Genetic Analysis of a Short-Petiolule-Type Soybean, LN89-3502TP

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An abnormal-leaf soybean [*Glycine max* (L.) Merr.] plant was observed in an $F_{4:8}$ line at Urbana, Illinois, in the summer of 1992. Petiolules of the plant were shorter than normal and leaflet margins curled uniformly upward forming a cupped-shaped leaf. All progeny of the single plant exhibited leaf cupping. Laboratory analysis showed an absence of soybean mosaic and tobacco ringspot virus in the plants. Seeds from the progeny were bulked and designated line LN89-3502TP. Further observation of LN89-3502TP revealed dense pubescence on the short petiolule plants. The objective of this study was to determine the inheritance of the short petiolule trait of LN89-3502TP. In F_2 populations derived from LN89-3502TP crossed with normal leaf-type cultivars, three petiolule phenotypes (short, intermediate, and normal) segregated in a 1:2:1 ratio. The 1:2:1 ratio was confirmed in the $F_{2:3}$ families. These ratios indicate the short petiolule trait is controlled by a single gene showing incomplete dominance that we designated *lc*.

Genetically controlled abnormalities of soybean petioles and leaves have been documented. Kilen (1983) published the inheritance of a short petiole-type soybean conditioned by a single recessive gene, *lps*. Rode and Bernard (1975a,b) concluded two leaf mutants, wavy and bullate, are each controlled by two recessive genes, lw_1, lw_2 and lb_1, lb_2, respectively. Tharp et al. (1994) determined that a sinuate leaf type was conditioned by two recessive genes.

In the summer of 1992, an abnormal-leaf plant, not resembling any previously documented plant type, was observed at Urbana, Illinois, in an $F_{4:8}$ line derived from the cross Hobbit 87 (Cooper et al. 1991) × Asgrow 3205. Petiolules, which connect the leaflets to the petiole, were shorter than normal and the leaflet margins curled uniformly upward forming a cup-shaped leaf. All progeny of the single plant exhibited the short petiolule trait and leaf cupping. Also, dense pubescence was observed on all plants. Seeds from the progeny were bulked and designated LN89-3502TP. Leaf cupping is apparent on the first trifoliolate and every subsequent leaf until maturity.

An enzyme-linked immunosorbent assay (ELISA) test (Hancock and Evan 1992) performed in the laboratory of Dr. Glen Hartman, USDA Plant Pathologist at the University of Illinois, showed an absence of soybean mosaic or tobacco ringspot virus. The absence of virus and the uniformity of the phenotype suggested that short petiolules and leaf cupping are under genetic control.

The objective of this study was to determine the inheritance of the short-petiolule trait of LN89-3502TP.

Materials and Methods

In 1994, LN89-3502TP was crossed with four normal-leaf type cultivars, LN89-5322-2 (Stephens and Nickell 1992), LN89-5699 (Nickell et al. 1994), Hartwig (Anand 1992), and Thorne (McBlain et al. 1993). The reciprocal cross also was made with Thorne. Six F_1 seeds from each cross were planted in 1995. Seeds from each F_1 plant were harvested in bulk. In 1996, F_2 populations of each cross were grown in two 3.5 m rows spaced 76 cm apart with a seeding rate of approximately 100 seeds per row.

Terminal petiolule lengths of the short-petiolule type, LN89-3502TP, ranged from 5 to 15 mm, with a mean of 9 mm. Mean terminal petiolule lengths were 38, 34, 40, and 38 mm for normal-leaf types, LN89-5322-2, LN89-5699, Hartwig, and Thorne, respectively. In the F_2 populations, three distinct leaf types were observed. Plants exhibiting the phenotype of the parent, LN89-3502TP (petiolule lengths less than 15 mm and cupped leaflets), were classified as short. Plants exhibiting the phenotype of the normal-leaf cultivars (petiolule lengths greater than 30 mm and normal leaflets) were classified as normal. Plants having petiolule lengths between 16 and 29 mm and semicupped leaflets were classified as intermediate. Classification of F_2 plants was made from growth stages V4 through R3 (Ritchie et al. 1994).

Each F_2 plant was harvested individually. In 1997, seed from 40 randomly selected F_2 plants from each cross were grown as $F_{2:3}$ families in rows 1.5 m long spaced 76 cm apart with a seeding rate of approximately 60 seeds per row. Each row was classified by phenotype as short, intermediate (segregating), or normal. Individual plants were classified and counted within segregating $F_{2:3}$ families. Chi-square tests were used to measure the goodness-of-fit for expected genetic ratios at the .05 probability level.

Results and Discussion

All F_1 plants exhibited leaf cupping and shortened petiolule length, but not as pronounced as the parental short petiolule line. This suggested incomplete dominance for the gene controlling short petiolule. All F_1 plants had dense pubescence, which suggested dominance for pubescence type. Segregation in the F_2 populations fit a 1:2:1 ratio for short, intermediate (heterozygous), and normal length petiolules (Table 1). Short-petiolule plants could be easily identified at the V4 growth stage. Distinction between intermediate and normal-leaf types was difficult prior to the R1 growth stage. Terminal petiolule lengths of plants classified as short and normal were similar to parental types. Terminal petiolule lengths of intermediate leaf plants ranged from 20 to 28 mm in length. Chi-square tests for each population fit a 1:2:1 ratio (Table 1). Nearly identical chi-square values for the two reciprocal Thorne crosses indicate the short petiolule trait is not maternally inherited.

The petiolule classification of the 40 randomly selected F_2 plants and the $F_{2:3}$ families also fit a 1:2:1 ratio (Table 1). All randomly selected short or normal F_2 plants produced short and normal $F_{2:3}$ families. Additional $F_{2:3}$ families classified as short or normal were produced by misclassified intermediate F_2 plants. A total of 12 of the 200 randomly selected F_2 plants were misclassified. The majority, 9 of the 12, were normal plants misclassified as intermediate. The misclassification of plants can be attributed to environmental factors causing some plants within F_2 rows to be less developed than others and therefore more difficult to correctly classify. Plants classified within heterozygous $F_{2:3}$ rows also fit a 1:2:1 ratio for short, intermediate, and normal petiolule length. Data from the $F_{2:3}$ families confirm that the short petiolule trait is controlled by a single incompletely dominant gene. When short and intermediate (heterozygous plants) petiolule classes are combined, there is a good fit to a 3 (short and intermediate) : 1 (normal) ratio which further supports single-gene conditioning of the short petiolule trait. We propose the genotype of short petiolule plants for leaf type is *lclc* and the genotype for the intermediate (heterozygous) individuals is *Lclc*.

Cupped, intermediate, and normal-leaf types were directly correlated with the

Table 1. Classification of soybean plants by leaf type derived from crosses of LN89-3502TP and four normal leaf type cultivars

Cross	Generation	Leaf type			χ^2 (1:2:1)	*P*
		S[a]	I[b]	N[c]		
LN89-5322-2 × LN89-3502TP	F_2	30	71	32	0.67	.72
	Ran[d]	9	21	10	0.15	.93
	$F_{2:3}$[e]	12	18	10	0.60	.74
LN89-5699 × LN89-3502TP	F_2	33	76	39	0.59	.74
	Ran	10	21	9	0.15	.93
	$F_{2:3}$	10	19	11	0.15	.93
Thorne × LN89-3502TP	F_2	43	115	50	2.80	.25
	Ran	7	22	11	1.20	.55
	$F_{2:3}$	7	19	14	2.55	.28
LN89-3502TP × Thorne	F_2	52	116	44	2.49	.29
	Ran	9	22	9	0.40	.82
	$F_{2:3}$	9	22	9	0.40	.82
LN89-3502TP × Hartwig	F_2	21	66	37	4.65	.10
	Ran	8	21	11	0.55	.76
	$F_{2:3}$[f]	8	17	14	2.49	.29

[a] Short (petiolule lengths less than 15 mm and cupped leaflets).

[b] Intermediate (petiolule lengths between 16 and 29 mm and semicupped leaflets).

[c] Normal (petiolule lengths greater than 30 mm and normal leaflets).

[d] Leaf type classification of 40 randomly selected F_2 plants.

[e] Actual leaf type of $F_{2:3}$ families derived from the 40 randomly selected F_2 plants. $F_{2:3}$ families classified as short or normal were derived from F_2 plants classified the same. Additional $F_{2:3}$ families classified as short or normal were produced by misclassified intermediate F_2 plants. $F_{2:3}$ families classified as I were segregating for leaf type.

[f] One $F_{2:3}$ row was lost due to planting error.

short, intermediate, and normal petiolule lengths. All short petiolule plants had fully cupped leaves, all intermediate plants had intermediately cupped leaves, and all normal petiolule plants had normal leaves. This indicates a possible pleiotropic effect by the gene *lc*.

The dense pubescence observed on the parental line LN89-3502TP was also present on all plants classified as short or intermediate in the F_2 populations and $F_{2:3}$ families. All plants classified as normal had normal pubescence. Trichrome counts of LN89-3502TP were not significantly different from counts of an isoline having the *Pd1* gene for dense pubescence. This may indicate a close linkage between the genes *lc* for short petiolule and *Pd1* for dense pubescence. However, the expected recombination of a short petiolule plant with normal pubescence was not observed in 5,000 plants observed in the F_2 populations and $F_{2:3}$ families. We are in the process of doing an allelism test with LN89-3502TP and an isoline having *Pd1*.

The results of this study demonstrate the short petiolule trait of LN89-3502TP is controlled by a single gene showing incomplete dominance that we designate *lc*. However, the association of dense pubescence and cupped leaves with the short petiolule trait is not fully understood and provides an area for further study.

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References

Anand SC, 1992. Registration of 'Hartwig' soybean. Crop Sci 32:1069–1070.

Cooper RL, Martin RJ, Schmitthenner AF, McBlain BA, Fioritto RJ, St. Martin SK, and Calip-DuBois A, 1991. Registration of 'Hobbit 87' soybean. Crop Sci 31:1093.

Hancock DC and Evan GI, 1992. Production and characterization of antibodies against synthetic peptides. In: Methods in molecular biology, immunochemical protocols (Manson MM, ed). Totwa, New Jersey: Humana Press; 33–41.

Kilen TC, 1983. Inheritance of a short petiole trait in soybean. Crop Sci 23:1208–1210.

McBlain BA, Fioritto RJ, St. Martin SK, Calip-DuBois A, Schmitthenner AF, Cooper RL, and Martin RJ, 1993. Registration of 'Thorne' soybean. Crop Sci 33:1406.

Nickell CD, Noel GR, Bernard RL, Thomas DJ, and Frey K, 1994. Registration of soybean germplasm line LN89–5699, resistant to soybean cyst nematode. Crop Sci 34: 1133–1134.

Ritchie W, Hanway JJ, Thompson HE, and Benson GO, 1994. How a soybean plant develops. ISU/CES Special Report no. 53. Ames, Iowa; 12–14.

Rode MW and Bernard RL, 1975a. Inheritance of bullate leaf. Soybean Genet Newslett 2:44–46.

Rode MW and Bernard RL, 1975b. Inheritance of wavy leaf. Soybean Genet Newslett 2:42–44.

Stephens PA and Nickell CD, 1992. Inheritance of pink flower color in soybean. Crop Sci 32:1131–1132.

Tharp JE Jr, Nickell CD, and Lohnes DG. 1994. Agronomic evaluation of a wavy type soybean. In: Agronomy abstracts. Madison, Wisconsin; 106.

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Isolation of Microsatellite Loci from a Social Lizard, *Egernia stokesii,* Using a Modified Enrichment Procedure

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We report a modified microsatellite enrichment technique that was used to isolate tetranucleotide (AAAG) repeat loci from a group living Australian lizard, *Egernia stokesii.* The enrichment method is based on magnetic/biotin capture of repetitive sequences from restricted genomic DNA. The technique can be performed rapidly and recovers microsatellite loci with both flanking sequences intact. Twenty unique microsatellite loci (16.7% of white colonies screened) containing 10 or more tetranucleotide repeats were identified. Eleven loci were further analyzed in 10 unrelated individuals and had heterozygosities ranging from 0 to 90%. These loci will be used to investigate genetic relationships within and among crevice homesites of *E. stokesii* and also genetic differentiation among *E. stokesii* populations. At least four of the microsatellite loci can be amplified in related taxa, including other *Egernia* species and the closely related genera *Corucia, Cyclodomorphus,* and *Tiliqua.*

Various forms of complex social organization have been described in mammals, birds, and insects, while, until recently, social organization in lizards has been considered relatively simple (Bull 1994). A number of lizard species are known to form aggregations, but with the exception of mating, these are thought to result from mutual attraction to environmental features rather than any particular social function (Graves and Duvall 1995). However, in several species of the Australian skink genus *Egernia,* social aggregations are found consistently, regardless of the time of year, and consequently have been described as families or colonies (Hutchinson 1993). In the gidgee skink (*E. stokesii*), for example, individuals form temporally stable groups in crevice refuges and show evidence for parent-offspring recognition (Duffield G, personal communication; Main and Bull 1996).

Central to a thorough understanding of this observed sociality is knowledge of the genetic relationships within and among supposed family groups. We have chosen

to use microsatellite markers to assess these relationships in the communal *E. stokesii*. Use of microsatellite loci has become widespread in studies of relatedness, paternity, and population structure due to their abundance in eukaryotic genomes, high polymorphism, and amenability to polymerase chain reaction (PCR) technology (Queller et al. 1993). We chose to isolate tetranucleotide microsatellite loci because they can be scored less ambiguously and are less likely to suffer from slippage errors than the more commonly used dinucleotide repeats (Schlötterer and Tautz 1992). In addition, tetranucleotide loci of the form $(AAAG)_n$ are known to exist in a related skink species (*Tiliqua rugosa*) and have been PCR amplified in *E. stokesii* (Cooper et al. 1997).

Tetranucleotide repeat loci are often less abundant in the genome than dinucleotide repeat loci (Queller et al. 1993) and as such can be difficult to isolate using traditional cloning and colony hybridization techniques. The use of an enrichment technique can alleviate this problem and several methods are available (e.g., Armour et al. 1994; Kandpal et al. 1994; Kijas et al. 1994; Ostander et al. 1992; Refseth et al. 1997). We originally tried a recent technique developed by Refseth et al. (1997) which is similar to existing methods by Kandpal et al. (1994) and to a lesser extent Kijas et al. (1994). The Refseth et al. method eliminates the need for constructing a genomic library prior to microsatellite enrichment, and involves simple hybridization and cloning procedures which can be completed in several days. However, when we followed this procedure all of the clones containing microsatellites had only one flanking region and a single linker, a problem we also observed in a high proportion of clones isolated by Refseth et al. (1997). Recently, and subsequent to the research we report here, Li et al. (1997) published an enrichment procedure that is similar to that described by Refseth et al. (1997) but resulted in low cloning efficiencies. In this article we report a modification of the Refseth and Kandpal methods to isolate tetranucleotide repeat loci from the lizard *E. stokesii*. We show that this enrichment technique isolates microsatellite sequences with both flanking regions intact. We also report a simple PCR-based microsatellite screening technique as an alternative to traditional colony hybridization approaches.

