locus *Li* regulate the presence or absence of linamarase, a β-glucosidase that hydrolyzes linamarin and lotaustralin. The loci segregate independently according to Mendelian ratios.

After discovering the pederin polymorphism in *Paederus,* Kellner and Dettner (1995) hypothesized that this polymorphism might also be explained by genetic differences. Heterozygous (+) females could then produce homozygous (−) females, which were surmised because some females descended from (+) females had not accumulated pederin when they were analyzed several months after imaginal eclosion. As pederin is present in the hemolymph all the time and not only after liberation by an enzyme after predation as in cyanogenesis, one locus could suffice for the distinction between (+) and (−) females. Analyzing the progeny of known specimens reared in the laboratory, this study aims at finding evidence for or against such a genetic basis of pederin polymorphism.

Materials and Methods

Beetles

Adult rove beetles (*Paederus riparius*) are found in central Europe mainly in spring and autumn (Horion 1965). The beetles reproduce in spring and imagoes of the new generation hibernate (Boháč 1985). Therefore beetles collected in northeastern Bavaria, Germany, from autumn 1992 to spring 1996 were grouped according to their expected season of reproduction, that is, the 1992 autumnal catch was combined with the beetles collected in spring 1993 under the label 1993 and so forth. Nine sites in northeastern Bavaria were visited, some repeatedly, to collect *P. riparius*: two sites in 1993, four in 1994, six in 1995, and two in 1996. The sites lie up to 100 km apart.

In the laboratory the beetles were isolated according to sex and site. Those collected in autumn had to be hibernated artificially by placing them for at least 3 months in a dark climate chamber at 6°C. After that period or upon collection in spring, pairs were founded and kept separately as described by Kellner and Dettner (1995) in order to obtain eggs of particular females. The eggs were taken out of the breeding cages three times per week and the larvae reared on moist absorbent paper in 24-cell wells (1.7 cm diameter of the wells). Frozen *Drosophila melanogaster* flies were supplied twice a day. One feeding during each larval stage (first and second stadium) consisted of a piece of either a *Tenebrio molitor* larva or a *Calliphora* pupa, which reduces larval mortality to about 22% (Kellner 1998).

The first-generation laboratory-reared imagoes were kept singly in petri dishes (9 cm diameter) with moist absorbent paper where they were fed with live *D. melanogaster* (strain vg). After artificial hibernation (2–4 months in a climate chamber at 12°C with a 9:15 h day/night photoperiod) pairs reproduced to give a second generation (20°C, 15:9 h day/night). The pairs were fed with pieces of *T. molitor* larvae or *C.* pupae as well as *D. melanogaster.* By trying to continue this breeding, only a few imagoes of the third generation were obtained which were not kept long enough to be included in this study (see below). The progeny of F_1 females, that is, generation F_2, is important because only then are both parents known. In contrast, females from the natural population could have copulated prior to collection, that is, their mate in the laboratory need not be the progeny's father.

Pederin Analysis

Pederin contents of single specimens were determined using the method described in detail by Kellner and Dettner (1995). Crude (of eggs) or purified (of imagoes) ethyl acetate extracts were chromatographed on HPTLC plates (0.2 mm silica gel 60, Merck) with ethyl acetate as solvent and stained in anisaldehyde-sulfuric acid-acetic acid (1:2:100 v/v/v, 2 min at 90°C). Pederin spots at $R_f = 0.22$ were quantified with a computer program (BASys 1D, Biotec Fischer GmbH, Reiskirchen) using calibration lanes on each plate where known amounts of authentic pederin had been applied.

Data Analysis

The females were classified as (+) females or (−) females according to the following rules:

1. Females laying eggs lacking pederin are obviously (−) females. Likewise, females whose eggs contain more than 0.4 μg pederin are (+) females. As already noted by Kellner and Dettner (1995), (−) females may transfer small amounts of pederin (<0.1 μg) into their first few eggs. This occurs especially in laboratory-reared (−) females descended from (+) females, who have thus received maternal pederin and transfer it into their own eggs. Therefore females that transferred less pederin into all their eggs than they had received maternally are classified as (−) females.
2. Females that could not be stimulated to lay eggs or were not kept long enough to do so were preserved by freezing and extracted. In this case only females 2 months or more of age are considered, as females accumulate pederin within 60 days after imaginal eclosion (Kellner 1998). The (+) females' descendants were classified as (+) females if they had more than 2.5 μg of pederin in their whole-body extract, that is, more than they could have received maternally (Kellner and Dettner

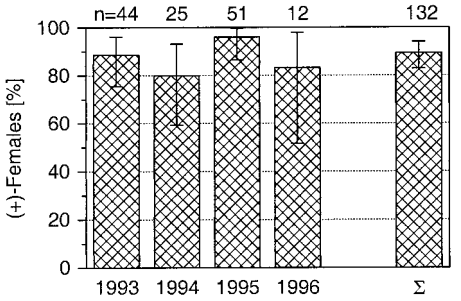

Figure 1. Percentage of (+) females (with 95% confidence interval) in *P. riparius* samples collected from several sites in northeastern Bavaria during four consecutive years. The females were caught during the preceding autumn or in spring of the respective years and laid eggs in the laboratory containing or lacking pederin.

1995). Most of them possessed more than 5 μg of pederin. Those classified as (−) females because they had not accumulated the substance had only up to 0.5 μg of pederin. There is a clear gap between (+) and (−) females. Classification of (−) females' daughters is even more straightforward, as they have received (almost) no maternal pederin. Therefore all females containing detectable amounts of the substance must have the ability to biosynthesize it and are regarded (+) females. Females without pederin are obviously (−) females.

Many females were classified according to both methods described above. No inconsistencies between these two sets of data were observed. Analysis of the eggs, however, is the prime method given in the results section, with whole-body extracts shown only for specimens without eggs available.

Statistical analyses were performed using CSS (StatSoft Inc., Tulsa, OK, version 2.1). Ninety-five percent confidence intervals of frequencies were calculated according to the method described by Sachs (1984). The binomial distribution, described by Sokal and Rohlf (1995), is applied to the data of (−) females' progeny.

Results

Collected Females

In *P. riparius* females collected from outdoors and reproducing in the laboratory, there is a small but persisting percentage of (−) females. Most of the females (nearly 90%), however, are (+) females (Figure 1). There is no significant change in the percentage of (+) females over the years ($\chi^2 = 5.22$, df = 3). The proportion of the two types of females thus appears to be rather

Table 1. (+) and (−) females in the progeny of 17 *P. riparius* (+) females

Mother	Progeny						
		Females laying eggs		Whole-body extracts		All females	
Female no.	*n*	(+)	(−)	(+)	(−)	(+)	(−)
1	6	—	—	0	6	0	6
2	14	2	4	0	8	2	12
3	4	0	1	1	2	1	3
4	32	5	8	4	15	9	23
5	6	0	1	2	3	2	4
6	5	1	1	2	1	3	2
7	8	3	1	2	2	5	3
8	11	3	1	4	3	7	4
9	8	3	0	4	1	7	1
10	5	3	0	2	0	5	0
11	7	3	0	4	0	7	0
12–17	11	4	1	0	6	4	7
1–17	117	27	18	25	47	52	65

Daughters were classified as (+) or (−) females according to the eggs they laid or from their whole-body extract if no eggs were obtained. Females (1–11) with at least four daughters are given separately.

stable in the natural population. In the hypothesized single gene, two allele system $p_{(+)}$, the gene frequency for pederin biosynthesis, could be calculated from $(1 - p_{(+)})^2 = 0.1$, thus $p_{(+)} = 0.7$.

The proportion obtained by analyzing the eggs laid in the laboratory could be biased if one type of female is more likely to lay eggs under these conditions. Therefore all females failing to reproduce in 1995 (the year with most cases), although kept in pairs for several weeks, were extracted after the unsuccessful breeding trial and classified. This resulted in 17 (+) females and 1 (−) female, which does not differ from the proportion obtained from egg-laying females (49:2, $\chi^2 = 0.09$, df = 1). Laboratory breeding thus does not favor one type of female.

Progeny of (+) Females

The progeny of (+) females reared in the laboratory is split into (+) females and (−) females (Table 1). Indeed, 18 (−) females laying eggs without pederin could be found. Moreover, there is no clear predominance of one type of female. One female (no. 1 in Table 1) produced exclusively (−) females, while two females (nos. 10 and 11) had only (+) females in their progeny. A whole array of proportions spans between these two extremes, with four (+) mothers producing predominantly (−) females, three (+) mothers nearly no (−) females, and four (+) mothers in between. There is thus no pattern of proportions emerging from these data on (+) and (−) females descended from

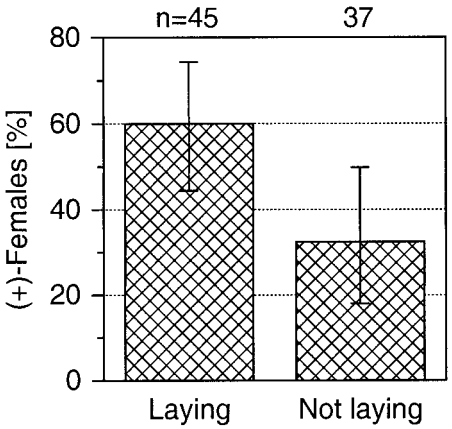

Figure 2. Percentage of (+) females (with 95% confidence interval) in laboratory-reared *P. riparius* females descended from (+) mothers and kept in breeding cages together with a male. Part of the females laid eggs while the others did not reproduce.

(+) mothers (Table 1). Given $(p_{(+)})^2 = 0.47$, which has been approximated from the data on collected females, about half of the (+) mothers (52%) would have been expected to produce only (+) females and the other half (48%) a 1:1 ratio of (+) and (−) females. The data in Table 1 do not fit that hypothesis, as only 2 of 11 (+) mothers produced exclusively (+) females ($\chi^2 = 5.04$, df = 1, $P < .05$). Furthermore, if the hypothesis was correct, there should have been no (+) mothers producing only (−) females (as no. 1).

Compared to the proportions in the natural population, (+) females give rise to remarkably many (−) females in the laboratory. When given the opportunity to reproduce, however, (+) females reared in the laboratory will succeed more often than (−) females ($\chi^2 = 6.19$, df = 1, $P < .05$; Figure 2). This differs from the equal breeding performance of both types of collected females.

Analysis of (+) females' progeny according to generation number reveals a decline of (+) females from generation to generation (Table 2A: F_1 = progeny of (+) mothers paired with collected males, F_2 =

Table 2. Summary of (+) and (−) females descended from 17 *P. riparius* (+) mothers and 10 (−) mothers

Mothers		Progeny	
n	paired with	(+)	(−)
A) (+) Mothers			
13	collected males	51	53
4	sons of (+) females	1	12
B) (−) mothers			
4	collected males	0	28
4	sons of (+) females	0	21
2	sons of (−) females	0	8

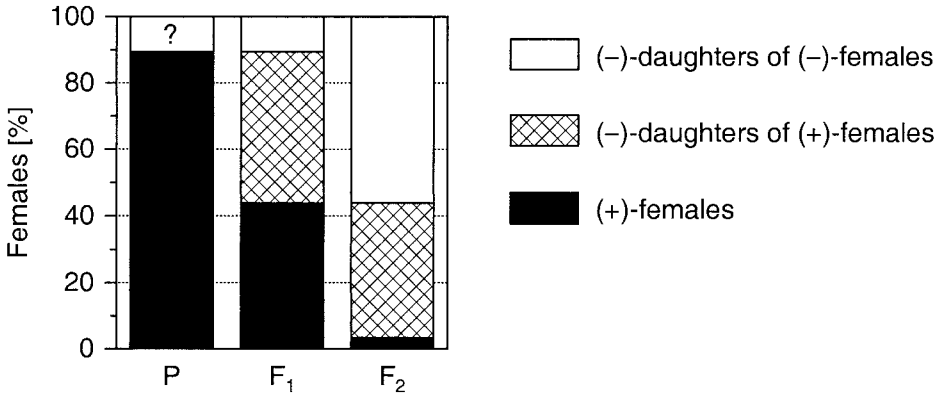

Figure 3. Percentage of (+) and (−) females in successive laboratory generations of *P. riparius.* Collected (−) females (P) are of unknown descent (?).

progeny of (+) mothers paired with sons of (+) females). Twelve additional F_2 females had not accumulated pederin until they were analyzed at 30 days old. These data are not included in the analysis, however, because identification of (+) females is not reliable at this early age, although some increase in pederin content should have occurred in (+) females within this lifespan (Kellner 1998). Nevertheless, the data obtained show a reduction of (+) females in F_2 as compared to F_1 ($\chi^2 = 8.00$, df = 1, $P < .01$), although all F_1 females had been crossed with sons of (+) females. Even F_1 females have a clearly reduced proportion of (+) females with regard to collected females ($\chi^2 = 46.60$, df = 1, $P < .001$).

Progeny of (−) Females

Contrary to the progeny of (+) females, the progeny of (−) females is not split into (+) females and (−) females (Table 2B). Not only did the collected (−) females, which were paired with collected males, produce only (−) females, but the progeny of (−) females reared in the laboratory and paired with males of known descent is exclusively (−) females. Three of the (−) females whose progeny are analyzed here were themselves descended from (+) females, suggesting that there is no effect if the maternal grandmother or both grandmothers were (+) females.

In summary, there are 57 (−) females and no (+) female descended from (−) females. According to the binomial distribution, the probability of obtaining such a result is $\binom{57}{0}(1-q)^0 q^{57} = q^{57}$, where q stands for the probability of (−) females in the progeny. Suppose q should be the same as in the (+) females' progeny ($q \sim 0.5$). This would give a practically impossible observed result (probability $< 10^{-17}$). Therefore q must be much higher indeed. Calculating the other way round, the observed result would be expected in 5% of cases if $q = 0.948$. The probability of (−) females producing (+) females can thus be only 0.052 or lower, because higher values would have led to the detection of (+) females regarding conventional significance criteria.

Overall Performance of Laboratory Population

Including the progeny of both (+) and (−) females, Figure 3 shows a rapid decline in the percentage of (+) females in the laboratory. Although fewer and fewer females are able to biosynthesize pederin, the amount of substance accumulated in one specimen is not affected (Table 3). Laboratory-reared specimens that are able to biosynthesize the toxin accumulate large amounts. The maxima observed indicate a positive nutritional effect for specimens kept in the laboratory as compared to specimens analyzed immediately after collection. That means, the amide is not diluted and lost due to possibly inadequate food quality or supply.

The frequency of females is reduced in laboratory-reared imagoes as compared to collections from outdoors ($\chi^2 = 7.09$, df = 1, $P < .01$; Table 4). This difference is mainly due to the sex ratio found in the progeny of (−) mothers, as they produce fewer daughters than (+) mothers ($\chi^2 = 7.80$, df = 1, $P < .05$). Such a shift could be caused by differences in mortality during the laboratory rearing.

Discussion

The analysis of laboratory reared specimens of *P. riparius* reveals several remarkable and even unexpected insights into females' pederin polymorphism. First, (−) females can be descended both from (+) females and (−) females. This could be important for maintenance of the polymorphism in natural populations. As larvae without pederin are selectively preyed on by wolf spiders (Kellner and Dettner 1996), the progeny of (−) females might not become adult in the field. In the long term, this would lead to extinction of the polymorphism if (+) females only produced (+) females. From this observation, the initial hypothesis based on heterozygous (+) females might be true.

Second, (−) females had only (−) females in their progeny. Thus the males cannot contribute to their daughters' ability to accumulate the toxin and protect their grandchildren against spider attacks. Transition in succeeding generations between the two polymorphic characters seems to be possible only in one direction, from (+) females to (−) females and not vice versa. Taking this into account, pederin biosynthesis appears to be a matrilineal trait.

Third, the (+) females produced a high proportion of (−) females. In view of the rather stable high percentage of (+) females collected, the occurrence of pederin in diverse species of *Paederus,* and the (−) females' inability to give rise to (+) females, this is a most unexpected result. The ability to accumulate pederin would be lost within a few generations. In the natural environment, however, the proportional reduction of (+) females could be counteracted by (heavy) selection against (−) females. But a selection pressure as high as required in this case is not known. Spiders, the only predators proved to be

Table 3. Amount of pederin determined in *P. riparius* (+) females

		Pederin (μg)	
(+) Females	*n*	Mean ± SEM	Range
Collected	29	10.8 ± 0.62	4.5–16.8
Kept in laboratory	16	12.5 ± 1.05	5.3–21.0
Laboratory reared	39	14.7 ± 0.92	4.5–24.8

Dead (+) females are excluded from the analysis.

Table 4. Frequency of females and males in collected and laboratory-reared imagoes of *P. riparius*

Origin	Females	Males	Significance[a]
Collected	282	230	*
Laboratory reared	257	291	ns
from (+) mothers	159	153	ns
from (−) mothers	98	138	**

[a] Chi-square test for deviation from 1:1 ratio, *$P < .05$, **$P < .01$, ns = not significant.

deterred by pederin (Kellner and Dettner 1996) cannot be blamed for that because they reject all progeny of (+) females, that means future (−) females as well. Abiotic factors such as a distinct hibernation rate between (+) and (−) females can be ruled out, as the females collected in autumn and hibernated artificially gave no indication of such a factor's importance.

Regarding the data discussed, it is clear that the initial hypothesis is not supported because the ability to biosynthesize pederin cannot be inherited from the father and furthermore no Mendelian proportions are found in the progeny of (+) mothers. The sudden drop of the percentage of (+) females in F_1 could be explained by approaching equilibrium of gene frequencies (Falconer and Mackay 1996), but data on F_2 do not support this possibility as there is a further drop. The results thus indicate a completely different mode of transmission from one generation to the next. This might involve characteristics of the egg that are supplied only by the mother, such as cytoplasmic genes, distinct cell compartments with their own genome as in mitochondria, or even microorganisms. Microorganisms, for example, could also be involved in sex ratio distortion, as described in other beetles (e.g., Werren et al. 1994). Furthermore, they could be transmitted horizontally among unrelated members of a population. Since *Paederus* beetles are known to prey on conspecifics (Pickel 1940; Ramírez 1966), such a horizontal transmission could account for the high percentage of collected (+) females. In the laboratory breeding scheme, specimens were intentionally kept apart, which prevents them from eating others. This was important for individual recognition of specimens, which was the aim of this study. Other experiments are needed to address the open questions of which quality of the egg could be responsible for transmission of biosynthetic capabilities and how important preying on conspecifics might be.

From the Lehrstuhl für Tierökologie II, Universität Bayreuth, D-95440 Bayreuth, Germany. I am indebted to Professors K. Dettner (Bayreuth) for research facilities and reading of an earlier version of the manuscript, D. Ghiringhelli (Milan) and P. Kocieński (Southampton) for samples of authentic pederin, and E. Beck (Bayreuth) for utilization of BASys 1D. Thomas Meise and Nigel Dobbin bred most of the collected pairs in 1994 and 1996, respectively. Marc Ehnert and Sandra Kellner kindly took care of the beetles when I was abroad. Address correspondence to Rupert L. L. Kellner at the address above or e-mail: rupert.kellner@uni-bayreuth.de.

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Received May 24, 1999
Accepted October 18, 1999

Corresponding Editor: Ross MacIntyre

Systematic Implications of Chromosomal Data from Two Insular Species of *Peromyscus* from the Gulf of California

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G- and C-banded karyotypes for two insular species of deer mice, *Peromyscus slevini* and *P. sejugis,* are described and analyzed relative to the evolutionary relationship of these species to and their inclusion within the *P. maniculatus* species group. The chromosomal phenotype of *P. slevini* is unique among all banded karyotypes reported for *Peromyscus,* and comparison with published karyotypes suggests that *P. slevini* has systematic affinities with either the *P. boylii* or *P. mexicanus* species groups. The karyotypic data for *P. sejugis* clearly align these mice with *P. maniculatus* and provide a diagnostic character that supports the specific distinction between these taxa.

Of the 13 currently recognized groups in the genus *Peromyscus* (Carleton 1989), none is more widely distributed or intensively studied than is the *P. maniculatus* species group. With the exception of the addition of two insular species (*P. slevini* and *P. sejugis*) from the Gulf of California, Osgood's (1909) initial constitution of this group (*P. maniculatus, P. polionotus, P. melanotis,* and *P. sitkensis*) has remained remarkably stable. Recent analyses, however, have raised questions concerning the circumscriptions of some of the inclusive species and stimulated reconsiderations of the systematic affinities and composition of this group. Analyses of genetic and morphologic characters resulted in the recognition of *P. keeni,* subsuming *P. sitkensis* and including most of the subspecies of *P. maniculatus* from the Pacific northwest (Allard et al. 1987; Allard and Greenbaum 1988; Calhoun and Greenbaum 1991; Gunn 1988; Gunn and Greenbaum 1986; Hogan et al. 1993; Sullivan et al. 1990). Chromosomal data have also pro-

vided support for specific differentiation of the historically recognized grassland (short-tailed) and forest (long-tailed) ecophenotypes in the northeastern range of *P. maniculatus* (Myers Unice et al. 1998). In addition, analyses of variation in mtDNA (Hogan et al. 1997) indicate that *P. slevini* (Isla Santa Catalina) is independent of the *P. maniculatus* group and raise questions about the specific status and systematic relationships of *P. sejugis* (Isla Santa Cruz and Isla San Diego) and populations of *P. maniculatus* from Baja California.

Despite the maintenance of a constant diploid number of 48, analyses of chromosomal variation and differentiation resulting from pericentric inversions have facilitated the resolution of a variety of systematic and taxonomic questions within *Peromyscus* (for a review see Greenbaum et al. 1994). Cladistic analyses of banded chromosomal data (Rogers et al. 1984; Smith 1990; Stangl and Baker 1984) have provided phylogenetic hypotheses for the majority of the approximately 60 species of *Peromyscus*. For the *P. maniculatus*-group species for which data are available (*P. maniculatus, P. polionotus, P. melanotis,* and *P. keeni*), the chromosomal phylogeny is entirely congruent with implications from analyses of morphologic and allozymic data (for a review see Carleton 1989). Herein we present G- and C-banded chromosomal data for *P. slevini* and *P. sejugis* and interpret these data relative to the systematic and taxonomic relationships of the *P. maniculatus* species group.

Materials and Methods

Specimens of *P. slevini* and *P. sejugis* were live trapped in January 1992 from natural populations in Baja California del Sur, Mexico. The animals were transported to and maintained (for periods not exceeding 1 year) in the AAALAC certified small-animal vivarium in the Department of Biology at Texas A&M University. The specimens were preserved as skin and skull, or skin, skull, and partial skeleton and deposited in the Texas Cooperative Wildlife Collections (TCWC) at Texas A&M University. The individuals included in this report, their locality of capture, and their respective TCWC catalog numbers are *P. slevini,* Isla Santa Catalina (n = 16, 55782–55797); and *P. sejugis,* Isla Santa Cruz (n = 9, 55768–55773, 55778–55780), Isla San Diego (n = 11, 55746, 55747, 55749–55752, 55754–55758).