Materials and Methods

Samples and Oligonucleotides Used

Seven unrelated individuals used for microsatellite isolation were obtained from different geographic locations over the range of *E. stokesii* in Australia. Another 10 unrelated individuals and two mother/offspring groups (litters of four and five), used for characterization of loci, were obtained from sites around Hawker, near the Flinders Ranges, in South Australia. Other samples used in the cross-species amplification tests were obtained from the South Australian Museum. Collection location details for all specimens are available upon request to the authors. The following oligonucleotides were synthesized for use in the enrichment procedure: linker oligo A (S61): 5′-GGC CAGAG ACCCC AAGC TTCG-3′ (Refseth et al. 1997); linker oligo B (S62): 5′-GATCC GAAGCTT GGGGTCT CTGGCC-3′ (Refseth et al. 1997); vector oligo (S4): 5′-TAATACGACTCACTATA GGG-3′ (T7 promoter, Promega protocols); vector oligo (S15): 5′-TCA CACAGG AAACAG CTAT GAC-3′ (M13 reverse sequencing primer, Promega protocols); biotinylated oligo (S64): 5′-$(AAAG)_6$GCAC[Biotin]A-3′; microsatellite oligo(S6): 5′-$(AAAG)_6$-3′.

An approximate time in days is given for each of the methods described below. For a schematic representation of the strategy used to isolate microsatellites directly from genomic DNA we refer the reader to Figure 1 in Refseth et al. (1997).

Generation of a Phosphorylated Adapter and Ligation to *Sau*3A Cut Genomic DNA (Refseth et al. 1997 and Wu et al. 1987 with minor modifications) (3–4 days)

Total DNA was extracted from frozen liver tissue of the seven unrelated *E. stokesii* individuals using a standard phenol/chloroform extraction procedure with RNase (20 μg/ml) digestion (Sambrook et al. 1989). The DNA samples were pooled and 5 μg digested in a total volume of 20 μl with 5 units of the restriction endonuclease *Sau*3A (Biolabs) at 37°C for 3 h, followed by heat inactivation of the *Sau*3A at 65°C for 30 min.

The oligonucleotide S62 (1.5 nmol) was heat denatured at 90°C for 2 min, quickly chilled on ice, and phosphorylated by incubation at 37°C for 1 h in a solution containing 1× polynucleotide kinase buffer, 0.05 mM adenosine triphosphate, and 12 units T4 polynucleotide kinase enzyme (Promega) in a total volume of 25 μl. This solution was heated to 80°C for 5 min to inactivate the kinase. To generate an adapter, the solution was then hybridized to an equal amount (1.5 nmol) of S61 in a heating block set at 80°C and removed to room temperature to allow the mixture to cool slowly over 1 h.

Sau3A cut *E. stokesii* DNA (5 μg) was ligated to 0.9 nmol of the adapter in 1× DNA ligase buffer containing 40 units T4 DNA ligase (Promega) in a total volume of 200 μl at room temperature. This reaction mix was cooled slowly by placing the tube in 1 L of room temperature water at 4°C overnight. The DNA was ethanol precipitated, resuspended in 20 μl TE and electrophoresed on a 2.5 % NuSieve GTG (FMC Bioproducts) agarose gel. DNA in the size range 300–1,000 bp was excised, gel purified (BresaClean kit, Bresatec), and eluted with 50 μl of water.

Magnetic Isolation of AAAG Microsatellites (modified from Kijas et al. 1994) (0.5–1 day)

In order to isolate AAAG repeat microsatellite loci we synthesized an oligonucleotide with six AAAG repeats and a six-base noncomplementary region with biotin at the second base from the 3′ end (S64). The noncomplementary region prevents incompletely synthesized oligonucleotides and biotinylated oligonucleotides, copurified with target DNA during magnetic isolation, acting as primers in subsequent PCR reactions. One hundred microliters of Streptavidin MagneSphere® Para-Magnetic particles (PROMEGA) were resuspended and washed as per manufacturers recommendations and resuspended in 100 μl 5× SSC (1× SSC = 0.15 M NaCl, 15 mM trisodium citrate) containing 200 pmol of the biotinylated oligo S64.

This bead mixture was incubated for 15 min at room temperature then washed three times in 5× SSC and resuspended in 50 μl of 1× hybridization solution (0.5 M NaCl, 4% w/v polyethylene glycol 8000) at 55°C. In a separate tube, 10 μl of the ligated DNA/adapter solution was added to 40 μl of 1× hybridization solution which includes 20 pmol of S61. The S61 oligo is included to block terminal priming sites to limit the formation of concatamers (see Kijas et al. 1994). This solution was heat denatured at 95°C for 5 min and cooled to 55°C before adding all the resuspended bead mixture and incubated for 20 min at 55°C. The beads were then washed four times (in 100 μl of 2× SSC, 10 pmol S61) at room temperature and then washed four times in 100 μl 1× SSC, 10 pmol S61 at 30°C to remove unbound DNA frag-

Table 1. ***Egernia stokesii*** **microsatellite loci isolated in this study**

Locus name	Primer sequences (5′–3′)	GenBank numbers	(Repeat unit)$_n$ in clone	Number of alleles[b]	H_o[b] (%)	Product size (bp)	Annealing temperature (°C)
EST1	GCATTCTGTATTTTAGTGGTTC GTAGACAACACAGCCATCTCA	AF069696	$(AAAG)_{32}$	7	80	234–266	55
EST2	CAGTGAAACTTTGAGTGTGAAG CCTGAGCTAGCATGACTATTCT	AF069697	$(AAAG)_{28}$	9	80	205–257	52
EST3	CCAGCATAAAGCAAGAACTG CATTTATCCATCTGTCACATTG	AF069698	$(GATG)_9$ $(AAAG)_{17}$	10	90	280–332	60
EST4	ATCCAATACTCTGGCTTCCTA GCAAAGGGATTTACTTCTAAG	AF069699	$(AAAG)_{18}$	11	90	146–190	60
EST6	TCTTGTAATAGGGCAGTGGTC GAGCACACCAGATAACCTCA	AF069700	$(AAAG)_{13}$	8	80	163–189	52
EST8	GCTTATGTTCTCATGGAGCA GCCCTCTGCCTCTATCTCTC	AF069701	$(AAAG)_{12}$	8	80	101–141	60
EST9	CACTGGGGGGAGAATGTTAGA AACTCGCAGCACCAGGAGG	AF069702	$(AAAG)_{10}$ $(AGAG)_1$ $(AAAG)_9$	9	60	235–263	54
EST12	CAGGATTGCATGTTGGTCT CATTACACTTCTGCTTGATGTG	AF069703	$(AAAG)_{20}$	8	70	296–332	55
EST14	CTGGCATTCTGACATAATCT GTAATAGGGCAGTGGTCTTC	AF069705	$(AAAG)_{13}$	7	0[a]	114–178	55
EST15	CTCTTAGAACAGTGTAAAGCAGC GCATGAAAACTGGTGACTGC	AF069706	$(AAAG)_{17}$	2	10	129–141	55
EST16	CATGTGACTTGGTCCACTGT ATTGATTTTCCAGCATGAC	AF069707	$(AAAG)_{23}$	5	40[a]	156–184	55

[a] May contain nonamplifying alleles.

[b] Number of alleles and heterozygosity (H_0) values were determined from 10 individuals.

ments. The captured DNA fragments were eluted from the beads by denaturing for 20 min at room in 20 μl 0.15 M NaOH. The solution was neutralized with 1.3 μl 1.25 M acetic acid, and 2.2 μl 10× TE (pH 8.0) and DNA was purified using a Qiagen column (QIAquick PCR purification kit, QIAGEN) and eluted in 50 μl of 10 mM Tris-HCl (pH 8.0).

PCR Amplification of Captured Fragments and Cloning into *pGEM* T Vector (2–3 days)

The captured DNA (~280 ng) was used as template in a 50 μl volume containing 1× PCR buffer (Promega), 4 mM $MgCl_2$, 0.2 mM of each dNTP, and 30 pmol of S61. The reaction was hotstarted by addition of 1 μl of a solution containing 0.7 units *Taq* polymerase (Promega) in 1× PCR buffer after incubation of reaction mix at 94°C for 1 min. The amplification was carried out in a Corbett FTS 320 thermal cycler with one cycle of denaturing at 94°C for 3 min, annealing for 45 s at 60°C, extension for 1 min at 72°C followed by 39 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s, ending in one cycle of 72°C for 5 min. The PCR product was purified using glass milk (BresaClean kit, Bresatec), eluted in 20 μl of H_2O, and cloned into *pGEM* T vector (Promega) following the manufacturers recommendations. Recombinant clones were detected using blue/white screening (Sambrook et al. 1989).

Detection of Microsatellite-Containing Clones Using PCR (2–3 days)

White colonies were transferred to tubes containing 20 μl of 10 mM Tris-HCl pH 8.5 and incubated for 10 min at 95°C, and 0.5 μl was used as a template in PCR amplifications with two vector primers (S4 and S15) and the nonbiotin-labeled $(AAAG)_6$ primer (S6). The PCR reactions were performed using a hotstart approach in a total volume of 10 μl with 1× buffer (Amplitaq Gold buffer from Perkin Elmer), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 2 pmol of each primer, and 0.25 U of Amplitaq Gold. The reaction conditions were one cycle of 9 min at 95°C, 45 s at 60°C, 2 min at 72°C; 34 cycles of 45 s at 94°C, 45 s at 60°C and 2 min at 72°C; followed by 1 cycle of 5 min at 72°C. The products were visualized by agarose gel (1.5%) electrophoresis. Clones giving two (or more) bands were considered likely to contain a microsatellite and a 0.5 μl volume of these colonies lysates were PCR amplified with the two vector primers (S4 and S15) using similar conditions to those given above, except reactions were performed in a total of 50 μl with 30 cycles and 1 min extensions at 72°C. The products were purified with glass milk (BresaClean kit, Bresatec) and eluted in 20 μl of H_2O. Both strands of this product were cycle sequenced using 5 pmol of either primer (S4 or S16) and ABI Prism™ (Perkin Elmer), on a Corbett FTS1 thermal sequencer with procedures specified by the manufacturer. DNA sequences were determined using an ABI 373A autosequencer. Primers for PCR amplification of each microsatellite locus were designed using the program OLIGO (National Biosciences).

Assessment of Variation in Microsatellite Loci

For microsatellite analyses of unrelated individuals and mother/offspring groups, DNA was extracted from whole blood using Chelex 100 (Walsh et al. 1991). PCR amplifications were carried out in 10 μl reaction volumes in a Hybaid (OMN-E) thermocycler with similar conditions as given above for amplification of colony lysates, except that one of the primers was end-labeled with γ-^{33}P-ATP and annealing temperatures were optimized for each locus (see Table 1). The products were electrophoresed on standard sequencing gels (6% acrylamide, 8 M urea, in 1× TBE) and visualized by autoradiography on X-ray film (Fuji).

Results and Discussion

After cloning PCR-amplified DNA enriched for tetranucleotide repeat microsatellites, 303 white colonies were obtained. Using the PCR-based technique, 120 white colonies were screened and 34 clones (28%) were identified by the presence of two or more strong bands on an agarose gel to potentially contain AAAG repeat motifs.

The two bands are likely to correspond to products amplified using the two vector primers and a vector primer with the microsatellite primer. Additional bands are possibly heteroduplexes of these two fragments or may indicate extra microsatellites in the clone. The 34 clones were sequenced and 25 clones (21% of colonies screened) contained tetranucleotide microsatellites with a minimum of 10 repeats. Of the nine remaining clones, four had small imperfect repeats, two did not contain an obvious microsatellite, and three sequences were unreadable. Of the 25 clones containing microsatellites, 20 were unique. Importantly, all microsatellite clones contained both flanking sequences intact. This is likely to have resulted from modification of the biotin-labeled primer incorporating the biotin and a noncomplementary region, at the 3′ end rather than at the 5′ end of the primer.

The enrichment efficiency we report here (16.7% unique, flanking sequences intact, and of length greater than 10 repeats) is similar to that reported by Kijas et al. (1994) (~20%), but lower than that of Li et al. (1997) (35%) and Refseth et al. (1997) (33%). Microsatellite clones from the Kandpal et al. (1994) method were not fully sequenced and therefore their enrichment efficiency could not be directly determined. The Kijas et al. (1994) method involves the construction of genomic libraries prior to the enrichment step and is therefore likely to be more time consuming than the method we report here. The Li et al. (1997) method involved two enrichments and is not directly comparable with our method. However, their low cloning efficiency is likely to have resulted from the majority of their PCR products, prior to cloning, only containing one flanking sequence and a single linker. Their method could be improved by using the biotin-labeled primer reported here. The enrichment efficiency reported by Refseth et al. (1997) is likely to be inflated by an unknown proportion of loci with only one flanking sequence.

Primer pairs were designed for 11 loci (Table 1) and amplification products of expected size were obtained from the appropriate clone for all 11 loci. Amplification products similar to the length of the clone sequence were obtained from a sample of *E. stokesii* DNA for all these loci. Trials on 10 unrelated *E. stokesii* individuals at each of the 11 loci showed heterozygosity levels between 0% and 90% (Table 1). Microsatellite alleles showed Mendelian patterns of segregation through one generation for 10 of these loci by analysis of two mother/offspring families. Two loci (*EST14, EST16*) failed to amplify in all individuals and may contain null alleles (Brookfield 1996).

Several of the loci isolated in *E. stokesii* were trialed to assess their utility in related taxa. Of five loci trialed (*EST2, EST3, EST4, EST8, EST9*), four (except *EST3*) were successfully amplified in closely related species and genera, including *Egernia, Corucia, Cyclodomorphus,* and *Tiliqua* (data not shown). Several other species of *Egernia* exhibit gregarious behavior (Hutchinson 1993) and these loci should be useful for studies of sociality in these species. One related species (*Tiliqua adelaidensis*) is endangered (Hutchinson 1993) and the loci are currently being used to determine population parameters and management units for this species.