Metaphases were prepared by a modification of the method described by Baker et al. (1982). Nondifferentially (Giemsa)-stained metaphases were used to determine diploid and autosomal arm numbers ($2n$ and *FN,* respectively). Chromosomes were G-banded following the GTG technique of Verma and Babu (1995) and identified and numbered according to the standardized karyotype of *Peromyscus* (Greenbaum et al. 1994). C bands were produced by a modification of the method of Sumner (1972). Chromosomal localization of heterochromatin was determined from comparisons between G- and C-banded karyotypes and confirmed by analysis of sequentially G-/C-banded chromosomes.

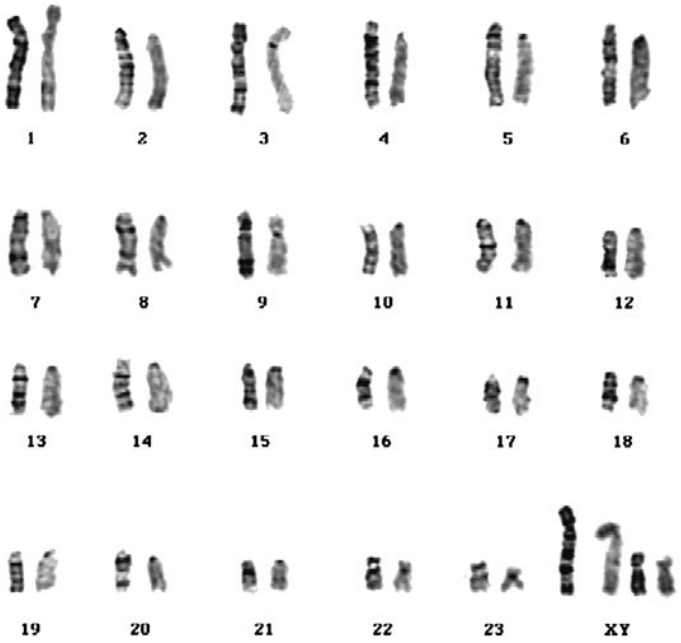

Figure 1. Composite G-banded (on the left) and C-banded (on the right) karyotypes of a male *P. slevini* (*FN* = 56). Chromosomes 1, 3, 9, 22, 23, and the sex pair are submetacentric or metacentric; the remaining chromosomes are acrocentric. Heterochromatin is restricted to the centromeric region and the sex pair.

Results

All specimens had $2n$ = 48. Karyotypes of the specimens of *P. slevini* were autosomally invariant and characterized by *FN* = 56. G-banded karyotypes of *P. slevini* indicated metacentric or submetacentric conditions of the X and Y chromosomes and of autosomes 1, 3, 9, 22, and 23 (Figure 1); the remaining autosomes were acrocentric. Variation was not observed among either the X or Y chromosomes. In this taxon, C-band-positive heterochromatin was limited to the centromeric regions of all chromosomes, the short arm of the X chromosome, and the entire Y chromosome (Figure 1).

Karyotypes of the specimens of *P. sejugis* from Isla Santa Cruz and Isla San Diego presented *FN* = 76. Chromosomes 4, 6, 7, 8, 12, 15, 16, and 17 were acrocentric, and the remaining autosomes and the X and Y chromosomes were metacentric or submetacentric (Figure 2). C-band-positive heterochromatin was located at the centromeric region of all the autosomes, the short arm of the X, and the entire Y chromosome. Heterochromatin was also present on the distal portion of the short arms of chromosomes 11, 18, and 21 from both populations and on chromosome 13 of the individuals from Isla Santa Cruz.

Discussion

The species-group affinity of *P. slevini* is historically problematic (for a review see Carleton 1989). Originally aligned (Maillaird 1924) with the *P. californicus* group of the subgenus *Haplomylomys,* comparisons of cranial morphology (Burt 1934) resulted in the tentative inclusion of *P. slevini* in the *P. maniculatus* group (Hooper 1968). Based on comparative analysis of the nucleotide sequence of the mtDNA genes *ND3, ND4L,* and *ND4,* Hogan et al. (1997) concluded that *P. slevini* should not be included in the *P. maniculatus* species group and is extremely divergent from both the *P. maniculatus* and *P. leucopus* species groups. The chromosomal data for *P. slevini* are entirely consistent with these conclusions. Including this report, descriptive data on G-band homologies are now available for all taxa that have been associ-

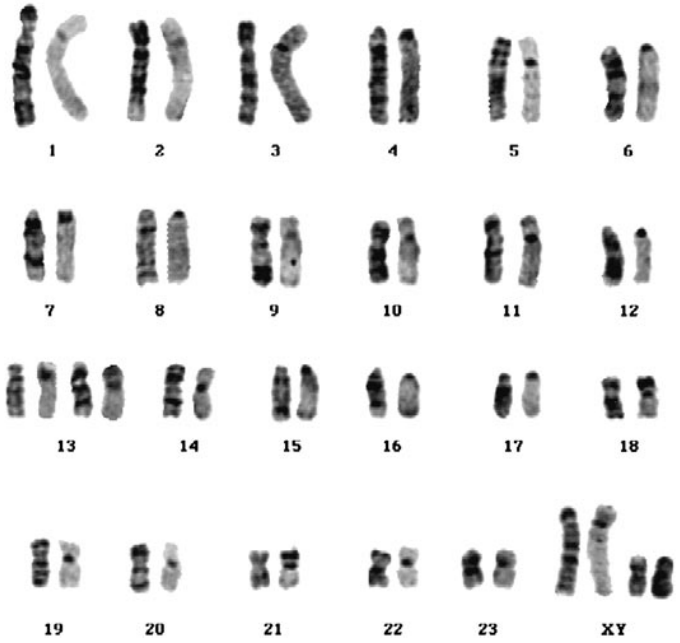

Figure 2. Composite G-banded (on the left) and C-banded (on the right) karyotypes of a male *P. sejugis* (*FN* = 76). With the exception of chromosome 13, all individuals had indistinguishable karyotypes. Karyotypes from specimens collected on Isla San Diego (the pair on the left) exhibited an alternate form of the submetacentric chromosome 13, designated 13B′. Karyotypes from individuals from Isla Santa Cruz (the pair on the right) have a heterochromatic addition to that chromosome, 13B′+. For all individuals, noncentromeric heterochromatin is present on chromosomes 11, 18, and 21.

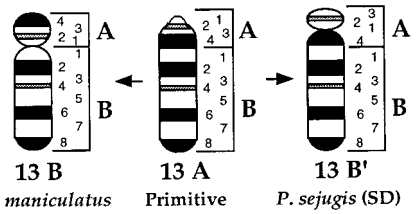

Figure 3. Ideogram of various chromosome 13 conditions within the genus *Peromyscus.* The primitive chromosome 13 is the acrocentric condition. The derived biarmed chromosome 13 in *P. maniculatus* (13B) comes from a pericentric inversion between bands A4 and B1. A pericentric inversion between bands A3 and A4 results in the alternative biarmed condition (13B′) found in karyotypes of *P. sejugis* from Isla San Diego (SD). The presence of heterochromatin on the euchromatic short arm of the latter chromosome forms the 13B′+ condition indicative of karyotypes of *P. sejugis* from Isla Santa Cruz (not shown).

ated with the *P. maniculatus* species group. Excluding *P. slevini,* the taxa in the *P. maniculatus* species group share indistinguishable biarmed inverted and derived conditions of chromosomes 2, 3, 9, and 20, with the latter character state occurring only in this group and convergently in one cytotype of *P. leucopus* (Stangl 1986). The karyotype of *P. slevini* exhibits acrocentric conditions of both chromosomes 2 and 20. Further, the composite array of chromosomal conformations in the karyotype of *P. slevini* is unique among all reported G-banded karyotypes of deer mice; no other species of *Peromyscus* is known to exhibit the combination of an acrocentric chromosome 2 and biarmed chromosomes 3 and 9.

Although comparisons of the G-banded karyotypes among species of *Peromyscus* do not yield an unambiguous species-group association of *P. slevini,* these data do provide initial hypotheses for studies designed to resolve the phylogenetic position of this species. The karyotype of *P. slevini* is most similar to those that generally characterize taxa in the *P. boylii* and *P. mexicanus* species groups. From the *FN* = 52 karyotype (biarmed chromosomes 1, 22, and 23) typical of *P. boylii, P. banderanus,* and *P. crinitus,* the karyotype of *P. slevini* differs by having biarmed chromosomes 3 and 9. Compared to the *FN* = 58 karyotype (biarmed chromosomes 1, 2, 3, 9, 22, and 23) of *P. mexicanus*-group species, the karyotype of *P. slevini* differs by the acrocentric condition of chromosome 2. From the cladistic-based assumption (Rogers et al. 1984; Smith 1990; Stangl and Baker 1984) that the acrocentric condition of chromosome 2 is plesiomorphic for *Peromyscus* and predates the inversions which result in the biarmed conditions of chromosomes 3 and 9, an equal number of inversion events would be needed to explain the differences between the karyotype of *P. slevini* and those of the *P. boylii* and *P. mexicanus* groups, respectively. Cranial similarities of the supraorbital shelf (Carleton 1989), however, support the phylogenetic association of *P. slevini* and the *P. mexicanus*-group as the more likely hypothesis.

The karyotypes of *P. sejugis* from both islands exhibit *FN* = 76 but are distinguished by the presence of distal heterochromatin on the short arm of chromosome 13 in all individuals from Isla Santa Cruz (Figure 2). The apparent alternate fixation for the presence/absence of this heterochromatic segment between the populations of *P. sejugis* from Isla Santa Cruz and Isla San Diego suggests the lack of effective gene flow between these populations. However, the absence of genetic divergence in the sequences of the *ND3, ND4L,* and *ND4* genes between these populations (Hogan et al. 1997) suggests that the acquisition of the heterochromatic addition to chromosome 13 and the restricted gene flow between the two island populations of *P. sejugis* are recent in origin.

The inclusion of *P. sejugis* within the *P. maniculatus*-species group was originally based on morphologic similarity and geographic proximity of *P. sejugis* to mainland (southern Baja California) *P. maniculatus coolidgei.* This association has been supported by analyses of phallic morphology (Hooper and Musser 1964), allozymes (Avise et al. 1974, 1979), and mtDNA sequences (Hogan et al. 1997). The karyotypic data, particularly the shared-derived biarmed conditions of chromosomes 2, 3, 9, and 20, strongly support the inclusion of *P. sejugis* within the *P. maniculatus*-species group. In addition, the shared-derived condition of chromosomes 5, 10, 11, 14, 18, and 21 among *P. sejugis, P. maniculatus,* and *P. keeni* is unambiguous evidence of a close phylogenetic relationship among these taxa. Indeed, the only character state that distinguishes the banded chromosomes of *P. sejugis* from those reported for *P. maniculatus* is an alternative biarmed condition of chromosome 13. In *P. sejugis* (Figures 2 and 3), the centromere of chromosome 13 is located between bands A3 and A4 (B′ condition), whereas in *P. maniculatus* (Greenbaum et al. 1994) the centromere of chromosome 13 is situated between bands A1 and B1 (B and B+ condition). This difference is most parsimoniously explained by independent pericentric inversions of the primitive acrocentric condition of chromosome 13 (Figure 3). The chromosomal condition in the mice from Isla Santa Cruz (13B′+) presumably resulted from the addition of heterochromatin to the distal portion of the short arm of the 13B′ condition.