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References

Armour JAL, Neumann R, Gobert S, and Jeffreys AJ, 1994. Isolation of human simple repeat loci by hybridisation selection. Hum Mol Genet 3:599–605.

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

Bull CM, 1994. Population dynamics and pair fidelity in sleepy lizards. In: Lizard ecology: historical and experimental perspectives (Vitt LE and Pianka ER, eds). Princeton, New Jersey: Princeton University Press; 159–174.

Cooper SJB, Bull CM, and Gardner MG, 1997. Characterization of microsatellite loci from the socially monogamous lizard *Tiliqua rugosa* using a PCR-based isolation technique. Mol Ecol 6:793–795.

Graves BM and Duvall D, 1995. Aggregation of squamate reptiles associated with gestation, oviposition, and parturition. Herpetol Monogr 9:102–119.

Hutchinson MN, 1993. Family Scincidae. In: Fauna of Australia, vol. 2A, Amphibia and reptilia (Glasby CJ, Ross GJB, and Beesley BL, eds). Canberra: Australian Government Publishing Service; 261–279.

Kandpal RP, Kandpal G, and Weisman SM, 1994. Construction of libraries enriched for sequence repeats and jumping clones, and hybridization selection for region-specific markers. Proc Natl Acad Sci USA 91:88–92.

Kijas JMH, Fowler JCS, Garbett CA, and Thomas MR, 1994. Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16:657–662.

Li SH, Huang YJ, and Brown JL, 1997. Isolation of tetranucleotide microsatellites from the Mexican jay *Aphelocoma ultramarina.* Mol Ecol 6:499–501.

Main AR and Bull CM, 1996. Mother-offspring recognition in two Australian lizards, *Tiliqua rugosa* and *Egernia stokesii.* Anim Behav 52:193–200.

Ostrander EA, Jong PM, Rine J, and Duyk G, 1992. Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. Proc Natl Acad Sci USA 89:3419–3423.

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

Refseth UH, Fangan BM, and Jakobsen KS, 1997. Hybridization capture of microsatellites directly from genomic DNA. Electrophoresis 18:1519–1523.

Sambrook J, Fritsch EF, and Maniatis T, 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schlötterer C and Tautz D, 1992, Slippage synthesis of simple sequence DNA. Nucleic Acids Res 20:211–215.

Walsh PS, Metzger DA, and Higuchi R, 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10: 506–513.

Wu R, Wu T, and Ray A, 1987. Adapters, linkers and methylation. Meth Enzymol 152:343–348.

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Sex-Linked Inheritance of a Cuticular Pigmentation Marker in the Marine Isopod, *Paracerceis sculpta* Holmes (Crustacea: Isopoda: Sphaeromatidae)

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Cuticular pigmentation is highly variable in *Paracerceis sculpta,* a Gulf of California isopod. Individuals bearing the distinctive pattern we call *Str* (*Stripe*) exhibit a longitudinal band of dark pigmentation on the proximal portion of each dorsal body segment and appear "striped" when viewed from above. In field samples collected over a 10 year period, over 90% of all individuals scored as *Str* ($N = 62$) were females ($G = 21.3$, $P < .001$, $N = 9598$). Three generations of laboratory-reared *Str* females, when crossed to unmarked males, yielded 1:1 sex ratios, 98% *Str* daughters (46/47) and no *Str* sons ($N = 56$). Sons from these families never produced *Str* daughters. The sex-limited expression of this cuticular marker in three consecutive generations indicates that sex determination in *P. sculpta* involves female heterogamety (ZW = females, ZZ = males) and that *Str* is W-linked. Our results

are consistent with studies documenting female heterogamety in flabelliferan and oniscoidean isopods, and suggest that chromosomal sex determination may be common within the Isopoda.

Crustacean sex determination mechanisms are varied and diverse (Bull 1983; Hurst 1993). Combinations of allelic and chromosomal sex factors are widespread (review in Ginsburger-Vogel and Charniaux-Cotton 1982), and genetic as well as extrachromosomal factors are known to affect family sex ratios in peracarids (Bull 1983; Heath and Ratford 1990; Hurst 1993; Juchault and Rigaud 1995; Juchault et al. 1992; Legrand et al. 1987; Rigaud and Juchault 1993; Rousset et al. 1992; Shuster and Sassaman 1997). Sex factors appear to be dispersed throughout the genome in certain marine and terrestrial isopods, and may be sensitive to epistatic or environmental variation (Heath and Ratford 1990; Juchault et al. 1992; Sassaman 1978). The sex-limited expression of cuticular pigmentation patterns has been documented in two genera of flabelliferan isopods to date (Legrand et al. 1987).

Cuticular pigmentation markers in isopods appear to be controlled by dominant Mendelian alleles at autosomal loci, which exist at low frequency in natural populations (Heath 1979; Legrand-Hamelin 1976; Shuster 1989). This observation leads to three predictions: most individuals bearing cuticular markers in nature are expected to be heterozygous at the marker locus; both males and females are expected to bear such markers in equal frequency; and marked individuals are expected to produce 1:1 ratios of marked : unmarked progeny when crossed to unmarked individuals.

When cuticular marker loci are located on heterochromosomes influencing sex determination, only members of the heterogametic sex are expected to express the marker. Most studies demonstrating chromosomal sex determination in isopods suggest that females are the heterogametic sex (ZW = females, ZZ = males; Ginsberger-Vogel and Charniaux-Cotton 1982; Juchault and Rigaud 1995; Legrand et al. 1987). In particular, females are heterogametic in *Dynamene bidentata,* a sphaeromatid isopod inhabiting European and north African coasts (Legrand-Hamelin 1976).

Paracerceis sculpta is a sphaeromatid isopod crustacean inhabiting the northern Gulf of California (Figure 1). Inheritance of sex in this species is consistent with female heterogamety (Shuster and Sassaman 1997). However, chromosomal sex determination has not been confirmed due to a lack of known sex-linked markers. In this article we document a sex bias in the expression of a cuticular pigmentation marker (*Str* = striped; Figure 1) in population samples of *P. sculpta* collected over a 10 year period, and we document the inheritance of this marker in three generations of laboratory-reared isopods. Our results indicate that sex determination in *P. sculpta* involves female heterogamety (ZW = females, ZZ = males) and that *Str* is W linked.

Materials and Methods

Field Collections

Isopods were collected from the spongocoels of the intertidal sponge *Leucetta losangelensis* in the northern Gulf of California between 1984 and 1994. All individuals were sexed, measured to the nearest 0.125 mm, and identified by cuticular pigmentation pattern (Shuster 1989). We tabulated these observations by sex and month, summed all observations, and using a *G* test (Sokal and Rohlf 1995), compared the number of individuals of each sex bearing *Str* (striped) with the numbers of individuals that were unmarked or which bore some marker other than *Str* (indicated "+").

Laboratory Experiments

A field-collected, sexually mature female (Shuster 1991) bearing *Str* was crossed with an unmarked α male (Figure 1) from a laboratory lineage (α-1-1) that consistently produces families with 1:1 sex ratios. Individuals from the α-1-1 lineage are homozygous for the Ams^{α} allele at *Ams* (*Alternative mating strategy*), an autosomal locus that controls male maturation rate, male external morphology, and male mating behavior (Shuster and Sassaman 1997). α-1-1 individuals are also homozygous for the Tfr^{1} allele at *Tfr* (*Transformer*), another autosomal locus that causes sex reversal, depending on an individual's genotype at *Ams* and at primary sex determination loci (Shuster and Sassaman 1997). Since Ams^{α} and Tfr^{1} alleles do not interact, the use of $Ams^{\alpha}Ams^{\alpha}$,$Tfr^{1}Tfr^{1}$ sires in this and in subsequent crosses (see below) minimized the possibility of sex-ratio distortion within families. Progeny were separated from the female at parturition, placed into individual, sterilized glass petri dishes, and reared to maturity at 24°C on coralline algae (*Amphiroa* sp.) and brine shrimp flakes, with seawater changes every 4 days, as described in Shuster and Sassaman (1997).

Five of the F_1 females were crossed to unmarked α-1-1 sires, and the F_2 generation was reared to maturity as described above. Five F_2 females were crossed to unmarked α-1-1 sires, three F_2 males were crossed to unmarked α-1-1 females, and the F_3 generation was reared to maturity as well. For each generation, all *Str* individuals were recorded at birth as well as at maturity, when all surviving individuals were measured and sexed.

We investigated Mendelian inheritance of *Str* by comparing the frequency of marked and unmarked individuals at birth and at maturity, within and among families. In all comparisons we expected 1:1 ratios of marked : unmarked individuals. We also investigated the inheritance of family sex ratio by comparing the number of male and female individuals within and among families. Under chromosomal sex determination, we expected 1:1 sex ratios in all families. All comparisons were performed using heterogeneity *G* tests (Sokal and Rohlf 1995).

We investigated the association of *Str* and sex by pooling all laboratory-reared adult males and females into *Str* and unmarked (+) classes. If *Str* and sex were unlinked, we expected equal frequencies of *Str* and unmarked individuals in both sexes. We tested the deviation from this expectation using an exact chi-square test (Toquenaga Y, personal communication). Lastly, we investigated the possible transmission of *Str* through male lineages by examining the number of *Str* individuals produced when sons of *Str* females were crossed to unmarked α-1-1 females.

Results and Discussion

Field Collections

In monthly samples collected over a 10 year period, the frequency of *Str* in the northern Gulf of California *P. sculpta* population never exceeded 8% within 1 month, and was less than 0.02 overall (mean ± 95% CI = 0.015 ± 0.006, N = 9598). These results are consistent with cuticular pigmentation markers reported in other isopod species (Heath 1979). Over 90% of all *Str* individuals collected were female (0.903; N = 62; G = 21.3, $P <$ .001, N = 9598), indicating a significant sex bias in the expression of *Str* in nature. Similar sex biases in cuticular marker expression are reported in *Idotea* and *Dynamene* (Legrand et al. 1987).

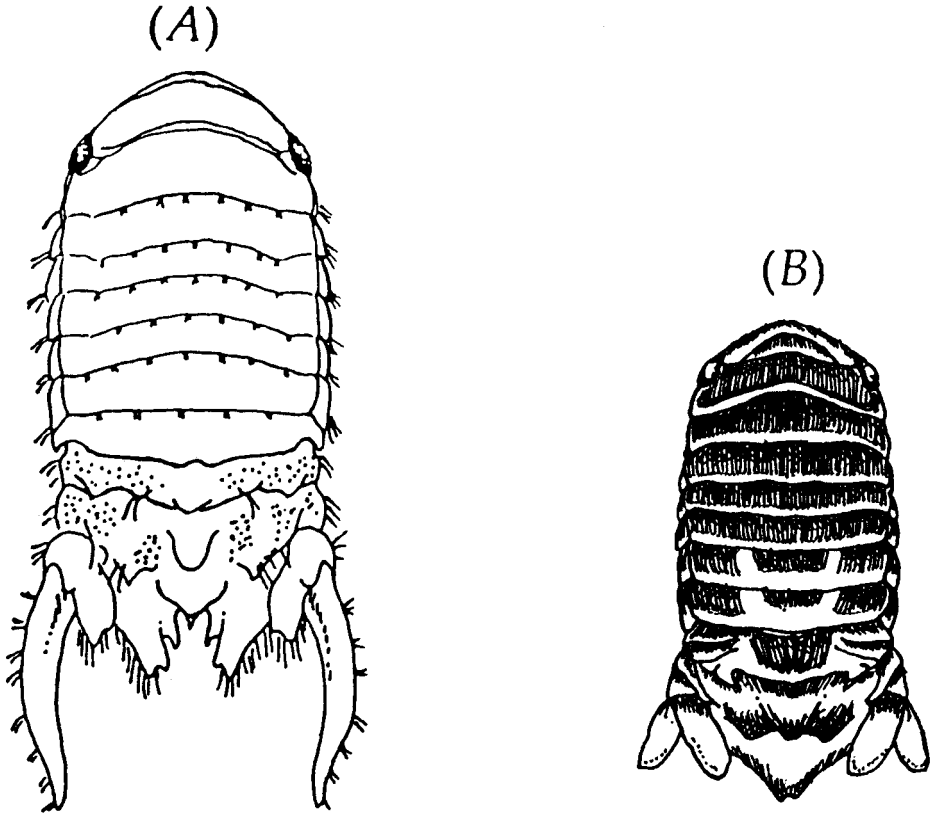

Figure 1. **(A)** Male and **(B)** female *Paracerceis sculpta*; the pigmentation pattern on the female represents *Str* (striped); redrawn after Shuster 1991).

Laboratory Experiments

In the three generations in which *Str* females were crossed to unmarked males, *Str* showed Mendelian inheritance at birth, within and among all families ($\Sigma G_{i[df=11]} = 8.93$, $P > .50$; $G_{Pooled[df=1]} = 1.26$, $P > .20$; $G_{Heterogeneity[df=10]} = 7.67$, $P > .50$; Table 1). Among adults, both *Str* and family sex ratio showed Mendelian inheritance within and among all families ($\Sigma G_{i[df=11]} = 7.75$, $P > .70$; $G_{Pooled[df=1]} = 0.79$, $P > .50$; $G_{Heterogeneity[df=10]} = 6.96$ and 6.57, $P > .70$, respectively; Table 1). In all but one case, in the F_3 generation, all females expressed *Str,* whereas all males were unmarked, indicating close linkage between *Str* and sex (Exact chi-square probability = 2.55×10^{-19}, $N = 103$). Unmarked F_2 males ($N = 3$) crossed to unmarked females produced no *Str* progeny of either sex ($N = 44$), indicating that *Str* is not transmitted through male lineages. These results are consistent with Mendelian inheritance of *Str* and with chromosomal sex determination involving female heterogamety in this species.

The decreased fecundity of F_{2-3} families compared to the F_1 family is explained by the negative relationship between body size and fecundity in *P. sculpta* (Shuster 1991). Isopods maintained in incubators at 24°C (F_{2-3}) were smaller in size and less fecund than the field-collected parental *Str* female, who had matured at a cooler temperature (17°C–20°C in March; Shuster and Guthrie, in press). An episode of overfeeding caused higher mortality among F_3 individuals compared to F_{1-2} individuals. Despite these differences in fecundity among generations, no differential mortality was detectable between *Str* and non-*Str* individuals, or between males and females (Table 1). Although genetic factors are known to cause reversal of sexual phenotype in *P. sculpta* and in other isopods (Juchault and Rigaud 1995; Legrand et al. 1987; Shuster and Sassaman 1997), our use of α sires from families with unbiased sex ratios (lineage α-1-1) and the lack of biased sex ratios within and among our F_{1-3} families indicate that factors responsible for sex reversal were not present in these crosses.