Although the specific recognition of allopatric populations is problematic, the unique chromosome 13B′ conditions in the populations from Isla Santa Cruz and

Isla San Diego provide a character state which would establish *P. sejugis* as a phylogenetic species (see Nixon and Wheeler 1990 and references therein) and support the morphologically based specific distinction of this taxon relative to *P. maniculatus* (Burt 1932). Based on the apparent alternate fixation for 13B′ and 13B′+ conditions, a similar argument could be made for a phylogenetic species-based distinction of the two island populations of *P. sejugis.* However, considering the lack of morphologic (Burt 1932), allozymic (Avise et al. 1979) and molecular (Hogan et al. 1997) divergence between these populations we see little value in recommending revision of their current taxonomy.

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Received May 26, 1999
Accepted October 24, 1999

Corresponding Editor: Oliver A. Ryder

The Rift Valley Complex as a Barrier to Gene Flow for *Anopheles gambiae* in Kenya: The mtDNA Perspective

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Descriptions of *A. gambiae* population structure based on microsatellite loci and mitochondrial DNA (mtDNA) were incongruent. High differentiation of populations was measured across the Rift Valley by microsatellites, but no differentiation was detected based on mtDNA. To resolve this conflict, we compared the old data to new mtDNA data using the same specimen previously genotyped in microsatellite loci. Analysis of a larger number of mtDNA sequences resulted in high and significant differentiation between populations across the Rift Valley. We developed a method to assess whether differentiation across the Rift Valley was generated by pure drift rather than mutation-drift, based on DNA sequence data. Applying this method to the mtDNA data suggested that pure drift was the primary force generating differentiation between the populations across the Rift, while mutation-drift generated differentiation across the continent. Given adequate sample size, mtDNA provided congruent results with microsatellite loci.

Different molecular markers (and loci) do not necessarily reflect the same evolutionary processes and sometimes produce inconsistent results. Such discrepancies can provide valuable insights into processes that affect different markers in different ways. For example, a lower rate of gene flow measured by mitochondrial DNA (mtDNA) compared with autosomal or Y-linked markers may reflect a larger dispersal distance for males (e.g., Avise 1994: 227–230). Recent studies on the population structure of *Anopheles gambiae,* the principal vector of malaria in Africa (Collins and Besansky 1994; Coluzzi 1992), revealed a discrepancy between results based on nuclear loci and those based on mtDNA. High differentiation was measured between populations from eastern and western Kenya by restriction fragment length polymorphisms (RFLPs) of the rDNA (McLain et al. 1989) and by microsatellites (Kamau et al. 1998b, 1999; Lehmann et al. 1998). Differentiation between those Kenyan populations (700 km

Table 1. Polymorphism at the 599 bp segment of the mitochondrial *ND5* gene of *A. gambiae* populations

	Asembo		Jego	
Collection year	1987	1994	1987	1996
N (haplotypes)	8 (7)	45 (25)	5 (3)	13 (8)
Haplotype diversity	0.96	0.94	0.90	0.92
Variable sites	7	24	7	10
Nucleotide diversity	0.0041	0.0042	0.0057	0.0052
Tajima's *D*	-0.48^{NS}	-1.81^{*}	0.08^{NS}	-0.15^{NS}

$^{*}P < .05$ (individual test level).
$^{NS}P > .05$.

apart F_{ST} = 0.07–0.1; Lehmann et al. 1998) was considerably higher than that measured by the same microsatellite loci between populations across the continent (6000 km apart, F_{ST} = 0.02; Lehmann et al. 1996b). Subsequent analysis based on additional populations demonstrated that the Eastern Rift Valley and associated areas act as a barrier to gene flow (Lehmann et al. 1999). Generally mtDNA results (Besansky et al. 1997) agreed well with those based on microsatellite and allozyme loci (Lehmann et al. 1996b, 1997), but no differentiation was detected across the Rift Valley using mtDNA (Besansky et al. 1997).

The simplest explanation for this discrepancy is that random noise, due to small sample size, masked the differentiation. Alternatively, a biological process such as low mutation rate or selective constraints could be involved. The level of polymorphism measured in the *ND5* gene was moderate to high (33 haplotypes of 65 individuals and nucleotide diversity of 0.004; Besansky et al. 1997), suggesting that low mutation rate is not a good explanation. Three out of 28 nucleotide substitutions resulted in amino acid replacements and all were singleton polymorphic sites (i.e., substitutions segregating in haplotypes that were observed only once). Tajima's and Fu's tests were consistent with neutral mutations in most populations. Moreover, significant differentiation (F_{ST} = 0.085) was detected based on the mtDNA data between populations from Kenya and Senegal, suggesting that those processes did not prevent divergence of these populations (Besansky et al. 1997). An additional explanation involves the high similarity of mtDNA between *A. gambiae* and *A. arabiensis,* and the possibility that introgression of mtDNA between these species occurs at a substantially higher rate than nuclear introgression, as was proposed for *Drosophila pseudoobscura* and *D. persimilis* (Powell 1983). MtDNA gene flow from *A. arabiensis* to *A. gambiae* can "dilute" differentiation between populations of the latter. Female *A. gambiae-arabiensis* hybrids are fertile and were observed at a frequency of approximately 0.2% (reviewed in Coluzzi et al. 1979). Low differentiation between these mostly sympatric species at the mtDNA (F_{ST} = 0.09; Besansky et al. 1997) was considered evidence for introgression between them. In contrast to mtDNA, higher differentiation was measured between these species by microsatellites (F_{ST} = 0.25; Kamau et al. 1998a; Lanzaro et al. 1998) and allozymes (F_{ST} = 0.07–0.19; Besansky et al. 1997; Nei's (1987) distance = 0.15; Cianchi et al. 1985), lending support for selective introgression of mtDNA.

To resolve this conflict, we assessed mtDNA variation using the same specimens previously analyzed at nine microsatellite loci (Lehmann et al. 1998, 1999). Thus different results can be attributed to differences between markers rather than to the source populations or the samples themselves.

Materials and Methods

Study localities and sample collection were described previously (Lehmann et al. 1998). In short, collections were made in Asembo Bay (hereafter, Asembo), located on the shores of Lake Victoria in western Kenya, and Jego, located 700 km away on the coast of the Indian Ocean near the Tanzanian border. Indoor resting female mosquitoes were collected from both localities in May 1987. Subsequent collections were made in Jego (July 1996) using the same method, and in Asembo (June 1994) using bed net traps hung over the beds of volunteers. In each sample, mosquitoes were collected from an area smaller than 10 km in diameter within a period of 2 weeks.

Sequencing protocols and mtDNA sequences from Asembo and Jego 1987 were described by Besansky et al. (1997) and those from Asembo 1994 were described by Lehmann et al. (1997). New sequences include the sample from Jego 1996. All these specimens were sequenced using the ABI Big Dye Sequencing Kit and the ABI 377 sequencing system (Applied Biosystems). The *ND5* sequences included positions 6912–7510 in the *A. gambiae* reference sequence (Beard et al. 1993; GenBank accession L20934). Sequence alignment was done using GCG software (Genetics Computer Group 1994) and basic sequence statistics were computed using MEGA (Kumar et al. 1993). Tajima's test was computed using DnaSP 2.5 (Rozas and Rozas 1997). All other computations were carried out by programs written in SAS language (SAS Institute 1990) by T. Lehmann as described previously (Besansky et al. 1997) or as described below. All permutation and bootstrapping tests were based on 2000 pseudoreplicates.

Results and Discussion

Moderate to high levels of polymorphism across the 599 bp at the *ND5* gene were observed in all samples (Table 1). The pooled dataset was comprised of 71 individuals, 34 haplotypes, and 29 variable sites. Similar to previous studies (Besansky et al. 1997; Lehmann et al 1997), the frequency of singletons (haplotypes observed only once) was high (27%, 19 of 71), while the number of substitutions between sequence pairs was moderate (0.47% per site or 2.8 per sequence in the pooled data). No insertion/deletion was found and all substitutions were silent, based on the *Drosophila* mtDNA code. Tajima's test of neutral polymorphisms was insignificant, except in the 1994 collection from Asembo (Table 1). The latter was not significant at the multitest level using the sequential Bonferroni procedure (Holm 1979).

Genetic differentiation between populations was estimated by F_{ST} (Hudson et al. 1992b) and its significance was assessed by a permutation test (Hudson et al. 1992a). Because sample sizes varied considerably, we also calculated a weighted F_{ST} in which the within-population component was a weighted mean based on sample size, as was previously described (Besansky et al. 1997). While no differentiation was observed between the 1987 collections from Asembo and Jego (Table 2; Besansky et al. 1997), high and significant differentiation was measured between collections from Asembo 1994 and Jego 1996 (Table 2). High and significant differentiation was also measured be-

Table 2. Differentiation between samples from different localities and time points

	Unweighted F_{ST}	Weighted F_{ST}
Asembo 87 vs. Jego 87	−0.041[NS]	−0.001[NS]
Asembo 94 vs. Jego 96	0.123***	0.176***
Asembo vs. Jego (pooled)	0.089***	0.142***
Asembo 87 vs. Asemo 94	0.026[NS]	0.018[NS]
Jego 87 vs. Jego 96	−0.016[NS]	0.005[NS]

*** $P < .001$.
[NS] $P > .05$.

Table 3. "Lack-of-fit test" with the mutation-drift model using comparison of haplotype F_{ST} and individual F_{ST} (calculated with weights according to sample sizes)

Populations (sample sizes)	Haploytpe F_{ST} (P estimated by permutation test)	Bootstrapped[b] individual F_{ST} (95% CI)
Asembo 94 vs. Jego 96 (25/8)	0.061 ($P > .13$)	0.171*** (0.072–0.285)
Asembo vs. Jego pooled (32/12)	0.058 ($P > .10$)	0.138*** (0.070–0.209)
Senegal vs. western Kenya[a] (17/16)	0.046 ($P < .029$)	0.073*** (0.031–0.134)

*** $P < .001$.
[a] Data from Besansky et al. (1997).
[b] Sample sizes were the same as used for the haplotype F_{ST} calculation (shown in first column).

tween localities in the pooled (over time) data. This level of differentiation and derived estimates of gene flow measured by mtDNA, which were adjusted to the difference in N_e between markers, closely agreed with those measured by microsatellites (Lehmann et al. 1998, 1999). The temporal variation between samples taken 7 (Jego) and 9 (Asembo) years apart from the same localities was minimal (Table 2), suggesting that the difference between the original (Besansky et al. 1997) and the present studies were not due to temporal changes in allele frequencies, nor that the 2 years separating the samples from Asembo (1994) and Jego (1996) contributed much to the differentiation between localities. It can be concluded therefore that the discrepancy between the results based on microsatellites and the original mtDNA study was merely a small sample size effect.

The lack of unique alleles in eastern populations and higher F_{ST} than R_{ST} values measured at nine microsatellite loci suggested that pure drift was the main process generating differentiation between these populations (Lehmann et al. 1999). To distinguish between pure drift and mutation-drift using mtDNA data, a test was developed based on the fact that pure drift affects haplotype frequencies but does not systematically affect the number of pairwise substitutions between haplotypes. Accordingly, if two populations became isolated from each other a few generations ago, and one population has experienced a bottleneck and lost several alleles as part of the rapid change in allele frequencies, then allele frequencies will differ markedly between these populations, but the average mutational distance between two different alleles is expected to be the same, regardless of whether they were taken both from a single population or each from a different population. Independent mutations, in addition to drift, must occur in each population to increase the expected mutational distance between two different alleles, each sampled from one population. Therefore we calculated F_{ST} on haplotypes instead of individuals (haplotype F_{ST}), which estimates the between-population variation in the number of substitutions (i.e., mutations) per haplotype disregarding the haplotype frequency. If differentiation was generated by pure drift, then the haplotype F_{ST} is expected to be zero. Permutation and bootstrapping tests were used to determine the significance of the results and to evaluate whether an insignificant haplotype F_{ST} reflects low statistical power due to smaller sample size.