The single non-*Str* female observed in the F_3 generation may represent a female whose maternal *Str*-bearing W chromosome had undergone recombination with a non-*Str*-bearing segment of its corresponding Z chromosome. This hypothesis cannot be confirmed because no *Str* male was found in the F_3 family in which the unmarked female appeared (Table 1). However, six (0.0006; $N = 9598$) *Str* males were observed in field collections. The relative rarity of these males is consistent with their identity as recombinant individuals. Alternatively, these individuals could be the result of misclassification of cuticular markers in the field. One particular pattern, "scale," observed primarily in α males, resembles *Str* but involves a somewhat different distribution of cuticular pigmentation (i.e., a longitudinal band of dark pigment on the *distal* portion of each dorsal body segment, with regular, anterior-directed projections; this pattern gives its bearers a "scaled" as well as "striped" appearance; Johnson K, unpublished data). The inheritance of scale, as well as the frequency of recombination of *Str* in labora-

Table 1. Heterogeneity *G* tests for Mendelian inheritance of *Str* and sex ratio in *Paracerceis sculpta* (F_1–F_3)

Generation	At birth[a]				At maturity					
					Str		+			Sex ratio[b]/*Str*[c]
	Str	+	*N*	*Gi*	F	M	F	M	*N*	*Gi*
F_1	34	38	72	0.22	14	0	0	15	29	0.03
F_2	13	9	22	0.73	8	0	0	5	13	0.70
	6	8	14	0.29	1	0	0	3	4	1.50
	9	20	29	4.28[d]	2	0	0	5	7	1.33
	17	21	38	0.42	1	0	0	5	6	2.91
	4	8	12	1.36	2	0	0	4	6	0.68
F_3	18	24	42	0.86	8	0	1	8	17	0.06
	27	21	48	0.75	2	0	0	2	4	0.00
	18	18	36	0.00	2	0	0	3	5	0.20
	14	14	28	0.00	4	0	0	3	7	0.14
	21	22	43	0.02	2	0	0	3	5	0.20
Total	181	203	384	8.93	46	0	1	56	103	7.75

[a] $G_P = 1.26$, $P > .10$, $G_{H[df=10]} = 7.67$, $P > .50$.
[b] $G_P = 0.79$, $P > .10$; $G_{H[df=10]} = 6.96$, $P > .10$.
[c] $G_P = 1.18$, $P > 0.10$, $G_{H[df=10]} = 6.57$, $P > .50$.
[d] $P < .05$.

tory populations are presently under investigation.

Our results demonstrate that *Str* in *P. sculpta* is a sex-linked trait. In particular, the appearance of *Str* in three *consecutive* generations of daughters, but *never* in sons, indicates that females in this species are heterogametic and that *Str* is located on the W chromosome (ZW = female, ZZ = male). Our results confirm existing evidence of chromosomal sex determination and female heterogamety in this species (Shuster and Sassaman 1997) and are consistent with reports of these mechanisms in two other species of flabelliferan isopods (reviews in Ginsberger-Vogel and Charniaux-Cotton 1982; Legrand et al. 1987). The fact that sex determination in oniscoidean isopods also involves female heterogamety (Juchault and Rigaud 1995) suggests that this form of sex determination may be an ancestral characteristic within the Isopoda (Brusca and Wilson 1991), and thus that female heterogamety may be common within this crustacean order.

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References

Bull JJ, 1983. Evolution of sex determining mechanisms. Menlo Park, California: Benjamin Cummings.

Brusca RC and Wilson GDF, 1991. A phylogenetic analysis of the Isopoda with some classificatory recommendations. Mem Queensl Mus 31:143–204.

Ginsberger-Vogel T and Charniaux-Cotton H, 1982. Sex determination. In: The biology of Crustacea. Vol. 2: Embryology, morphology and genetics (Abele L, ed). New York: Academic Press; 257–283.

Heath DJ, 1979. Colour polymorphism in the salt marsh isopod, *Sphaeroma rugicauda*; evidence for stable equilibrium frequencies. Oceologia 44:95–97.

Heath DJ and Ratford JR, 1990. The inheritance of sex ratio in the isopod, *Sphaeroma rugicauda.* Heredity 64: 419–425.

Hurst LD, 1993. The incidences, mechanisms and evolution of cytoplasmic sex ratio distorters in animals. Biol Rev 68:121–193.

Juchault P and Rigaud T, 1995. Evidence for female heterogamety in two terrestrial crustaceans and the problem of sex chromosome evolution in isopods. Heredity 75:488–471.

Juchault P, Rigaud T, and Mocquard J-P, 1992. Evolution of sex-determining mechanisms in a wild population of *Armadillidium vulgare* Latr. (Crustacea: Isopoda): competition between two feminizing parasitic sex factors. Heredity 69:382–390.

Legrand JJ, Legrand-Hamelin E, and Juchault P, 1987. Sex determination in Crustacea. Biol Rev 62:439–470.

Legrand-Hamelin E, 1976. Sur le polychromatisme de l'isopode Flabellifere *Dynamene bidentata* (Adams) III. Relations entre les genes responsables de phenotypes *bimaculata* et *lineata.* Arch Exp Genet 117:325–343.

Rigaud T and Juchault P, 1993. Conflict between feminizing sex ratio distorters and an autosomal masculinizing gene in the terrestrial isopod, *Armadillidium vulgare* Latr. Genetics 133:247–252.

Rousset F, Bouchon D, Pintureau B, Juchault P, and Solignac M, 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in Arthropods. Proc R Soc Lond B 250:91–98.

Sassaman C, 1978. Dynamics of a lactate dehydrogenase polymorphism in the wood louse *Porcellio scaber* Latr.: evidence for partial assortative mating and heterosis in natural populations. Genetics 88:591–609.

Shuster SM, 1989. Male alternative reproductive behaviors in a marine isopod crustacean (*Paracerceis sculpta*): the use of genetic markers to measure differences in fertilization success among α-, β- and γ-males. Evolution 34:1683–1698.

Shuster SM, 1991. Changes in female anatomy associated with the reproductive molt in *Paracerceis sculpta* (Holmes), a semelparous isopod crustacean. J Zool 225:365–379.

Shuster SM and Sassaman C, 1997. Genetic interaction between male mating strategy and sex ratio in a marine isopod. Nature 388:373–376.

Shuster SM and Guthrie EE, in press. The effects of temperature and food availability on adult body size in natural and laboratory populations of *Paracerceis sculpta,* a Gulf of California isopod. J Exp Mar Biol Ecol.

Sokal RR and Rohlf JF, 1995. Biometry, 3rd ed. San Francisco: W.H. Freeman.

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High Levels of Conservation at Microsatellite Loci Among Ictalurid Catfishes

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The potential of microsatellite sequences as genetic markers in channel catfish (*Ictalurus punctatus*) was investigated with respect to their variability, inheritance, and usefulness in related species. Six small insert genomic DNA libraries enriched for six families of microsatellites of channel catfish were constructed. We describe here the isolation, characterization, and PCR amplification of 32 microsatellites from channel catfish. The flanking primer regions of microsatellite loci were highly conserved between channel catfish and blue catfish (*I. furcatus*). Of the 32 loci, 29 were amplified from blue catfish using primers designed from channel catfish, indicating conservation of primer binding sequences. Most of the amplified alleles from channel catfish and blue catfish were polymorphic. White catfish (*Ameiurus catus*) and flathead catfish (*Pylodictus olivaris*) loci were also amplified. The microsatellite markers are highly polymorphic for all catfish species tested and are inherited as codominant markers. They should be highly useful for construction of genetic linkage maps of catfish and for marker-assisted selection.

Polymorphic DNA markers are crucial to genome mapping. Segregation analysis of polymorphic markers allows assignment of DNA fragments to chromosomes, ordering of DNA fragments, estimation of genetic distances, and mapping of important genes. Among various types of polymorphic DNA markers, microsatellite markers are highly useful. Microsatellites are tandem repeats of 1–6 bp. They are abundant, evenly distributed and highly polymorphic. Microsatellite loci are short in size, facilitating genotyping by polymerase chain reaction (PCR). They are codominant markers allowing generation of maximum genetic information. Genetic linkage maps have been constructed using microsatellites in various animal and plant species (Bell and Ecker 1994; Bishop et al. 1994; Dietrich et al. 1992; Gyapay et al. 1994; Knapik et al. 1996; Lee and Kocher 1996; Rohrer et al. 1994).

To construct a genetic linkage map of catfish, we have exploited channel catfish (*Ictaluras punctatus*) × blue catfish (*I. furcatus*) hybrids (Argue 1996; Argue and Dunham 1998; Liu et al. 1997). Although the hybrid system offers many advantages such as drastic phenotypic differences and high levels of marker polymorphism (Liu et al. 1998a,b), their application using microsatellite markers awaits demonstration of evolutionary conservation of microsatellite flanking sequences between the two species.

Using the interspecific hybrid system, Liu et al. (1992) initially mapped isozyme markers and six linkage groups were established (Morizot D and Dunham RA, unpublished data). Recently we demonstrated the feasibility of using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers for linkage analysis using the interspecific hybrid system (Liu et al. 1998a,b). Several hundred microsatellite loci have been isolated and sequenced

from the channel catfish genome (Liu et al. 1998c, Waldbieser GC, personal communication). If the microsatellites are conserved between the two species, it is possible to exploit all types of polymorphic markers to make a unified catfish map. Twenty-two microsatellite loci containing tri- and tetranucleotide repeats were previously reported for channel catfish (Waldbieser and Bosworth 1997). Here we report the isolation and characterization of 32 microsatellite loci containing dinucleotide repeats from channel catfish. We demonstrate that the vast majority of microsatellite loci can be amplified in both channel catfish and blue catfish, suggesting evolutionary conservation between the two catfish species. In addition, we report conservation of microsatellites within the family *Ictaluridae* among the genera *Ictalurus, Ameiurus,* and *Pylodictus.*

Materials and Methods

Fish, Blood Collection, and Isolation of DNA

Channel catfish (*I. punctatus*), blue catfish (*I. furcatus*), white catfish (*Ameiurus catus*), and flathead catfish (*Pylodictus olivaris*) were obtained either from the Fish Genetics Facility of Auburn University, Auburn, Alabama, or from the Gold Kist Aquaculture Center, Inverness, Mississippi.

Blood samples (0.2–0.5 ml) were collected in 1 ml syringes and immediately expelled into 50 ml tubes containing 20 ml of DNA extraction buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA, 0.5% SDS, and freshly added proteinase K at 0.1 mg/ml) and DNA was isolated as previously described using standard protocols (Liu et al. 1998a,b; Strauss 1989).

Library Construction

Genomic DNA (10 μg) was digested with 50 units of *Eco*RV, *Rsa*I, and *Hae*III. The digested DNA was size fractionated on a 1.5% agarose gel and DNA fragments 300–800 bp in size were excised from the gel. The DNA was recovered from the gel using a fragment isolation kit from Qiagen (Los Angeles, California) according to the supplier's protocol.

The small size DNA library was constructed in pBluescript (Strategene, LaJolla, California). Plasmid vector was digested by restriction endonuclease *Eco*RV and dephosphorylated with calf intestinal alkaline phosphatase (Boerhinger Manheim, Indianapolis, Indiana). The vector DNA and the recovered DNA fragments were ligated using the T4 DNA ligase at 8°C for 2 days and then transformed into the *ung*$^-$/*dut*$^-$ mutant strain of *E. coli,* CJ236 (BioRad, Hercules, California). This mutant clone allows incorporation of uracil triphosphate into DNA, which will be degraded upon transformation into a wild-type *E. coli* strain, such as DH5α. Transformation into CJ236 was performed by electroporation according to supplier's instructions. Transformants were plated at a calculated density to obtain confluent plates after titering the efficiency of the transformation. Each plate of the confluent bacterial cells was added with 10 ml of LB medium and the bacteria were collected by scraping with a glass spreader. The collected bacteria from 20 plates were combined and mixed. This primary small insert DNA library was used to prepare single-stranded plasmid DNA.

Microsatellite-enriched libraries were made according to Ostrander et al. (1992). Single-stranded phagemid DNA was primed by oligonucleotide primers containing $(CA)_{15}$, $(GA)_{15}$, $(AAT)_8$, $(CCA)_8$, $(CAG)_8$, and $(CAA)_8$. Six microsatellite-enriched libraries were made. Plating and screening of microsatellite-containing clones were done with colony lifting hybridization protocol (Sambrook et al. 1989) using oligonucleotide primer probes: $(CA)_{15}$ and $(GA)_{15}$ labeled with γ-^{32}P ATP to isolate (CA)- and (GA)-containing clones. The enriched libraries contained about 50% of microsatellite-positive clones.

Sequencing of Microsatellite-Containing Clones and Primer Design

Plasmid DNA from clones containing simple sequence repeats (SSRs) was prepared using the alkaline lysis procedure (Sambrook et al. 1989). Double-stranded DNA was sequenced using the dideoxynucleotide chain termination method with the AmpliCycle™ cycle sequencing kit from Perkin-Elmer (Foster City, California) following the manufacturer's instructions.

Sequences were manually input into a computer using the DNA Star software package (DNA Star, Inc., Madison, Wisconsin). Sequences generated with the M13 universal primer and the reverse primer were aligned to find the overlapping region. SSRs often are very useful for a quick determination of overlapping. The two sequences were then combined into a single sequence by splicing the complement of one sequence onto the other sequence. Sequences were aligned using DNA Star software.

PCR primers were designed using the OLIGO software package (National Biosciences, Inc., Plymouth, Minnesota). In each case, primers were designed at the highest possible stringency. For analysis of small differences of the SSR polymorphism, PCR primers were designed to generate products of about 100 bp, but in some cases primers were designed to generate larger PCR products because of highly repetitive sequences flanking microsatellites. PCR product sizes were best controlled by limiting the regions for primer design flanking the SSR. In addition to the factors analyzed by the OLIGO software such as length of the PCR products, duplex formation, hairpin, Tm, and false priming, several other factors were considered for selection of a pair of primers after generation of a primer pair list from the computer to select primers with stronger annealing and fewer simple sequences.