The haplotype F_{ST} was calculated between Asembo 1994 and Jego 1996 samples, and in the pooled (over time) samples (Table 3). Haplotype F_{ST} values were approximately one-third of the corresponding individual F_{ST} values and were not significant ($P > 0.09$, permutation test), suggesting a lack of fit with the mutation-drift model. To verify that the lack of significance was not a result of weak statistical power due to smaller sample size, we calculated the 95% confidence interval (CI) of individual F_{ST} by bootstrapping over individuals from each population while using the same sample sizes as used for the haplotype F_{ST} calculation. The bootstrapped F_{ST} values were nearly identical to the original individual F_{ST} values (Table 2) and they were significantly higher than zero. Moreover, their lower 95% confidence limits were higher than the corresponding haplotype F_{ST} (Table 3), indicating that the lack of significance of the haplotype F_{ST} values was not due to reduced sample sizes.

In contrast to Kenyan populations across the Rift Valley, higher R_{ST} than F_{ST} values were measured between western Kenya and Senegal (6000 km apart, both west of the Rift Valley barrier) and unique alleles were observed in each population (Lehmann et al. 1996b), suggesting that differentiation between these populations was generated by the mutation-drift model. To test this interpretation, we analyzed the mtDNA data of these populations (from Besansky et al. 1997). Haplotype F_{ST} for this comparison was significantly larger than zero, and it was not significantly different from the individual F_{ST} based on the same sample sizes (Table 3). Therefore different genetic markers suggested that pure drift was the dominant process generating differentiation between western and eastern Kenyan populations, whereas mutation-drift was the dominant process generating differentiation between western Kenyan and Senegal populations. Pure drift implies a recent bottleneck-like event(s) rather than a long separation of stable populations. Possible scenarios include a recent colonization of the eastern localities by migrants from western populations, that is, a founder effect, or a bottleneck in populations which previously were similar genetically to western populations. Such an event must be invoked to reconcile the finding of large current (deme) N_e based on temporal variation between 1987 and 1996 in allele frequencies (Lehmann et al. 1998) with that of strong genetic drift generating differentiation between populations from eastern and western Kenya (Lehmann et al. 1999 and the present results).

Several studies called for caution in using microsatellite data to describe population structure because of the possibility of constraints on allele size (e.g., Garza et al. 1995; Lehmann et al. 1996a; Nauata and Weissing 1996) and because of uncertainty about the mutation process and consequential difficulties in selecting an adequate measure of differentiation (Paetkau et al. 1997; Perez-Lezaun et al. 1997). High concordance between results based on microsatellites and other markers is evidence that these potential complications have little effect, at least in *A. gambiae.* However, the congruence between markers may be more apparent than real if the population structure has been shaped primarily by pure drift because the elapsed time may be insufficient to detect the effects of mutation (Slatkin 1995) or constraints (Nauata and Weissing 1996).

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Received August 12, 1999
Accepted October 24, 1999

Corresponding Editor: Ross MacIntyre

Chromosome Evolution of the Blue Sheep/Bharal (*Pseudois nayaur)*

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A male dwarf blue sheep was collected 60 km south of Batang east to the Jinsha Jiang river, and a male Subei blue sheep (Greater form) was collected from Gansu, China, representing two geographically separated blue sheep forms. Chromosome preparations were prepared from fibroblast cultures. The dwarf blue sheep has a $2n = 54$ and a karyotype with three biarmed formations that resulted from acrocentric chromosome fusions (based on the $2n = 60$ *Capra* autosomal equivalents) 14p/5q, 27p/1q, and 29p/2q from the largest to the smallest biarmed chromosome, respectively. The 14p/5q fusion is metacentric, whereas the 27p/1q and 29p/2q are submetacentric. The Subei blue sheep had a $2n = 56$, with only the 27p/1q and 29p/2q biarmed chromosome fusions. The remainder of the chromosomes in both blue sheep are acrocentric; the X is the largest acrocentric chromosome and the Y is a minute biarmed chromosome. Our observation is one evidence showing that chromosome evolution within blue sheep has followed a series of centric fusions resulting in the reduction of chromosome number, which is typical of all extant genera within the tribe Caprini.

The blue sheep was originally given the scientific classification *Ovis nayaur* Hodgson based on the assumption that it was a true sheep (*Ovis*) rather than a true goat (*Capra*) (Lydekker 1898). The blue sheep resembles a true sheep with horns sweeping out and back and does not have a beard nor a potent body odor as in goats. It has goatlike characteristics as well, with a broad flat tail, black and white markings on its forelegs, and some skull features typical of the goat. True sheep have preorbital and interdigital glands on all feet, whereas goats lack preorbital glands and have interdigital glands only on their forefeet, if they have them at all. Blue sheep either have rudimentary preorbital and interdigital glands or none at all. Because of the varying morphologic characteristics that are common to both sheep and goats, the blue sheep was reclassified, given a genus of its own, and today it is scientifically referred to as *Pseudois nayaur* (Ellerman and Morrison-Scott 1965). The common name, blue sheep, also is inappropriately used and technically should be referred to by its Hindi name, bharal; however, it is more commonly recognized as the blue sheep and will be referred to in this article by that name.

Schaller (1973) studied the behavior of blue sheep and concluded that they are basically a goat. Their sheeplike traits are a consequence of convergent evolution as it settled into habitats usually occupied by

Figure 1. Dwarf blue sheep.

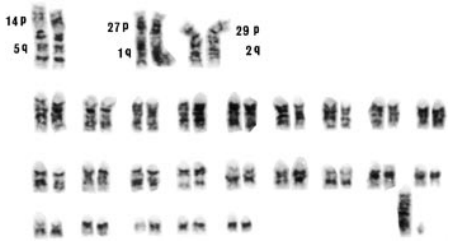

Figure 2. G-band karyotype of the dwarf blue sheep.

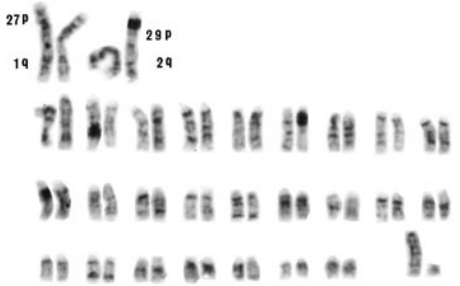

Figure 3. G-band karyotype of Subei blue sheep (Greater form).

true sheep. Schaller (1973) also stated that blue sheep probably split from goat stock shortly after sheep and goats diverged from ancestral stock and developed along separate evolutionary pathways.

The blue sheep inhabit very remote regions and ranges from Baltistian in Kashmir eastward across Tibet and into Yunnan, Szechwan (Sichuan), Kansu (Gansu), and Shensi (Shaanxi) provinces of the People's Republic of China (Schaller 1973). The northern distribution is bordered by the Kunlum Shan and Altyn-tag ranges and the southern border is the Himalayas.

There are two recognized forms of blue sheep: the dwarf and the Greater form (Groves 1978). In the Yangtze gorge near Batang the two forms are located within relatively close proximity where they are separated by only 1000 m of forest, yet they retain their distinctness. The Greater form occupies the upper region and the dwarf the lower.

The chromosome number and karyotype of the blue sheep were first described from a male specimen classified as a Himalayan blue sheep (*Pseudois nayura*) [nayaur is incorrectly spelled in this previous publication] (Hard 1969). It had a $2n = 54$ ($FN = 60$) represented by 6 submetacentric and 46 acrocentric autosomes. The X chromosome was not identified, but was thought to be acrocentric, and the Y chromosome was identified as a very minute acrocentric chromosome (Hard 1969). Bunch et al. (1978) reexamined the same specimen using G-banding techniques and reaffirmed a $2n = 54$. They identified the X chromosome as a large acrocentric and the Y chromosome as a small metacentric rather than a minute acrocentric chromosome. They also identified specific acrocentric chromosomes involved in the evolution of its $2n = 54$ karyotype.

Herein we report the chromosome number and G-banded karyotypes from specimens sampled from the northern and southern boundaries of the blue sheep range.

Materials and Methods

A male dwarf blue sheep was collected 60 km (29°37′N 99°02′E) south of Batang and 4 km east of the Jinsha Jiang river. A male blue sheep, commonly referred to as a Subei blue sheep (Greater form), was collected from the Gansu province, Peoples Republic of China. Skin biopsies were collected by Donald Cox, Bloomfield Hills, MI, and sent to the Key Laboratory of Cellular and Molecular Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Peoples Republic of China. Chromosome preparations were prepared from fibroblast cultures. G-banding followed the procedures of Wang and Federoff (1972) and the description of the karyotypes was based on the format of Menscher et al. (1989) and Ansari et al. (1999).

Results and Discussion

The dwarf blue sheep (Figures 1 and 2) has a $2n = 54$ and a karyotype similar to the description of Bunch et al. (1978) for the Himalayan blue sheep (*Pseudois nayura*). The three biarmed formations resulted from acrocentric chromosome fusions 14p/5q, 27p/1q, and 29p/2q [based on the goat acrocentric nomenclature system of Menscher et al. (1989) and sheep nomenclature of Ansari et al. (1999)] from the largest to the smallest biarmed chromosome, respectively. The 14p/5q fusion is metacentric, whereas the 27p/1q and 29p/2q are definitely submetacentric. The p arms in the *Pseudois* biarmed chromosomes 1–3 are equivalent to *Ovis* acrocentric autosome equivalents 11, 24, and 26 (Ansari et al. 1999; Menscher et al. 1989).

The Subei blue sheep (Greater form; Figure 3) had a $2n = 56$, with only the 27p/1q and 29p/2q biarmed chromosome fusions. Except for the biarmed chromosomes, the remainder of the chromosomes in both blue sheep are acrocentric; the X is the largest acrocentric chromosome and the Y is a minute biarmed chromosome.

Our observations in this study indicate that chromosome evolution within blue sheep have followed a series of centric fusions resulting in the reduction of chromosome number. Chromosome evolution involving centric fusions is common in the superfamily Bovoidea and has been well documented in *Ovis* with extant forms with a $2n = 58$, 56, 54, or 52. The Sebei blue sheep ($2n = 56$) has been separated from the range of the dwarf blue sheep ($2n = 54$) geographically and over an extended period of time. This separation has allowed for the $2n = 56$ karyotype to evolve into the $2n = 54$ karyotype. A $2n = 58$ population of blue sheep might still exist within the range of *Pseudois,* which supports the need for further cytogenetic analysis of *Pseudois* and closely related taxa.

Schaller (1973) suggested that the bharal probably split from goat stock shortly after sheep and goats diverged from ancestral stock to develop along separate evolutionary pathways. This split not only involved particular morphologic and behavioral characteristics, but most likely set the stage for a split in the pathway of chromosomal evolution.

There are five extant genera within the tribe Caprini. Among these taxa, *Hemitragus* (tahr) with a $2n = 58$ is generally regarded as the most primitive and is the probable link between Caprini and Rupicaprini (Bunch and Nadler 1980; Schaller 1977; Thenius and Hofer 1960). Wild goats of the genus *Capra* and domestic goats in general share a similar chromosomal complement of $2n = 60$. *Ammotragus* ($2n = 58$), commonly called the Barbary sheep or Aoudad, and *Pseudois* ($2n = 54$ and 56), are regarded as aberrant sheep with goatlike affinities. True sheep of the genus *Ovis* have $2n = 52$, 54, 56, or 58 (Bunch et al. 1976; Bunch and Nadler 1980; Nadler and Bunch 1977; Nadler et al. 1973).

Hemitragus, Ammotragus, Pseudois, and

Ovis evolved from a common evolutionary pathway. All arose from ancestral stock that shared a $2n = 60$ diploid chromosome number and a karyotype with 29 acrocentric chromosomes, a large acrocentric X, and a very small acrocentric or metacentric Y chromosome. The primitive-type karyotype is still maintained universally in *Capra* and has been maintained during the evolution of the wild and domestic goat and the ibex and markhor. The largest goat acrocentric autosome was involved in the first centric fusion or Robertsonian translocation and became biarm 1q in *Hemitragus, Ammotragus, Pseudois,* and *Ovis* lineage. The pathway for a common 1q arm was shared only in *Ammotragus* and *Ovis*. The fact that both genera share acrocentrics 1q and 3q in the evolution of the first biarmed chromosome suggests they share a common ancestor that arose after a split from goat stock. The *Ammotragus* and *Ovis* karyotype with a $2n = 58$ is shared by the Barbary sheep (*Ammotragus lervia*) and in what is considered the more primitive extant species of wild sheep, the urial (*Ovis vignei*). Based on chromosome fusions involving acrocentric autosome 1 in the *Capra* karyotype, *Hemitragus* and *Pseudois* would have split off from ancestral *Capra* stock separately from *Ammotragus* and *Ovis*.

It is not known which centric fusion occurred first in *Pseudois.* In all likelihood it may have been the 27p/1q translocation, since the 1q was the first centric fusion involved in the $2n = 58$ karyotypes of *Ammotragus* and *Ovis.* The karyotype of the Greater form, Sebei blue sheep ($2n = 56$), has 27p/1q and 29p/2q fusions. Assuming that karyotype evolution of *Pseudois* is toward the reduction of diploid number, then the 14p/5q fusion is the more recent translocation, although it is arranged first in the karyotype of Figure 2 because of its relative size being the largest of the biarmed chromosomes.

Chromosome evolution in Caprini may have set the stage for genetic isolation, which eventually led to speciation. Considering the potential chromosomal segregational problems during meiosis in F_1 hybrids with partial homology of biarmed chromosomes, fertility would be reduced if not totally impaired. F_1 hybrids resulting from a blue sheep ram and domestic goat ewe have been reported at the Henry Dorley Zoo, Omaha, Nebraska, although they were born dead (Bunch et al. 1978). No successful hybridization has been reported between blue sheep and true sheep.

Despite the divergent chromosome evolution in *Hemitragus, Ammotragus, Pseudois,* and *Ovis,* homologous G-banding patterns in all taxa examined by us and others indicate a conservatism in linear banding that can be traced back to a *Capra*-like karyotype. The evidence that acrocentric autosomes 1, 2, and 5 were preferentially selected for is born out in the karyotypes of all four genera (Bunch and Nadler 1980).

The blue sheep karyotyped by Bunch et al. (1978) from a zoological specimen had a $2n = 54$. The dwarf blue sheep reported in this study also has a $2n = 54$ and a G-banded karyotype that is similar to the zoological specimen. Whether the dwarf blue sheep is a neotenous form or a younger stage of the greater blue sheep is still problematic. Allen (1939) suggested that the small size of the dwarf blue sheep might be due to the effect of insufficient pasturage, which was supported by Schafer's (1937) observations that dwarf blue sheep become rather thin in winter, unlike the Greater form. Groves (1978), however, found no evidence for such a pronounced divergence in developmental plasticity. He observed that in addition to body size between the dwarf and the Greater form, the horns of the dwarf are smaller than would be predicted on a body-to-horn ratio, thinner, and with a consistently different shape. He also observed a difference in pelage color and pattern and therefore recommended as a provisional measure that the dwarf should be classified as a full species. Although we have no record of origin of the blue sheep sampled at the Henry Dorley Zoo, its diploid chromosome number and karyotype are similar to the dwarf in this study. Further research with fresh specimens may show whether there is enough morphologic diversity to justify the dwarf and Greater forms as separate species.

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Received May 10, 1999
Accepted October 24, 1999

Corresponding Editor: Leif Andersson

Genetics of Rough Seed Coat Texture in Cowpea

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Seed coat texture is an important trait in determining the acceptability of cowpea varieties in different regions. A rough seed coat is preferred in western and central Africa, since it permits easy removal of the seed coat which is essential for indigenous food preparations. On the other hand, a smooth seed coat is preferred in

eastern and southern Africa as well as in parts of South America where cowpea is consumed as boiled beans without removing the seed coats. This study was undertaken to elucidate the inheritance of seed coat texture so that cowpea breeders may adopt appropriate breeding strategy to develop cowpea varieties with preferred seed types for different regions. The F_1 plants between smooth- and rough-seeded parents as well as between rough- and rough-seeded parents produced smooth seeds, indicating a complementary gene action and dominance for smooth seed coat. The F_2 plants from the smooth × rough cross segregated into a 3 smooth:1 rough seed coat ratio, but the F_2 plants from rough × rough crosses segregated into a 9 smooth:7 rough seed coat ratio. The F_1 plants from backcross to the smooth parent were all smooth, while the F_1 plants from backcross to rough parent segregated in a 1 smooth:1 rough seed coat ratio. However, both the backcross populations in rough × rough crosses segregated into 1 smooth:1 rough seed coat ratio. These results indicate that two pairs of independent recessive genes confer rough seed coat texture in cowpea and the presence of at least one dominant gene at each of the two loci results into smooth seed coat. The gene symbols rt_1rt_1 and rt_2rt_2 are being assigned for rough seed coat texture in cowpea.

Cowpea [*Vigna unguiculata* (L.) Walp] is an important food legume in the semiarid tropics covering Asia, Africa, southern Europe, and parts of North and South America (Singh et al. 1997). However, the varieties and preferences for seed characteristics differ from region to region (IITA 1983). The cowpea seed coat has been classified into smooth, wrinkled, split, loose, or rough (IITA 1974). In western and central Africa, the preference is for rough seed coat, whereas in eastern and southern Africa and parts of South America smooth seeds are preferred (IITA 1983; Ojomo 1968). This is based on how cowpea is used in various food preparations. In western and central Africa, more than 50% of the cowpeas produced are used as snack foods (Kosai or Akara) or steamed food (Moin-Moin) for which the seed coat has to be removed before making a paste of the cotyledons (Ojomo and Chheda 1970; Steele 1972). In the absence of suitable mechanical devices to remove the seed coats, West African women soak cowpea seeds in water for a few min and rub off the seed coats. The rough seed coat absorbs water faster than cotyledons (Sefa-Dedeh and Stanley 1979), and a gentle rubbing easily removes the testa which is then floated off. It takes much longer to remove the smooth coats (Sefa-Dedeh and Stanley 1979). Only limited studies have been made on the inheritance of seed coat texture in cowpea (Fery 1985; Fery and Singh 1997). Krishnaswamy et al. (1945) reported that loose texture is controlled by a single recessive gene pair. Rajendra et al. (1979) studied cowpea seed coat texture by scanning electron microscopy and observed two anatomically different macrosclereid arrangements. The perpendicular arrangement to the cotyledon (Pec) was associated with smooth seed coat and the parallel arrangement to the cotyledon (Pac) was associated with rough seed coat. They further observed that the perpendicular arrangement (Pec) was dominant over the parallel arrangement (Pac), and this was controlled by a single dominant gene, *PC*. Fery (1985) adopted the gene symbol *pc* for the rough seed coat texture. Thus the previous genetic studies indicated that rough seed coat in cowpea is controlled by a single recessive gene pair. However, in our breeding program we have observed that whenever a white-rough cowpea variety is crossed to a brown-rough cowpea variety, the F_1 plants always produce brown smooth seeds. We have also noticed in the white × brown crosses that if the white-seeded parent has black hilum, the F_1 plants produce black smooth seeds; and if the white seeded parent has brown hilum, the F_1 plants produce brown smooth seeds, indicating the involvement of more than one pair of recessive genes for rough seed coat texture and dominance of brown and black colors in cowpea. This study was undertaken to confirm these observations and further elucidate the number of genes involved in controlling rough seed coat texture in cowpea.

Materials and Methods

This study was conducted at the International Institute of Tropical Agriculture (IITA) Kano Station, Kano, Nigeria, located at 12°03′N latitude and 8°34′E longitude. The origin and characteristics of the five parents used in this study are indicated in Table 1. The segregating populations were derived from three crosses. The first cross was between IT88DM-345 (red-smooth seeds) and Kanannado (white-rough seeds and brown hilum). The second cross was between IT87D-941-1 (brown-rough seeds) and Kanannado (white-rough seeds). The third cross was between IT93K-693-2 (brown-rough seeds) and IAR 1696 (white-rough seeds and black hilum). The parental, F_1, F_2, and backcross populations of the first two crosses were grown in the field, whereas the third cross was grown in the screenhouse, and plants from these populations were classified into smooth and rough seed categories. It may be added that since seed coat is a maternal trait, F_1 population refers to the seeds produced on F_1 plants and not F_1 seeds produced by direct crossing. Due to the fact that the inheritance of seed coat color and hilum color is rather complex (Fery 1985) and the population size in this study was relatively small, no attempt was made to study the inheritance of seed coat color and its relationship to seed coat texture.

Table 1. Origin and characteristics of the cowpea varieties used as parents

Variety	Origin	Characteristics
IT88DM-345	A single plant selection from a local variety from Togo	Small red-smooth seeds, extra-early maturity (50–55 days), photoinsensitive
IT87D-941-1	IITA breeding line	Medium brown-rough seeds, early maturity (65–70 days), photoinsensitive, resistant to aphid
IT93K-693-2	IITA breeding line	Medium brown-rough seeds, early maturity (65–70 days), photoinsensitive, resistant to aphid, bruchid, and *Striga*
Kanannado	Local variety from Nigeria	Large white-rough seeds with brown hilum, late maturity (110–120 days), photosensitive
IAR-1696	Local variety from Nigeria	Large white-rough seeds with black hilum, late maturity (120–130 days), photosensitive

Table 2 Segregation for seed coat texture in different populations of the cross involving IT88D-345 and Kanannado

Population	No. of plants with		χ^2	Probability
	Smooth seeds	Rough seeds		
IT88DM-345	18	—	—	—
Kanannado	—	36	—	—
F_1	18	—	—	—
F_1 × IT88D-345	19	—	—	—
F_1 × Kanannado	9	8	0.06 (1:1)	.8–.9
F_2	198	69	0.10 (3:1)	.7–.8