Genomic Amplification, Electrophoresis, and Southern Blot Analysis

Approximately 200 ng of genomic DNA was amplified in PCR reactions of 50 μl containing 50 mM KCl, 10 mM Tris (pH 9.0 at 25°C), 0.1% Triton X-100, 0.25 mM each of dNTPs, 1.5 mM $MgCl_2$, 20 μM each primer, and 2.5 units of *Taq* DNA polymerase. The general temperature profiles used in initial amplification trials were 94°C for 30 s, 45°C for 1 min, and 72°C for 2 min for 35–40 cycles. An initial denaturing period of 1 min at 94°C was used. Annealing temperatures were then changed to produce the most reproducible results as specified in Table 1. Following amplification, samples of 3 μl were mixed with 1 μl loading dye and electrophoresed on 10% acrylamide (19 acrylamide : 1 bis-acrylamide) gels. To analyze allelic amplification, PCR products were separated on agarose gels and transferred to nylon membranes. Sequencing reactions of previously sequenced clones were used as size standards on sequencing gels and a 100 bp ladder (GIBCO/BRL) was used on acrylamide gels.

Southern blot analysis was used to confirm allelic amplification because of the unexpected high levels of conservation at the microsatellite loci. If the amplification is allelic, all the amplified bands from all species should harbor the microsatellite repeats (though they may differ in repeat numbers) and thus should hybridize to the microsatellite probes. Southern blot analysis was conducted using standard protocols (Sambrook et al. 1989) to confirm allelic amplification. After gel electrophoresis, the DNA was transferred to ny-

Table 1. Characterization and conservation of 30 microsatellite loci isolated from channel catfish (*Ictalurus punctatus*)

Locus name	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Cloned SSR	Annealing temperature	Expected product (bp)	Conservation[a]
Ip30	ctaaagttggagaagagttcagc	aagacaaggacatctcaatgc	(CA)11	50	193	C/B/F/W
Ip34	tatggacgaaactgttaagtgg	aaagcgttgtgattatttctgac	(CA)17	55	122	C/b-
Ip41	ccgaccaccttctctgtcactac	ctcattaaaatggcctgtgtcc	(CA)12	50	112	C/B/F/W
Ip44	ggtcagaataccccttggatg	cactaaattgccccttggtg	(CA)15	50	98	C/B/F/W
Ip246	ccagcccattgcaggcacac	ctccccgagtccaaaaacac	(CA)14	55	121	C/B
Ip255	ttttgcaagatgtctttaaaacac	aggggtctcaataatttggtg	(CA)16	58	221	C/B
Ip270	actcaataaatcaaatcatgcg	atttgtgaaacaaaatgagtgg	(ATTT)10 (TC)18 (CA)18	60	266	C/B
Ip271	tggggaaaaagaaagtaataacg	cagtagagctttgaagccattc	(TG)18 (GA)19	60	177	C/B/F/W
Ip351	cctaccgttcatctgtgctca	cataccactgacctgaccctcac	(CA)15	60	139	C/b-
Ip357	cctgccaccatatcagtgaattt	gataatgagtctccggaggtgc	(CA)18	58	128	C/B/F/W
Ip365	taaaggatctgattcaccgtatc	aaaccgctaacctaccctct	(CA)13	58	124	C/B/F/W
Ip368	caaaacacagcagccacttcttc	ttggtggcacgtaaagtcctg	(CA)7G (CA)6	58	120	C/B
Ip372	ggcactgaggtttgggctgcac	tggcatcgctcctcatcatcctg	(CA)8	54	180	C/B/F/W
Ip374	tgttgacggaggcaggac	acaatcaatttcccaacagtgac	(CA)5AT (CA)31	54	188	C/B/F/W
Ip383	aagaagcgagcgtgttgac	tcaatttccaacagttgactgtg	(CA)37	55	195	C/B
Ip385	tcatcgctctgacctgaaacac	ctaatcacagctacagcgaccgt	(CA)23	55	108	C/B
Ip388	gcgtgacatgacacatgagcgag	cggtcattaaggttattctatc	(CTAT)6 A17 N3(CA)14	50	300	C/B
Ip391	caacagtaagcgactcacac	tatactagtcgaaatgtgctcac	(CA)20	55	132	C/B
Ip393	actgtatgacttatattaccac	gatcaggtcttccatgtggctgc	(CA)31	55	220	C/B
Ip394	cctgagtcgccagcattcacg	cctaatgaggctcagctttgatg	(GA)9 (CA)13	55	118	C/B
Ip396	cctgtgccaaatcctaacacac	cacactatacccacacccactgt	(CA)31	55	167	C/B
Ip397	cggacaatctacagcacaaagac	cagactgcacacgtgggtc	(CA)22	55	202	C/B
Ip398	agccaaaaatatacagctaacag	ccaatagtttcagaagggac	(CA)28 (AAAT)8	55	203	C/B
Ip399	acaattaatggctttgtttac	tcttagacccagacctatttaca	(CA)21 (N)8 (CA)5	55	180	C/B/F/W
Ip401	ccaacacactacagagtccc	cactgaaattgggcagttgtac	(CA)9	50	99	C/B/F/W
Ip403	ctgcttgatagaaatagcatgtg	cctgaaccatgtctggtagcc	(CA)14	50	179	C/B/F/w-
Ip438	gatctggccaagtgtaaacatag	cactgtgatgcactgtgatg	(CA)11	50	112	C/b-
Ip443	gcaataccacacccttcttac	gaagattcgttgaatacatattc	(CA)10	50	134	C/B/F/W
Ip466	cctcacatcgattggttgagag	ttttatgtatgcatgcatgtgtg	(AAT)15	55	153	C/B/F/W
Ip554	gagatgaagtgagatgaagacag	tgcttaaataatgacacggttc	(GA)20	54	235	C/B/F/W
Ip555	cagcgaacactggagcagttcac	catatgaagtgtgaatcttttctg	(GA)29	55	130	C/B
Ip557	cgcgcacgagcgagactgaccgg	gctcttacttgactcaggtccg	(GA)24	55	250	C/B

[a] C/B/F/W, microsatellite loci conserved among channel catfish (C), blue catfish (B), flathead catfish (F), and white catfish (W); C/B/F/w-, microsatellite loci conserved among channel, blue, and flathead catfish, but not white catfish; C/B, microsatellite loci conserved between channel and blue catfish; C/b-, channel catfish microsatellite loci that were not amplifiable from blue catfish. Microsatellite loci indicated by C/B or C/b- were not tested in white catfish or flathead catfish.

lon membranes and probed with end-labeled $(CA)_{15}$ oligonucleotide primers.

Results and Discussion

Isolation, Sequencing, and Characterization of 32 Microsatellite Loci

Microsatellite-containing clones were isolated by screening the small-insert, microsatellite-enriched libraries containing CA or GA repeats. Among the 32 characterized microsatellites, 24 were simple microsatellites containing only one type of repeat sequence (Table 1). Eight were composite microsatellites containing two or more types of repeat sequences. Most microsatellite clones contained high A/T-rich sequences immediately adjacent to the microsatellite repeats. PCR primers were designed based on the channel catfish microsatellite flanking region sequences and used to amplify genomic DNA of channel catfish, blue catfish, flathead catfish, and white catfish.

Although it was straightforward to identify, isolate, and characterize microsatellites by using the small-insert genomic DNA libraries, large numbers of clones were sequenced to achieve the 32 microsatellite markers. Among the 120 sequenced clones, 71 harbored enough nonrepetitive flanking sequences for primer design. Optimization of PCR primers and conditions for successful allelic amplification is another major step for microsatellite development. On average, about 50% of primer pairs produced clean and reproducible bands. In this study, sequencing was conducted manually where only less than 300 bp accurate sequences can be read. The number of clones with sufficient unique flanking sequences would be higher if larger insert (e.g., 600–800 bp) clones were made and sequenced by using an automated DNA sequencer.

High levels of heterozygosity were observed from both channel catfish and the blue catfish. Because of the high levels of heterozygosity, 25 of 32 pairs of primers generated two PCR bands with an individual channel catfish, indicating allelic variation (Figure 1). Similar results were observed from white catfish and flathead catfish (data not shown). Waldbieser and Bosworth (1997) previously characterized 22 microsatellite loci from channel catfish

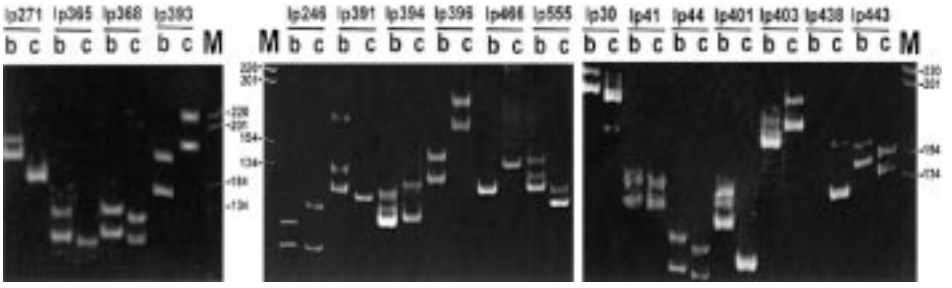

Figure 1. Conservation of the microsatellite loci between channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) genomes. PCR amplification products using channel catfish genomic DNA (c) and blue catfish genomic DNA (b) were analyzed on 10% acrylamide gels. PCR products from 17 loci are shown with locus names on the top (Ip271, Ip365, . . . through Ip443). M, 1 kb molecular markers with sizes labeled on the side immediately next to the marker lanes. Note that at locus Ip438, the channel catfish primers failed to amplify the blue catfish alleles.

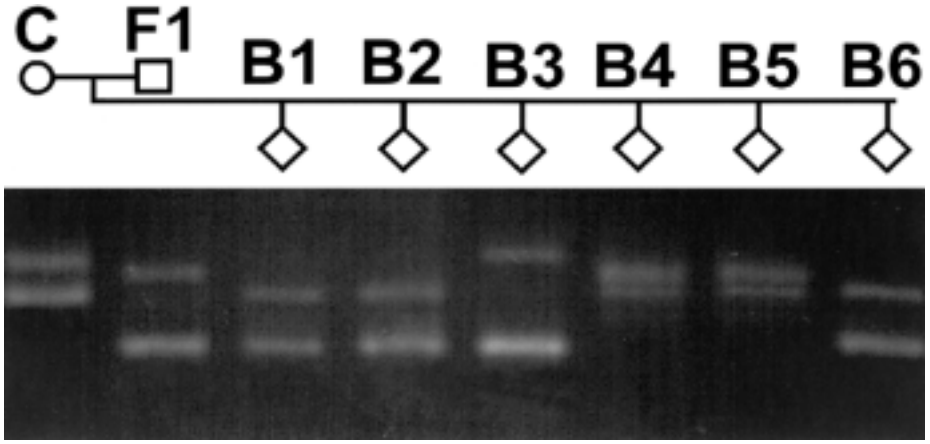

Figure 2. Inheritance of the microsatellite loci. PCR amplification products were analyzed on a 2% agarose gel. C, channel catfish female; F1, channel catfish × blue catfish F_1 hybrid male; B1–B6, six individuals of backcross progeny.

containing tri- and tetranucleotide repeats and showed high levels of heterozygosity. Our work here indicated that dinucleotide microsatellite repeats are similarly heterozygous and therefore can be useful for genetic analysis in channel catfish. For genetic linkage analysis, this would indicate that small numbers of reference families are sufficient for analysis of these microsatellite loci. High levels of polymorphism also suggest that microsatellite markers may be highly useful for population studies and for identification of fish strains or individuals using combinations of microsatellite markers.

We examined the inheritance of microsatellites in the interspecific hybrids of channel × blue catfish. Backcross progeny were produced from matings of F_1 hybrid × channel catfish and F_1 hybrid × blue catfish. Figure 2 demonstrates classic Mendelian inheritance of a marker locus in the channel catfish backcross. Similar results were obtained with the backcross to blue catfish. As in all reported cases, microsatellite markers in catfish are inherited as codominant markers.

Conservation of Microsatellite Loci Between Channel Catfish and Blue Catfish

Microsatellite clones were isolated from channel catfish using microsatellite-enriched, small-insert DNA libraries. PCR primers were designed based on the flanking sequences. Thirty-two pairs of primers (Table 1) were used to amplify microsatellite loci from channel catfish and from blue catfish. Among the 32 tested pairs of primers, 29 pairs of primers successfully amplified genomic DNA from both species (Figure 1, Table 1). Three criteria were used to judge the success of allelic amplification: efficient amplification from both species, similar sizes of amplified PCR products, and size variation of the amplified PCR products from the two alleles and from different individuals reflecting variation in repeat numbers of microsatellites. PCR products were similar in size in both species. High and similar levels of polymorphism were detected from channel catfish and blue catfish. This data indicates the evolutionary conservation of these loci between the two species.

Three of the 32 microsatellite loci (Ip34, Ip351, Ip438) could not be amplified in blue catfish. To distinguish the lack of conservation from experimental failure, the PCR was repeated three times to confirm divergence of sequence at these loci. For the loci that were not amplified at higher annealing temperature, PCR reactions were also performed under less stringent conditions (40°C). Allelic fragments of microsatellite locus Ip351 were amplified at 40°C, indicating conservation of the locus with minor base substitutions at the primer binding sites. Microsatellite loci Ip34 and Ip438 could not be amplified under the less stringent conditions.

The conservation of microsatellite loci between channel catfish and blue catfish is very important for practical application. Genetic linkage mapping of catfish has primarily used interspecific hybrids to exploit the polymorphism markers between the two species (Liu et al. 1992, 1998a,b; Morizot et al. 1994). Although polymorphism is not a problem with microsatellites, conservation of microsatellite loci between the two species allows construction of a unified map using the interspecific hybrids. Several types of markers can be mapped using the same reference families of the channel catfish × blue catfish interspecific hybrids.

Although conservation of microsatellite loci among closely related species is likely, successful amplification of these loci between species depends on the conservation of primer sequences. The fact that over 90% of the primers can amplify both channel catfish DNA and blue catfish DNA indicates high levels of genomic conservation between the two species, consistent with karyotype analysis (LeGrande et al. 1984).

Conservation of Microsatellite Loci Among Ictalurid Catfishes

To determine the conservation of microsatellite loci among catfishes, genomic DNA from channel catfish, white catfish, and flathead catfish were used to amplify microsatellite loci with 14 pairs of primers. The results demonstrate that the microsatellite loci are highly conserved among the three tested genera from the family *Ictaluridae* (Figure 2). All 14 pairs of channel catfish primers successfully amplified genomic DNA from flathead catfish. Thirteen of 14 pairs successfully amplified the genomic DNA from white catfish. Amplifiability, size, and polymorphic rates were initially used to determine allelic amplification and thus the conservation of the microsatellite loci. All amplified bands from channel catfish, flathead catfish, and white catfish hybridized to the $(CA)_{15}$ probe, confirming allele-specific amplifications (Figure 3). The high levels of conservation among the three genera within *Ictaluridae* predict that microsatellite loci may also be conserved, at lower levels, among more distant taxonomic groups.

Conservation of microsatellite loci across a broad species range has previously been described in various other species (Coote and Bruford 1996; de Gortari et al. 1997; Deka et al. 1994; FitzSimmons et al. 1995; Fredholm and Wintero 1995; Menotti-Raymond and O'Brien 1995; Moore et al. 1991; Rico et al. 1996; Schlotterer et al. 1991; Sun and Kirkpatrick 1996; Surridge et al. 1991; Zardoya et al. 1996). In most cases, primers designed from microsatellite flanking regions (MFRs) of one species were tested in closely related species. For instance, primers from human were tested and found to work in other primates (Coote and Bruford 1996), or primers from one member of a family were tested and found to work among other members of the family (Fredholm and Wintero 1995; Menotti-Raymond and O'Brien 1995). Recently Zardoya et al.

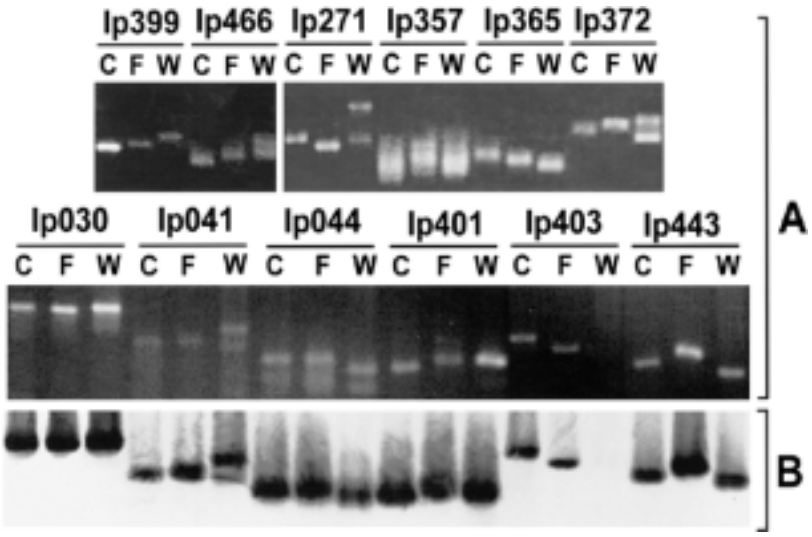

Figure 3. Conservation of microsatellite loci among Ictalurid catfishes. PCR products amplified from 12 microsatellite loci were analyzed on agarose gels (A, top two rows). Locus names are indicated on the top of each locus (e.g., Ip399). C, channel catfish, F, flathead catfish, W, white catfish. The third row (B) was a Southern blot analysis confirming allelic amplification using CA repeats $(CA)_{15}$ as probes. Their locus numbers are Ip30, Ip41, Ip44, Ip401, Ip438, and Ip443, same as the middle row.

(1996) tested conservation of MFRs of six microsatellite loci, and demonstrated that most primers were able to amplify some species from the same family in the cichlid fish, *Cichlidae.* Surprising results were recently obtained from studies using aquatic animals. Homologous microsatellite loci can persist for about 300 million years in turtle (FizSimmons et al. 1995) and for 470 million years in fish (Rico et al. 1996). Accumulating evidence indicates that microsatellite flanking sequences in aquatic organisms evolve at a slower rate than those in land animals.

The conservation of microsatellites across a broad range of taxa in fish has important applications. Microsatellite primers obtained from sequences of channel catfish are currently being tested to amplify the allelic fragments from several other important aquaculture species. If successful, it would be possible to map their genomes using microsatellite primers developed from various fish species (Lee and Kocher 1996; Knapik et al. 1996; Waldbieser and Bosworth 1997). Such high levels of conservation are also important for comparative gene mapping, especially considering the rare availability of type I markers in catfish (Liu ZJ, unpublished results). Comparative gene mapping would facilitate rapid progress in gene mapping programs of aquaculture species such as catfish, tilapia, salmon, and carp.

In conclusion, microsatellite markers are highly conserved among Ictalurid catfishes. Over 90% of the 32 examined microsatellite marker loci are conserved between channel catfish and blue catfish belonging to the genus *Ictalurus.* The intergeneric conservation rates of the microsatellite loci were also high among *Ictalurus, Ameiurus,* and *Pylodictus.* These results indicate similar genomic organization among catfishes and the feasibility of using the interspecific hybrid system for mapping the genomes of catfish. The availability of conserved microsatellite markers is important for gene mapping, marker-assisted selection, and evolutionary studies.

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References

Argue BJ, 1996. Performance of channel catfish *Ictalurus punctatus,* blue catfish *I. furcatus,* and their F1, F2, F3 and backcross hybrids (PhD dissertation). Auburn, Alabama: Auburn University.

Argue BJ and Dunham RA, 1998. Hybrid fertility, introgression and backcrossing in fish. Rev Fish Sci., in press.

Bell CJ and Ecker JR, 1994. Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis.* Genomics 19:137–144.

Bishop MD, Kappes SM, Keele JW, Stone RT, Sunden S, Hawkins GA, Toldo SS, Fries R, Grosz MD, Yoo J, and Beattie CW, 1994. A genetic linkage map for cattle. Genetics 136:619–639.

Coote T and Bruford MW, 1996. Human microsatellites applicable for analysis of genetic variation in apes and Old World monkeys. J Hered 87:406–410.

de Gortari MJ, Freking BA, Kappes SM, Leymaster KA, Crawford AM, Stone RT, and Beattie CW, 1997. Extensive genomic conservation of cattle microsatellite heterozygosity in sheep. Anim Genet 28:274–290.

Deka R, Shriver MD, Yu LM, Jin L, Aston CE, Chakraborty R, and Ferrell RE, 1994. Conservation of human chromosome 13 polymorphic microsatellite (CA)n repeats in chimpanzees. Genomics 22:226–230.

Dietrich W, Katz H, Lincoln S, Shin HS, Fiedman J, Dracopoli NC, and Lander ES, 1992. A genetic map of the mouse suitable for typing intraspecific crosses. Genetics 131:423–447.

FitzSimmons NN, Moritz C, Moore SS, 1995. Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. Mol Biol Evol 12: 432–440.

Fredholm M and Wintero AK, 1995. Variation of short tandem repeats within and between species belonging to the *Canidae* family. Mamm Genome 6:11–18.

Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau M, Marc S, Bernardi G, Lathrop M, and Weissenbach J, 1994. The 1993–1994 Genethon human genetic linkage map. Nat Genet 7:246–249.

Knapik EW, Goodman A, Atkinson OS, Roberts CT, Shiozawa M, Sim CU, Weksler-Zangen S, Trolliet MR, Futrell C, Innes BA, Koike G, McLaughlin MG, Pierre L, Simon JS, Vilallonga E, Roy M, Chiang PW, Fishman MC, Driever W, and Jacob HJ, 1996. A reference cross DNA panel for zebrafish (*Danio rerio*) anchored with simple sequence length polymorphisms. Development 123: 451–460.

Lee WJ and Kocher TD, 1996. Microsatellite DNA markers for genetic mapping in *Oreochromis niloticus.* J Fish Biol 49:169–171.

LeGrande W, Dunham RA, and Smitherman RO, 1984. Comparative karyology of three species of north American catfishes (*Siluriformes: Ictaluridae: Ictalurus*) and four of their hybrid combinations. Copeia 1984:873–878.

Liu Q, Goudi CA, Simco BA, Davis KB, and Morizot DC, 1992. Gene-centromere mapping of six enzyme loci in gynogenetic channel catfish. J Hered 83:245–248.

Liu ZJ, Li P, Argue B, and Dunham RA, 1997. Gonadotropin α subunit glycoprotein from channel catfish (*Ictalurus punctatus*) and its expression during hormone-induced ovulation. Mol Mar Biol Biotechnol 6:221–231.

Liu ZJ, Li P, Argue B, and Dunham RA, 1998a. Inheritance of RAPD markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*) and their F1, F2, and backcross hybrids. Anim Genet 29:58–62.

Liu ZJ, Nichols A, Li P, Dunham RA, 1998b. Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*) and their F1, F2 and backcross hybrids. Mol Gen Genet 258:260–268.

Liu ZJ, Tan G, Karsi A, Li P, Kucuktas H, Zheng X, Kim S, and Dunham RA, 1998c. Evolutionary conservation of microsatellite loci between channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). Abstract for the International Plant and Animal Genome Mapping Conference VI, Scherago International, New York.

Menotti-Raymond MA and O'Brien SJ, 1995. Evolutionary conservation of ten microsatellite loci in four species of *Felidae.* J Hered 86:319–322.

Morizot D, Schmidt M, and Carmichael G, 1994. Joint segregation of allozymes in catfish genetic crosses: designation of *Ictalurus punctatus* linkage group I. Trans Am Fish Soc 123:22–27.

Moore SS, Sargeant LL, King TJ, Mattick JS, Georges M, and Hetzel DJ, 1991. The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. Genomics 10:654–660.

Ostrander EA, Jong PM, Rine J, and Duyk G, 1992. Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. Proc Natl Acad Sci USA 89:3419–3423.

Rico C, Rico I, and Hewitt G, 1996. 470 million years of conservation of microsatellite loci among fish species. Proc R Soc Lond B Biol Sci 263:549–557.

Rohrer GA, Alexander LJ, Keele JW, Smith TP, and Beattie CW, 1994. A microsatellite linkage map of the porcine genome. Genetics 136:231–245.

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

Schlotterer C, Amos B, and Tautz D, 1991. Conservation of polymorphic simple sequence loci in cetacean species. Nature 354:63–65.

Strauss WM, 1989. Preparation of genomic DNA from mammalian tissue. In: Current protocols in molecular biology (Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, and Struhl K, eds). New York: John Wiley & Sons; 2.2.1–2.2.2.

Sun HS and Kirkpatrick BW, 1996. Exploiting dinucleotide microsatellites conserved among mammalian species. Mamm Genome 7:128–132.

Surridge AK, Bell DJ, Rico C, and Hewitt GM, 1997. Polymorphic microsatellite loci in the European rabbit (*Oryctolagus cuniculus*) are also amplified in other lagomorph species. Anim Genet 28:302–305.

Waldbieser GC and Bosworth BG, 1997. Cloning and characterization of microsatellite loci in channel catfish, *Ictalurus punctatus.* Anim Genet 28:295–298.

Zardoya R, Vollmer DM, Craddock C, Streelman JT, Karl S, and Meyer A, 1996. Evolutionary conservation of microsatellite flanking regions and their use in resolving the phylogeny of cichlid fishes (Pisces: Perciformes). Proc R Soc Lond B Biol Sci 263:1589–1598.

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The Yellow Color Inheritance in Rainbow Trout

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Yellow and wild-colored rainbow trout were used in crossbreeding experiments to determine the pattern of yellow color inheritance. The observed color phenotypes and their relative frequency in different progeny groups can be explained by a system of two gene loci with two alleles each. Yellow color (allele a) is caused by absence of the dominant allele A controlling wild color. Among the yellow fish (aa) the second gene locus allele B controls palomino and black eye color. Albino and red eye color (allele b) is caused by the absence of the dominant allele B controlling color development.

Table 1. Parental and progeny color phenotypes in families pooled into groups

Group (pooled families)	Female parent	Male parent	Progeny
1	Wild	Yellow	Wild
2	Yellow	Yellow	Yellow
3	Wild	Yellow	1 wild:1 yellow
4	Yellow-meiotic gynogenesis	—	All yellow uniform tint

Publications concerning yellow coloration in rainbow trout (*Oncorhynchus mykiss*) are rather scarce and sometimes controversial. Bridges and Limbach (1972) and Kohlmann and Fredrich (1986) described albinism in rainbow trout as a single-locus autosomal recessive trait. Clark (1970) found yellow coloration to be controlled by a recessive allele. Wright (1972; after Tave 1986) and Tave (1988; after Purdom 1993) described the inheritance of golden and normally pigmented phenotypes as a single-locus trait with two alleles that act with additive effects—the homozygous genotypes GG and G′G′ being normally pigmented and golden, respectively, while GG′ shows the intermediate palomino phenotype. Chourrout (1982) in experiments to produce gynogenetic rainbow trout, used males homozygous for a dominant depigmentation gene. The "albino-gold trout" reported by Klupp and Kaufmann (1979) showed dominant inheritance.

In our work considerable variability in coloration was observed, therefore the term "wild" was used instead of "normally pigmented." In addition, the description "yellow" covers a range of color tints from light to dark.

In Poland, the rainbow trout yellow line (Maliszewski 1987) started from a yellow female with big dark specks and a male which was dark yellow. The resulting progeny were half wild color and half yellow. This line has been reared in the Salmonid Research Laboratory Rutki since 1986. In these studies, at least two tints were observed in the yellow line. Therefore experiments were initiated to determine the pattern of yellow color inheritance.

Experiments and Results

Step 1

Preliminary test crosses were produced using several mating pairs chosen based on phenotype and progeny pooled into groups. Parental and progeny color phenotypes for each group are shown in Table 1. These results suggested that wild color was dominant to yellow color.

In groups 2 and 3, some families expressed a uniform yellow color while others showed different tints of yellow. For example, by the end of the first rearing season yellow fish from one family (group 3) expressed two body colors: part were light yellow and part were a brownish yellow which were described as albino and palomino, respectively. In addition, progeny (group 3) from a wild female and yellow (albino) male (F_0) were half wild and half brownish yellow (palomino).

Step 2

The F_1 full sibs—six females and six males (three wild color and three palomino in each sex)—were mated to create six families. In three families the palomino sisters were mated to wild brothers and in three other families wild sisters were mated to palomino brothers. Resulting F_2 families were incubated and reared separately. During this step we expected to identify palomino and albino fish based on body color at the fall fingerlings stage. The results (Table 2) suggested that wild, palomino, and albino coloration could be controlled by two gene loci according to the proposed model: allele A (wild) is dominant over allele a (yellow) and allele B (palomino) is dominant over allele b (albino), and locus A is epistatically dominant to the locus B. Thus segregation of alleles in consecutive generations would have been as shown in Table 3.