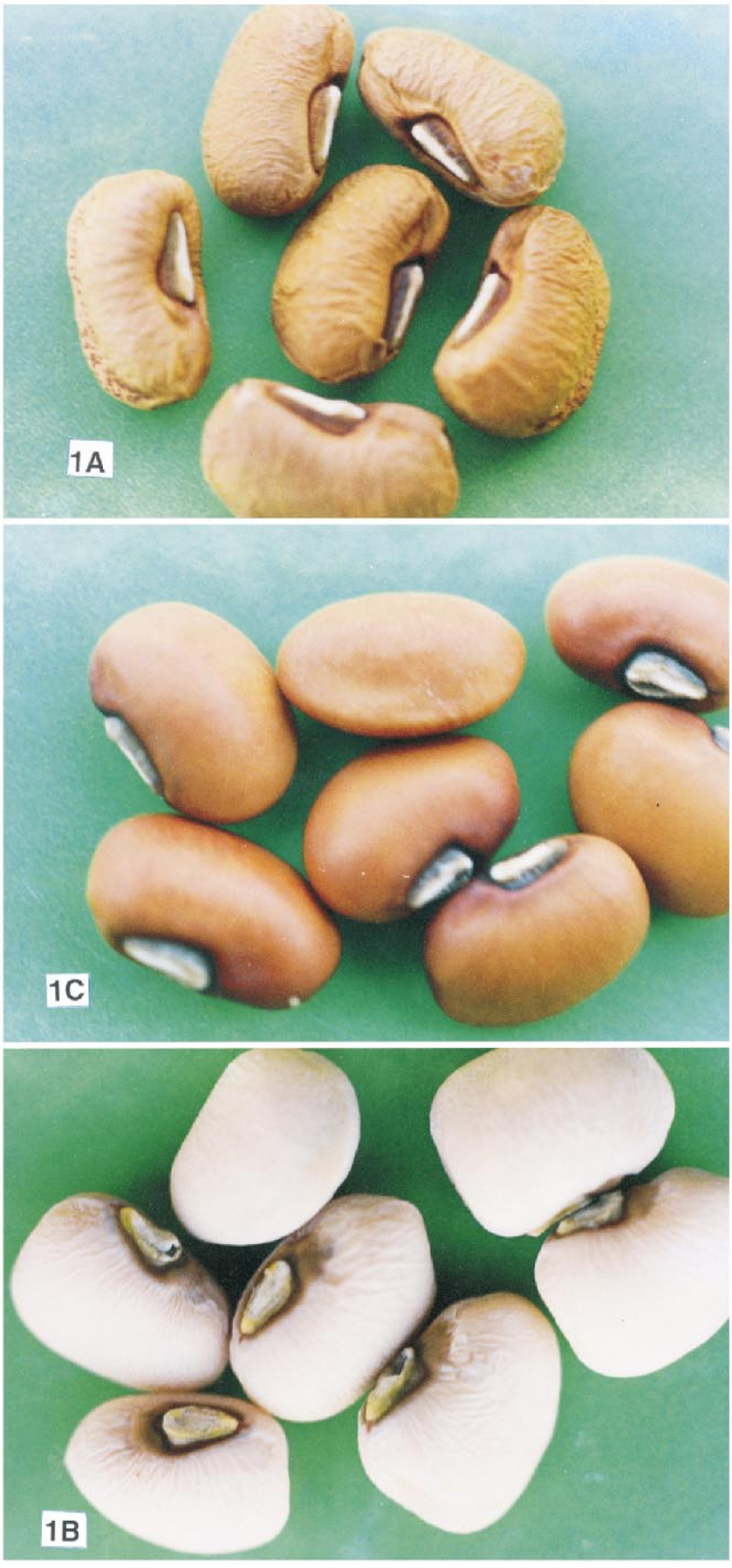

Table 3. Segregation for seed coat texture in different populations of the cross involving IT87D-941-1 and Kanannado

Population	No. of plants with		χ^2	Probability
	Smooth seeds	Rough seeds		
IT87D-941-1	—	66	—	—
Kanannado	—	48	—	—
F_1	32	—	—	—
$F_1 \times$ IT87D-941-1	26	23	0.18 (1:1)	.5–.7
$F_1 \times$ Kanannado	21	24	0.20 (1:1)	.5–.7
F_2	138	126	1.69 (9:7)	.1–.2

The observed segregation ratios for seed coat texture were subjected to chi-square tests to determine the goodness-of-fit to various genetic ratios.

Results and Discussion

The differences between smooth and rough seed coat textures were quite pronounced and could easily be observed with naked eyes in all the populations. Therefore classification of plants into smooth or rough seed groups was quite simple and without error. The results are presented separately for each cross.

Cross 1: Smooth × Rough

The segregation pattern in different populations involving IT88DM-345 (smooth) and Kanannado (rough) are presented in Table 2. As expected, all 18 plants of IT88DM-345 had smooth seeds and all 36 plants of Kanannado were rough seeded. The 18 F_1 plants derived from the cross of the two parents had brown smooth seeds, indicating a complete dominance of smooth seed coat over rough seed coat. All 19 backcross F_1 plants involving IT88DM-345 were smooth seeded, but the backcross F_1 plants involving Kanannado segregated into 9 smooth and 8 rough with a close fit to a 1:1 ratio. The F_2 segregated into 198 smooth-seeded and 69 rough-seeded plants, fitting very closely to a 3:1 ratio. The results indicated that rough seed coat in Kanannado is controlled by a single recessive gene pair.

Cross 2: Rough × Rough

The segregation pattern in different populations involving IT87D-941-1 with brown-rough seeds and Kanannado with white-rough seeds are presented in Table 3. All

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Figure 1. Seed coat texture and color of **(A)** IT87D-941-1 (brown-rough), **(B)** Kanannado (white-rough with brown hilum), and **(C)** their F_1 hybrid (brown-smooth).

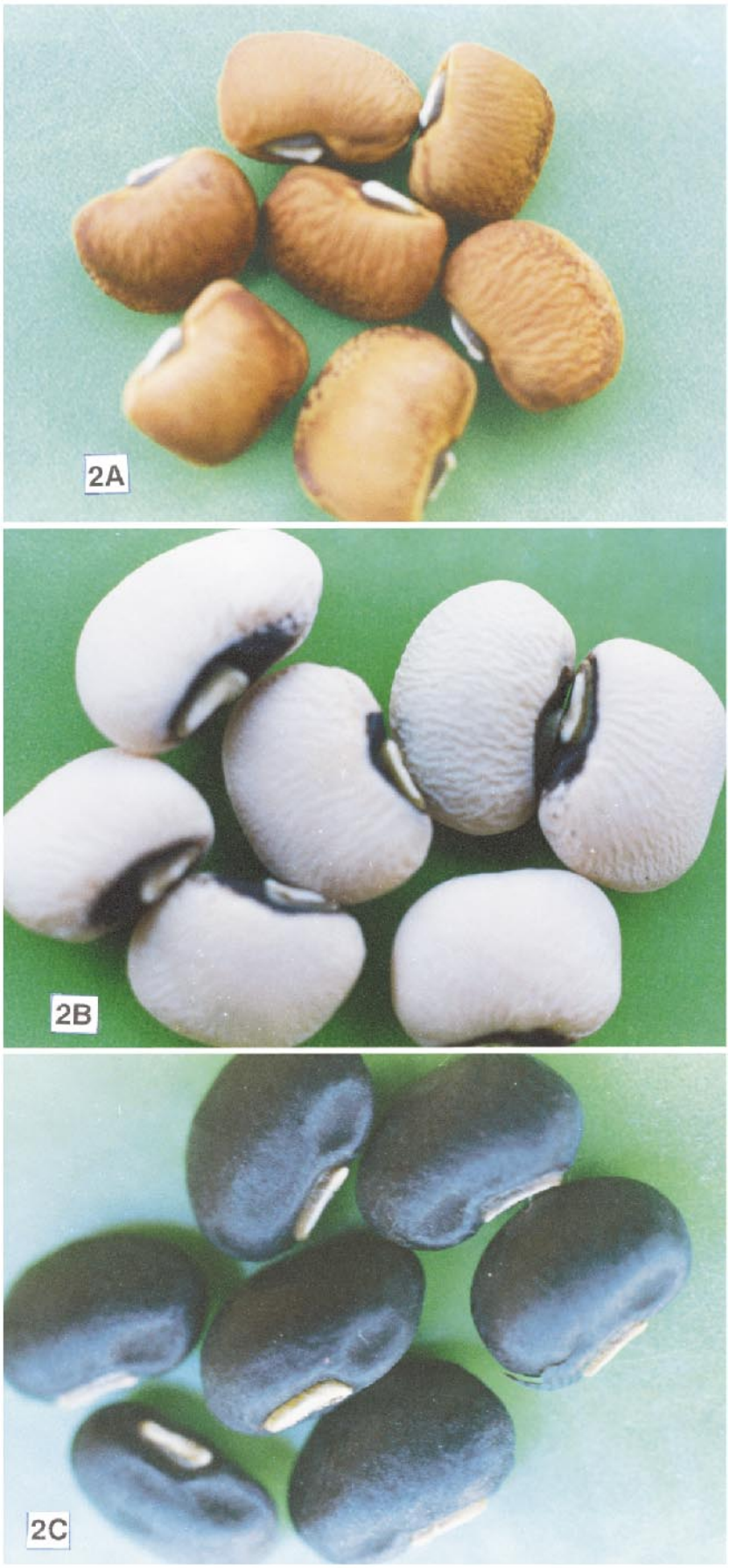

Table 4. Segregation for seed coat texture in different populations of the cross involving IT93K-693-2 and IAR-1696

Population	No. of plants with		χ^2	Probability
	Smooth seeds	Rough seeds		
IT93K-693-2	—	24	—	—
IAR 1696	—	17	—	—
F_1	26	—	—	—
$F_1 \times$ IT93K-693-2	9	7	0.25 (1:1)	.5–.7
$F_1 \times$ IAR 1696	35	32	0.13 (1:1)	.7–.8
F_2	46	37	0.02 (9:7)	.8–.9

the plants of both parents had rough seed coats, but the F_1 plants had smooth seed coats and brown color, indicating independent gene action for seed coat texture and complete dominance for the brown color (Figure 1). The backcross F_1 population involving IT87D-941-1 segregated into 26 smooth-seeded and 23 rough-seeded plants, and the backcross F_1 population involving Kanannado segregated into 21 smooth-seeded and 24 rough-seeded plants, both fitting closely to a 1:1 ratio. The F_2 population segregated into 138 smooth-seeded and 126 rough-seeded plants, showing close fit to a 9:7 ratio. These data indicate that rough seed coat is controlled by two independent recessive gene pairs, and the recessive gene pair for rough coat in IT89KD-941-1 is different from the gene in Kanannado.

Cross 3: Rough × Rough

This cross involved a brown-rough-seeded variety, IT93K-693-2, and a white-rough-seeded variety with black hilum, IAR 1696. The F_1 plants had smooth black seeds (Figure 2). The segregation pattern with respect to seed coat texture in backcross and F_2 populations is presented in Table 4. The 16 backcross F_1 plants involving IT93K-693-2 segregated into 9 smooth-seeded and 7 rough-seeded plants, and the 67 backcross F_1 plants involving IAR 1696 segregated into 35 smooth-seeded and 32 rough-seeded plants both showing close fit to a 1:1 ratio. The F_2 population segregated into 46 smooth-seeded and 37 rough-seeded plants fitting closely to a 9:7 ratio. These results are in close conformity with those observed in the IT87D-941-1 × Kanannado cross and further confirm that the rough seed coat is con-

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Figure 2. Seed coat texture and color of **(A)** IT93K-693-2 (brown-rough), **(B)** IAR 1696 (white-rough with black hilum), and **(C)** their F_1 hybrid (black-smooth).

trolled by two independent pairs of recessive genes.

The results of the three crosses reported here indicate that the recessive gene pair for rough seed coat in white-seeded varieties, Kanannado and IAR 1696, is different from the one in brown-seeded varieties IT89KD-941-1 and IT93K-693-2. The recessive gene pair for rough seed coat texture reported earlier by Rajendra et al. (1979) may be one of these two pairs of recessive genes because he used California Black Eye cowpea, which has white-rough seeds and black hilum. It is proposed that the earlier gene *pc,* based on macroscleroid arrangements (Fery 1985; Rajendra et al. 1979), should be changed to $rt_1\ rt_1$ and the second gene to $rt_2\ rt_2$ to represent rough seed coat texture since the trait is easily observed with naked eyes.

The independent gene action for smooth seed coat and the dominance for black and brown seed colors have important implications in selecting parents for breeding, if the desired cowpea variety must have brown or white seeds with rough seed coat, which is an essential requirement in western and central Africa. In that case it would be desirable to select parents for crosses having the same genes for rough seed coat and seed coat color. Otherwise either a large F_2 population would have to be screened or one or two backcrosses need to be made toward the desirable parents before evaluating the segregating populations. The genetic stocks used in this study are being maintained by IITA Kano Station and will be made available to interested researchers on request.