However, significant discrepancies from the proposed model were observed in two of six families (chi-square values in Table 2). Since mean mortality in experimental families from the start of feeding to the time of differentiation exceeded 50% and a continuous sequence of color intensity existed in palomino fish, the observed color ratio (Table 2) could be biased due to differential mortality of phenotype groups and inaccurate classification of palomino and yellow fish. These possibilities could not be ruled out due to the lack of within-family mortality records during incubation.

Step 3

To confirm the hypothetical model of wild, palomino, and albino color inheritance in

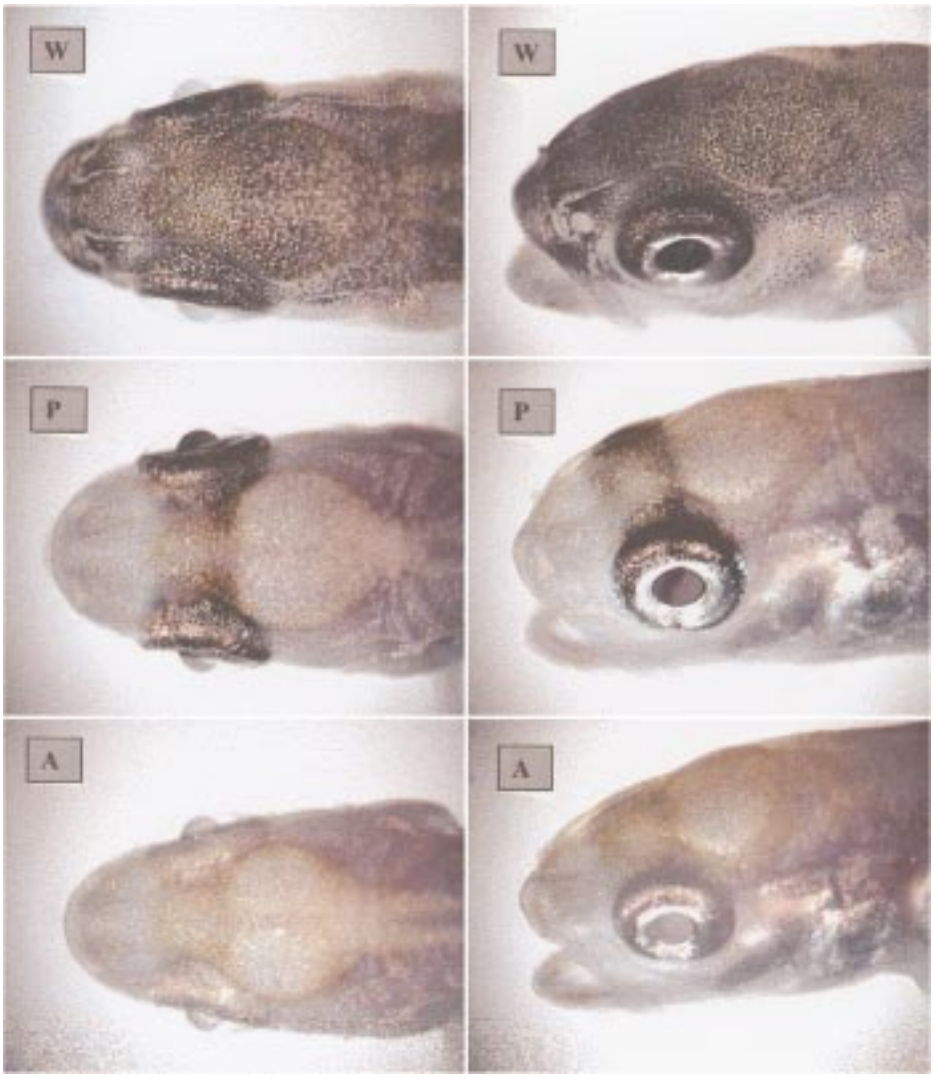

Figure 1. Rainbow trout alevins just before exogenous feeding (computer image): W, wild; P, palomino; A, albino phenotype.

our stock of rainbow trout, a third experiment was undertaken. The qualitative eye color character was considered at this step. It is noteworthy that palomino and albino phenotypes cannot be differentiated at the eyed stage of embryonic development due to the lack of eye pigment in both groups. The black (palomino) and red (albino) eye pigments develop in alevins when body pigment appears in wild-color fish. In this stage, phenotype is easily observed in almost transparent larvae (Figure 1). Based on this criteria, the following test crosses were evaluated: Eggs obtained from eight albino (red eyes) gynogenotes (double recessive homozygote—aabb) were mixed and divided into eight even lots. Full-sib males of three phenotypes (wild, palomino, and albino) originating from an albino aabb mother and a wild father of genotype AaBb (see above-mentioned model) were used. Thus the progeny had to be of the following genotypes: Aabb or AaBb (wild), aaBb (palomino), aabb (albino). Lots 1–5 were fertilized separately by five wild males. Lots 6–8 were fertilized separately by three palomino males. Lot 9 was fertilized by an albino male. The nine fertilized egg lots were incubated separately. At the eyed stage, dead and live eggs in each lot were counted. In live eggs, the number of embryos with pigmented and unpigmented eyes were recorded. Survival until the eyed egg stage and the ratio of embryo eye pigmentation in experimental lots are presented in Table 4. All wild-color males (lots 1–5) produced even numbers of embryos with pigmented and unpigmented eyes. All palomino (lots 6–8) and albino (lot 9) males produced only embryos with unpigmented eyes. Lot survival rates from fertilization to eye pigmentation stage varied from 28 to 84.2%.

A similar procedure was repeated with alevins just before the start of external feeding. The fish in each lot were counted and body and eye color recorded (see Figure 1). The pattern of inheritance of the three color phenotypes was assessed based on the pedigree and offspring phenotype ratio. For each lot (half sibs) the significance of differences between expected and observed counts of each phenotype was calculated using the chi-square test (Tables 4 and 5). At this stage of larval development a differentiation of eye color was observed among yellow fish in lots 4, 6, 7, and 8 (Figure 1, P and A).

In four of five lots (1, 2, 3, and 5) from wild-color males, almost equal numbers of wild (black eyes) and albino (red eyes) were observed (Figure 1, W and A). Chi-square values ranged from 0 to 2.02, indicating the sire genotype was Aabb (Table 5, lots 1–3 and 5). In lot 4, the wild-color male produced three phenotypes of offspring: wild (black eyes), palomino (black eyes), and albino (red eyes) (Figure 1, W, P, and A) with the ratio 2:1:1, respectively, and chi-square value 0.56, hence the sire genotype was AaBb (Table 5, lot 4). Within yellow fish in lots 6–8 the number of fish with red eyes was almost equal to the fish with black eyes. The differences were insignificant, with chi-square values ranging from 0.14 to 1.52 (Table 5). All alevins from lot 9 had red eyes.

These results confirm the proposed model for yellow color inheritance: gene A responsible for wild color dominates over gene a which produces yellow color (Tables 4 and 5). Gene B is requisite of palomino color. Its expression depends on the presence of homozygote aa (Table 5, lots 4, 6–8). Albino is the result of a recessive double homozygote aabb (Table 5, lot 9).

Discussion

The large variability in rainbow trout yellow color was of genetic origin. Complete dominance of yellow (Chourrout 1982) and albino-gold (Klupp and Kaufmann 1979) were described. The color inheritance in the rainbow strain reared and tested at the Inland Fisheries Institute, Salmonid Research Division, was different from the model described by Wright (1972; after Tave 1986 and Purdom 1993), who stated that in rainbow trout, yellow color was controlled by alleles at a single autosomal locus exhibiting incomplete dominance and acting additively to produce three phenotypes, unique for each genotype. These genotypes and pheno-

Table 2. Number of differently colored progenies in experimental families and chi-square test for their agreement with the expected ratio of 4:3:1 (wild, palomino, albino, respectively)

Family	Putative parents genotypes (females × males)	Wild	Palomino	Albino	χ^2
1	aaBb × AaBb	131 (121)	73 (90)	37 (30)	5.74
2	aaBb × AaBb	50 (52)	32 (39)	22 (13)	7.56*
3	aaBb × AaBb	201 (183)	127 (137)	38 (46)	3.85
4	AaBb × aaBb	104 (106)	78 (79)	30 (27)	0.53
5	AaBb × aaBb	136 (106)	58 (79)	18 (27)	17.03**
6	AaBb × aaBb	155 (145)	96 (109)	39 (36)	2.39

* $P < .05$, ** $P < .01$.

( ) = expected numbers.

Table 3. Segregation of alleles

F_0	Wild female (AaBB) × Albino male (aabb)		
F_1	Progeny: Segregation of alleles	Ratio	Phenotype
	AaBb, AabB	1	Wild
	aaBb, aabB	1	Palomino
F_1	Parents phenotype: Wild × Palomino or Palomino × Wild Parental genotype: AaBb × aaBb or aaBb × AaBb		
F_2	Progeny: Segregation of alleles	Ratio	Phenotype
	AaBB, AaBb, AabB, Aabb	4	Wild
	aaBb, aabB, aaBB	3	Palomino
	aabb	1	Albino

Table 4. Survival of embryos until eyed stage and numbers of embryos showing different eye pigmentation in experimental lots

Lot	Male color (putative genotype)	Number of eyed eggs	Survival (%)	Number of embryo with black eyes	Number of embryo with red eyes	χ^{2a}
1	Wild (Aa × b)	1084	84.2	535	549	0.18
2	Wild (Aa × b)	577	45.3	288	289	0.00
3	Wild (Aa × b)	355	28.0	187	168	1.02
4	Wild (Aa × b)	948	79.9	464	484	0.42
5	Wild (Aa × b)	921	78.0	445	476	1.04
6	1 Palomino (aaBb)	1051	83.4	—	1051	
7	2 Palomino (aaBb)	955	77.8	—	955	
8	3 Palomino (aaBb)	1018	81.4	—	1018	
9	1 Albino (aabb)	439	34.1	—	439	

Chi-square $P_{0.05} = 3.84$.

[a] Testing the ratio: wild (embryo with black eyes) to yellow (embryo with red eyes) as 1:1.

types are GG (normally pigmented), G′G′ (golden), and GG′ (palomino).

Our research indicates a complete dominance model with allele A dominate to a, controlling wild and yellow color, respectively, as reported by Clark (1970). Probably this same dominance pattern was described by Bridges and Limbach (1972) and Kohlmann and Fredrich (1986) for albino populations characterized by red eyes as was observed in our experiment in lots 1–3 and 5 (Table 4) and characterized by the lack of the dominant allele B at the B locus.

The genetics of yellow color in our rainbow trout strain is more complicated. The overall initial classification described as yellow contained both the palomino (variety of brownish-yellow tints) and the albino (light yellow) phenotypes. The interesting phenomenon of delayed development of black eye pigmentation in palomino fish, prevented differentiation of palomino and albino phenotype embryos at the eyed stage. Proper classification into palomino and albino is not feasible until the alevins stage based only on eye color. Based on body color, the distinction between these phenotypes is quite precise in fish larger than 40 g, however, it requires some skill.

Table 5. Survival from eyed eggs to the alevins stage and the alevins body and eyes' color in experimental lots

Lot	Male color (known genotype)	Number of alevins	Survival (%)	Number of wild alevins	Number of alevins with black eyes	Number of alevins with red eyes	χ^2
1	Wild (Aabb)	924	85.2	462	—	462	0.00
2	Wild (Aabb)	518	89.8	262	—	256	0.07
3	Wild (Aabb)	309	87.0	167	—	142	2.02
4	Wild (AaBb)	840	88.6	412	219	208	0.56
5	Wild (Aabb)	781	84.8	390	—	391	0.01
6	Palomino (aaBb)	948	90.2	—	455	493	1.52
7	Palomino (aaBb)	843	88.3	—	427	416	0.14
8	Palomino (aaBb)	915	89.9	—	450	465	0.25
9	Albino (aabb)	408	92.9	—	—	408	

Chi-square $P_{0.05} = 3.84$, 5.99 (1 or 2 df, respectively).

Conclusions

In the rainbow trout strain reared in SRL Rutki, yellow body color is controlled by two gene loci. The A locus is epistatically dominant to the B locus with the wild phenotype expressed in the presence of the A allele and the yellow phenotype expressed in the presence of the homozygous a allele. The B locus moderates the expression of yellow color to produce palomino in the presence of the B allele and albino in the presence of the homozygous b allele. In this system, it appears that A is completely dominant to a and B is completely dominant to b.

Palomino and albino phenotypes can be differentiated quite easily at the fry stage based on eye color (Figure 1) or as summer fingerlings and older based on body color.

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References

Bridges WR and Limbach B, 1972. Inheritance of albinism in rainbow trout. J Hered 63:152–153.

Chourrout D, 1982. Gynogenesis caused by ultraviolet irradiation of salmonid sperm. J Exp Zool 223:175–181.

Clark FH, 1970. Pleiotropic effects of the gene for golden color in rainbow trout. J Hered 61:8–10.

Klupp R and Kaufmann F, 1979. Farbverebung bei regenbogenforellen. Fischer Teichwirt 30:19–20.

Kohlmann K and Fredrich F, 1986. Albinismus bei re-

genbogenforellen (Salmo gairdneri). Z Binnenfischerei DDR 33:270–272.

Maliszewski R, 1987. Yellow-orange rainbow trout. Gosp Ryb 4:12–13 (in Polish).

Purdom CE, 1993. Genetics and fish breeding. London: Chapman & Hall.

Tave D, 1986. Genetics for fish hatchery managers. Westport, Connecticut: AVI Publishing.

Tave D, 1988. Body color in rainbow trout. Aquacult Mag 14:6566.

Wright JE, 1972. The palomino rainbow trout. PA Angler Mag 41:8–9.

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DNA Fingerprint Variability Within and Among the Silkworm *Bombyx mori* Varieties and Estimation of Their Genetic Relatedness Using Bkm-Derived Probe

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Genetic diversity within and among 13 silkworm varieties (6 diapausing and 7 nondiapausing) that differ in various quantitative and qualitative characters of economic importance was determined by DNA fingerprinting using Bkm-derived 2(8) probe. A high degree of genetic similarity was observed within each variety studied. Based on fingerprints of pooled DNA, the genetic similarity among various varieties was calculated. The dendrogram constructed using UPGMA resulted in the 13 varieties resolving into two major clusters. These two clusters were comprised of five nondiapausing as one group and five diapausing varieties as the other. The genetic similarity estimated within and among silkworms is consistent with the pedigrees and geographical distribution of the varieties. Our study has demonstrated that the variability of DNA fingerprints within and among silkworm can provide an essential basis on which breeders may plan crossbreeding strategies to produce potentially heterotic hybrids.