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Received April 29, 1999
Accepted September 14, 1999

Corresponding Editor: Susan Gabay-Laughnan

Polymorphism of α_1-Antitrypsin in North American Species of *Canis*

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α_1-Antitrypsin (A1AT) is a major protease inhibitor present in all mammalian sera that have thus far been investigated. A1AT is also highly polymorphic and is therefore a useful genetic marker. Previously reported A1AT polymorphism in domestic dogs consisted of two alleles designated as Pi^M and Pi^S which exhibited frequencies of 0.72 and 0.28, respectively, in a group of randomly collected mongrel dogs. North American species of *Canis,* which included gray wolves (n = 29), Mexican wolves (n = 2O), coyotes (n = 24), wolf-dog crosses (n = 9), and red wolves (n = 27) were tested for A1AT polymorphism. A1AT phenotypes were determined by isoelectric focusing, followed by direct immunoblotting using a specific antiserum. A1AT concentrations were determined by radial immunodiffusion. Concentrations of A1AT were similar to those found in domestic dogs (2.26 ± 0.3, ± SD mg/ml, ± SD) and tended to be higher in females than in males, possibly indicating that A1AT may be hormonally influenced in females. Three phenotypic band patterns were observed (M, MS, S). The allele frequencies for domestic dogs and gray wolves were very similar, 0.72 and 0.67 for Pi^M and 0.28 and 0.33 for Pi^S, respectively. The Mexican wolves had a significantly lower frequency of Pi^S = 0.10. Coyotes and red wolves were all found to be monomorphic for the Pi^S allele and were indistinguishable from each other in that respect.

α_1-Antitrypsin (A1AT) is a highly polymorphic glycoprotein in many mammalian species and may therefore be useful as a genetic marker (Patterson 1991). A1AT is a major proteinase inhibitor with activity against trypsin, elastase, chymotrypsin, cathepsin G, and probably against other serin proteinases. It is synthesized mainly by the liver. A major physiological role is the inhibition of neutrophil elastase and thus to protect tissues from enzymatic degradation (Koj et al. 1978; Travis and Salvesen 1983).

Canine A1AT was isolated and characterized by Abrams et al. (1978). A1AT polymorphism was reported in the domestic dog (*Canis lupus* var. *familiaris*) expressing a codominant mode of inheritance of two alleles at one locus (Kueppers et al. 1993). A1AT polymorphism in domestic dogs consisted of two alleles designated as Pi^M and Pi^S which exhibited frequencies of 0.72 and 0.28, respectively, in a group of randomly collected mongrel dogs. North American species of *Canis,* which included gray and Mexican wolves (*Canis lupus*), coyotes (*Canis latrans*), wolf-dog crosses, and red wolves (*Canis rufus*) were tested for A1AT polymorphism.

Materials and Methods

Blood samples of the following animals were included in this investigation: 29 captive and free-ranging gray wolves from Alaska, Canada, and Minnesota; 24 captive coyotes from Utah (these animals were collected in Utah or they were offspring from Utah coyotes); 27 captive red wolves and 20 captive Mexican wolves, and 9 privately owned wolf-dog crosses of varying lineage and recent wolf ancestry. Blood samples were collected by standard venipuncture of the cephalic vein. The blood was allowed to clot and serum was separated by centrifugation and frozen at −20°C until analysis.

The gene locus for A1AT has been designated as Pi (for proteinase inhibitor; Fagerhol and Gedde-Dahl 1969). The two alleles are written with superscripts as Pi^M

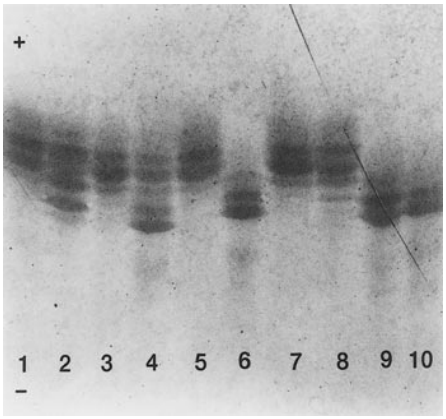

Figure 1. Canide A1AT phenotypes demonstrated by isoelectric focusing and immunoblotting. The bands are the stained immunoprecipitates of alpha 1 antitrypsin. Anode (+) is at the top. The types are 1:M, 2:MS, 3:M, 4:MS, 5:M, 6:S, 7:M, 8:M, 9:S, 1O:S. Samples in the following lanes are from the various canides: 1–3: gray wolf, 4–6: domestic dog; 7 and 8: Mexican wolf; 9: coyote; 10: red wolf.

and Pi^s for the common and the slower electrophoretic type, respectively. The phenotypes (band patterns) are called PiM, PiS, and PiMS.

Canine A1AT was isolated as described by Abrams et al. (1978) except that preparative electrophoresis (Muller-Eberhard 1960) was used as the final purification step following chromatography on Sephacryl S-200. Isoelectric focusing and immunoblotting were performed as described by Kueppers et al. (1993). Radial immunodiffusion for quantitation of A1AT in serum using isolated dog A1AT as standard was performed according to Mancini et al. (1965). Antiserum to canine A1AT was raised in New Zealand white rabbits. A high-titered antiserum was obtained by an initial injection of 1.0 mg of purified canine A1AT with Freund's adjuvent and booster injections of 0.4 mg administered 2 and 5 weeks following the initial injection.

Differences among allele frequencies were calculated by Fisher's exact test. The fit to Hardy–Weinberg equilibrium was calculated according to Haldane (1954).

Results

Three phenotypic band patterns were observed (M, MS, S) and were identical to those previously observed in domestic dogs (Figure 1). The prevalence of the three patterns in the six groups are shown in Table 1. The previously published frequencies in domestic dogs are included for comparison. Gray wolves and domestic dogs had similar ($P = .11$) allele frequencies (Pi^s: 0.33 and 0.28, respectively), while Mexican wolves differed significantly ($P = .014$) from both with an allele frequency of 0.1 for Pi^s.

Table 1. A1AT (Pi) phenotypes

Group	*n*	M	MS	S
Domestic dog	71	40 (56%)	22 (31%)	9 (13%)
Gray wolf	29	11 (38%)	17 (59%)	1 (3%)
Mexican wolf	20	16 (80%)	4 (20%)	
Red wolf	27			27 (100%)
Coyote	24			24 (100%)
Wolf-dog cross	9	4 (44%)	5 (56%)	

There was a significant ($P < .01$) deviation from Hardy–Weinberg equilibrium for the gray wolves. Domestic dogs and Mexican wolves did not show a significant deviation from equilibrium.

Coyotes and red wolves were found to be monomorphic for the Pi^s allele and were indistinguishable from each other in that respect. PiS bands of domestic dogs, wolves, red wolves, and coyotes were indistinguishable, and operationally we assume them to be identical.

The results of quantitative determination of A1AT by radial immunodiffusion are given in Table 2. There was a tendency for the females in all groups to have higher A1AT levels than males. This difference was statistically significant ($P < .01$ by paired *t* test).

Discussion

Gray wolves and domestic dogs were similar in allele frequencies. However, there was significant deviation from Hardy–Weinberg equilibrium for the gray wolves. A likely reason is that these animals, due to their different origins—some captive and some wild—do not fulfill the criteria for a random breeding population.

The domestic dog is an extremely close genetic relative of the wolf (Wayne 1993) and most authorities now consider the domestic dog a member of *lupus.* According to the fossil record, it is thought that dogs were domesticated from wolves approximately 12,000–14,000 years ago (Olsen 1985). However, Vila et al. (1997) recently suggested from the results of mtDNA control region sequence analysis that domestication may have occurred as much as 100,000–135,000 years ago, much earlier than previously thought. All available DNA sequences support the notion that only wolves were the ancestors of the domestic dog (for a recent review see Wayne and Ostrander 1999). According to the fossil record, it is thought that coyotes and wolves diverged approximately 1 million years ago (Nowak 1979). It is of interest that allele frequencies in the Mexican wolf differed significantly from those of the northern gray wolf and domestic dogs ($P = .014$), possibly due in part to the geographic separation of northern and southern wolf populations.

All animals in our red wolf group were monomorphic for the Pi^s allele, as were the coyotes. There has been much recent debate on the origin of the red wolf. The red wolf population has undergone a major contraction, initially due to hunting and habitat encroachment and then due to a captive breeding program. All red wolves presently in existence are direct descendants of the 13 founders of the captive breeding program (Waddell and Behrns 1996). Small populations that go through major contractions often are homozygous at multiple loci (Li 1955). Which allele eventually is fixed is unpredictable.

Extensive hybridization with coyotes is known to have taken place (Hill et al. 1987; Roy et al. 1996; Wayne and Jenks 1991). It is therefore impossible to decide whether the Pi^s allele was present in the original ancestral population or is there simply due to recent admixture from coyotes.

The quantitative determination of A1AT shows a similar range of concentrations for all groups tested. The higher concentrations of serum A1AT in females suggests the possibility of hormonal regulation by estrogens. Hughes et al. (1995) demonstrated that this difference disappears after ovariectomy of female domestic dogs.

In summary, our data show that the A1AT polymorphism previously demon-

Table 2. Concentrations of A1AT (in mg/ml) ± standard deviation

Group	Male	Female	Combined
Domestic dog	2.19 ± 0.38	2.65 ± 0.42	2.42 ± 0.41
Gray wolf	2.14 ± 0.18	2.34 ± 0.35	2.26 ± 0.30
Wolf-dog cross	2.28 ± 0.21	2.45 ± 0.34	2.37 ± 0.32
Mexican wolf	2.70 ± 0.15	3.06 ± 0.11	2.80 ± 0.22
Red wolf	2.70 ± 0.40	3.04 ± 0.32	2.88 ± 0.42
Coyote	2.66 ± 0.40	2.88 ± 0.38	2.72 ± 0.43

strated in domestic dogs is also present in gray wolves and that allele frequencies are statistically similar. Mexican wolves are also polymorphic, although the significantly lower Pi^S frequency suggests that they represent a separate population. Red wolves and coyotes are monomorphic for Pi^S. It is as yet undecided if this is due to extensive hybridization or results from common ancestry. Comparison of A1AT concentrations demonstrates the similarity of all animals tested. In addition, the quantitative dimorphism of males and females, known to be present in domestic dogs, was also found in all canids presently tested.

From the Patuxent Wildlife Research Center, Laurel, Maryland (Federoff) and Temple University School of Medicine, Pulmonary Disease Section, 3401 N. Broad St., Philadelphia, PA 19140 (Kueppers). The authors would like to thank the following individuals and institutions for providing blood samples: L. D. Mech (free-ranging Minnesota wolves); F. Knowlton and M. Roetto (Utah coyotes); M. Bush (National Zoo, Washington, DC); N. Reindl (Minnesota Zoo); M. Phillips, V. G. Henry, and J. Gilbreath (U.S. Fish and Wildlife Service red wolf recovery project); W. Waddell and S. Behrns (Point Defiance Zoo and Aquarium, Tacoma, WA); J. Davis (U.S. AWA); S. Johnston and D. Johnston; R. Stubbe; S. Williams and P. Ferrari; S. Lindsey (WCSRC, Eureka, MO); P. Siminski (AZ-Sonora Desert Museum); B. Snyder (Rio Grande Zoo, NM); R. Wack (Columbus Zoo, OH); T. Becker (Zoo America, PA); and S. Fain (U.S. Fish and Wildlife Service National Forensics Laboratory). N. E. Federoff is currently at the U.S. EPA, 401 M St. SW, Washington, DC.

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Received September 1, 1998
Accepted October 24, 1999

Corresponding Editor: Robert Wayne