The domesticated silkworm (*Bombyx mori*) comprises a large number of ecotypes and synthetic inbred lines that are distributed in temperate and tropical countries. These different varieties differ in their qualitative and quantitative traits that affect silk yield (Gamo 1983). The nondiapausing varieties available in tropical countries are poor silk yielders, although they are rapid breeders (Polyvoltine) and are hardy, that is, known to survive and reproduce efficiently under tropical conditions. The temperate varieties are invariably diapausing (uni- or bivoltine) and are endowed with higher silk yield of better quality. However, they fail to attain normal yield levels under tropical conditions (Nagaraja and Nagaraju 1995; Nagaraju et al. 1995). In other words, diapausing varieties are low silk yielders in the tropical conditions because of high levels of heat, humidity, diseases, and inadequate sanitary conditions during silkworm rearing (Datta and Nagaraju 1993; Goldsmith 1991). Although the classical silkworm breeding approaches, particularly crossbreeding of tropical and temperate varieties, have resulted in an overall increase in silk productivity, they have been unsuccessful in integrating the high-yielding traits of temperate varieties with the robustness of low-yielding tropical varieties.

Molecular marker-assisted breeding is expected to increase the speed and precision in silkworm breeding processes to integrate the desired characters from tropical and temperate varieties into elite varieties. However, until recently, there was a complete lack of information on the molecular analysis of the silkworm genome. In recent years attempts have been initiated to construct molecular linkage maps based on random amplified polymorphic DNAs (RAPDs; Pramboon et al. 1995) and restriction fragment length polymorphic DNAs (RFLPs; Shi et al. 1995).

DNA fingerprinting, first described by Jeffreys et al. (1985), is now commonly used to study genetic variability and to analyze pedigree relationships in a wide variety of organisms including insects (Blanchetot and Gooding 1994; Dallas 1988; Georges et al. 1988; Nybom 1991). It has been proven that PCR-based DNA fingerprinting using random arbitrary primers is a powerful tool in investigating the genetic diversity of silkworm varieties (Nagaraja and Nagaraju 1995). In addition, the potential use of a minisatellite probe, banded krait minor satellite DNA [Bkm-2(8); Aggarwal et al. 1994; Lang et al. 1993; Singh 1995; Singh and Jones 1986; Singh et al. 1980, 1984, 1988] in generating DNA fingerprints in silkworm has also been demonstrated (Nagaraju et al. 1995). Characterization and quantification of genetic diversity, both within and between populations, has long been a major goal in crop improvement programs. In silkworm breeding programs, information concerning the genetic diversity within a variety is essential for a rational use of genetic resources. It is particularly useful in the characterization of individual varieties and various ecotypes in detecting duplications in germplasm collection and serves as a general guide in the choice of parents for producing heterotic hybrids. The objectives of the present study were to examine genetic diversity within 13 silkworm varieties and to estimate genetic relatedness among them. The silkworm genotypes studied differ in the following characteristics: larval duration, cocoon weight, cocoon shell weight, silk filament length, and voltinism (refers to the number of life cycles in a year) (Nagaraja and Nagaraju 1995).

Materials and Methods

Silkworm Varieties

Six diapausing (Hu_{204}, Ka, NB_1, NB_7, NB_{18}, and NB_4D_2) and seven nondiapausing (C. nichi, Gungnong, Moria, Nistari, Pure Mysore, Diazo, and Sarupat) silkworm varieties, which differ from each other in a number of characteristics, were used in the present study. The characteristics of the varieties used are discussed in detail in Nagaraja and Nagaraju (1995).

Genomic DNA Extraction

For studying genetic variability within a variety, DNA from 8 male and 8 female moths of each of the 13 varieties was extracted separately. For analyzing genetic relatedness among the 13 varieties, DNA was isolated from silk glands of day 3 fifth instar larvae of each of the varieties (Suzuki et al. 1972). In brief, silk glands were ground in liquid nitrogen using a mortar and pestle. A buffer containing 100 mM Tris-HCl pH 8.0, 50 mM NaCl, 50 mM EDTA, and 1% SDS was added to it. The mixture was incubated at 37°C for 2 h with occasional swirling. The DNA was extracted once each with phenol, phenol : chloroform : isoamyl alcohol, and chloroform : isoamyl alcohol, ethanol precipitated, and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was treated with RNase A (100 μg/ml final concentration) at 37°C for 2 h, following which it was extracted with organic solvents, precipitated, and dissolved in TE as described above. DNA was quantified, using a known standard, on an agarose gel stained with ethidium bromide.

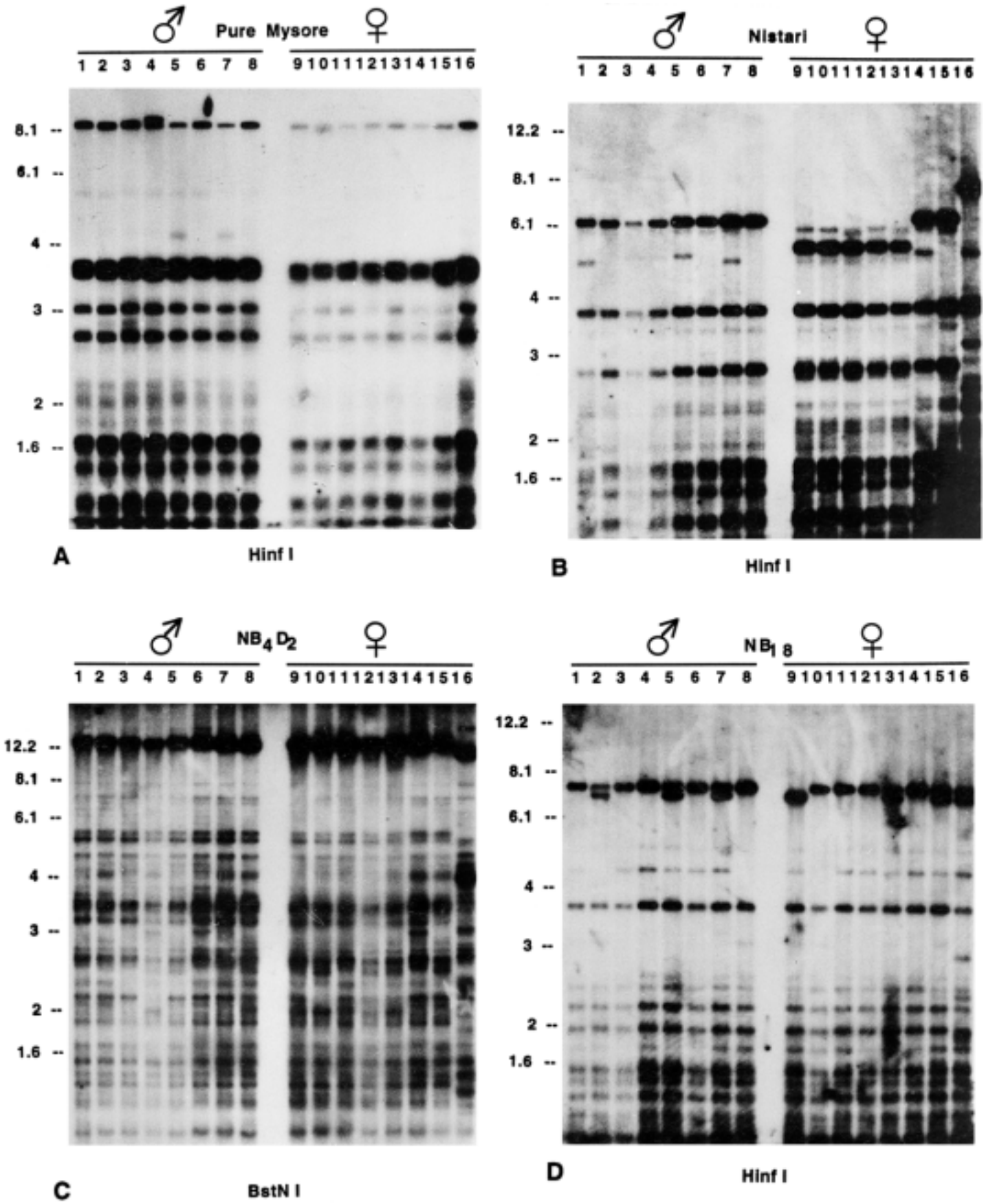

Figure 1. Bkm 2(8) hybridization pattern of DNA from **(A)** Pure Mysore, **(B)** Nistari, **(C)** NB_4D_2, and **(D)** NB_{18} silkworm varieties. Eight male and eight female moths of each variety were fingerprinted in a single gel. Males and females are indicated in the photograph. Restriction enzymes used were *Hin*fI (A, B, and D) and *Bst*NI (C). Note that individuals within a variety show more or less similar fingerprint profiles. Also note the absence of sex-specific hybridized bands. Each lane contains 8–10 μg of completely digested DNA. Numbers on the left indicate DNA fragment size in kilobase pairs.

Table 1. Similarity coefficients within various diapausing and nondiapausing varieties of *Bombyx mori* with respect to sex as well as restriction enzyme, sex, restriction enzyme, and irrespective of sex and restriction enzyme (mean)

	Sex and restriction enzyme								
	*Bst*NI		*Hin*fI		Sex		Restriction enzyme		
Varieties	Male	Female	Male	Female	Male	Female	*Bst*NI	*Hin*fI	Mean
HU_{204}	0.970 ± 0.02	0.968 ± 0.02	0.875 ± 0.05	0.866 ± 0.06	0.923 ± 0.06	0.917 ± 0.07	0.966 ± 0.02	0.870 ± 0.07	0.918 ± 0.06
KA	0.748 ± 0.14	0.774 ± 0.13	0.760 ± 0.11	0.905 ± 0.05	0.754 ± 0.13	0.837 ± 0.12	0.741 ± 0.14	0.805 ± 0.09	0.773 ± 0.12
NB_1	0.764 ± 0.12	0.801 ± 0.09	0.839 ± 0.12	0.725 ± 0.11	0.802 ± 0.13	0.763 ± 0.11	0.780 ± 0.10	0.739 ± 0.14	0.759 ± 0.12
NB_7	0.881 ± 0.05	0.908 ± 0.04	0.922 ± 0.04	0.954 ± 0.02	0.906 ± 0.05	0.928 ± 0.04	0.887 ± 0.05	0.923 ± 0.04	0.904 ± 0.05
NB_{18}	0.973 ± 0.02	0.989 ± 0.01	0.924 ± 0.04	0.900 ± 0.05	0.994 ± 0.04	0.945 ± 0.06	0.979 ± 0.02	0.908 ± 0.04	0.944 ± 0.05
NB_4D_2	0.885 ± 0.07	0.886 ± 0.07	0.946 ± 0.05	0.778 ± 0.34	0.916 ± 0.07	0.832 ± 0.25	0.872 ± 0.06	0.863 ± 0.26	0.867 ± 0.19
C. nichi	0.961 ± 0.03	0.947 ± 0.03	0.978 ± 0.02	0.980 ± 0.03	0.970 ± 0.03	0.964 ± 0.04	0.950 ± 0.03	0.970 ± 0.04	0.960 ± 0.03
Gungnong	0.886 ± 0.06	0.829 ± 0.09	0.900 ± 0.09	0.774 ± 0.17	0.893 ± 0.08	0.802 ± 0.14	0.848 ± 0.08	0.831 ± 0.14	0.840 ± 0.11
Moria	0.917 ± 0.05	0.826 ± 0.13	—	0.881 ± 0.09	0.917 ± 0.05	0.853 ± 0.12	0.835 ± 0.13	0.881 ± 0.08	0.847 ± 0.12
Nistari	0.841 ± 0.10	0.771 ± 0.15	0.887 ± 0.05	0.640 ± 0.31	0.864 ± 0.08	0.706 ± 0.25	0.717 ± 0.16	0.672 ± 0.24	0.695 ± 0.20
Pure Mysore	0.968 ± 0.01	0.962 ± 0.03	0.953 ± 0.04	0.895 ± 0.07	0.961 ± 0.03	0.929 ± 0.06	0.948 ± 0.03	0.921 ± 0.06	0.935 ± 0.05
Diazo	1.000 ± 0.00	0.722 ± 0.16	0.915 ± 0.06	0.728 ± 0.16	0.958 ± 0.06	0.725 ± 0.16	0.787 ± 0.16	0.796 ± 0.15	0.791 ± 0.16
Sarupat	0.925 ± 0.05	0.902 ± 0.04	0.836 ± 0.08	0.873 ± 0.10	0.880 ± 0.08	0.888 ± 0.08	0.888 ± 0.05	0.831 ± 0.08	0.860 ± 0.07

DNA Fingerprinting

For each gel lane, 8–10 μg of *Bst*NI- or *Hin*fI-digested DNA was loaded. Digested samples were electrophoresed in 30 cm long, 5 mm thick, 0.8% agarose gels at 60 V for 16–18 h in TPE buffer (15 mM Tris-HCl, 18 mM NaH_2PO_4, 0.5 mM EDTA, pH 7.8). Marker X (Boehringer Mannheim) was used as molecular weight markers. For analyzing similarity within a variety, DNA of all the 16 individuals (8 males and 8 females), digested with either *Bst*NI or *Hin*fI, were run in a single gel. Similarly, pooled DNA samples digested with either *Bst*NI or *Hin*fI were run in a single gel to estimate among variety differences. Gel fractionated DNA samples were transferred onto Hybond-N membrane (Amersham, UK) using a vacuum blotting assembly at 30 mm Hg (Olszewska and Jones 1988). The membranes were baked at 80°C for 2 h under vacuum. The blots were prehybridized in 7% SDS, 0.5% sodium phosphate buffer (pH 7.5) at 60°C for 2–3 h, and then hybridized with 1–2 × 10^6 cpm/ml of Bkm probe in the same but fresh buffer at 60°C for 14–18 h. The Bkm-2(8) DNA (Aggarwal et al. 1994; Lang et al. 1993; Singh 1995; Singh and Jones 1986; Singh et al. 1980, 1984, 1988) containing a 545 bp sequence consisting mainly of GATA repeats was used as a probe. Single-stranded ^{32}P-labeled probe was prepared to a specific activity of 0.7–3.0 × 10^8 cpm/μg (Hu and Messing 1982), using ^{32}P-dATP (specific activity 3000 Ci/mmol; Jonaki, BARC, India). After hybridization, blots were washed in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 sodium citrate, pH 7.2) con-

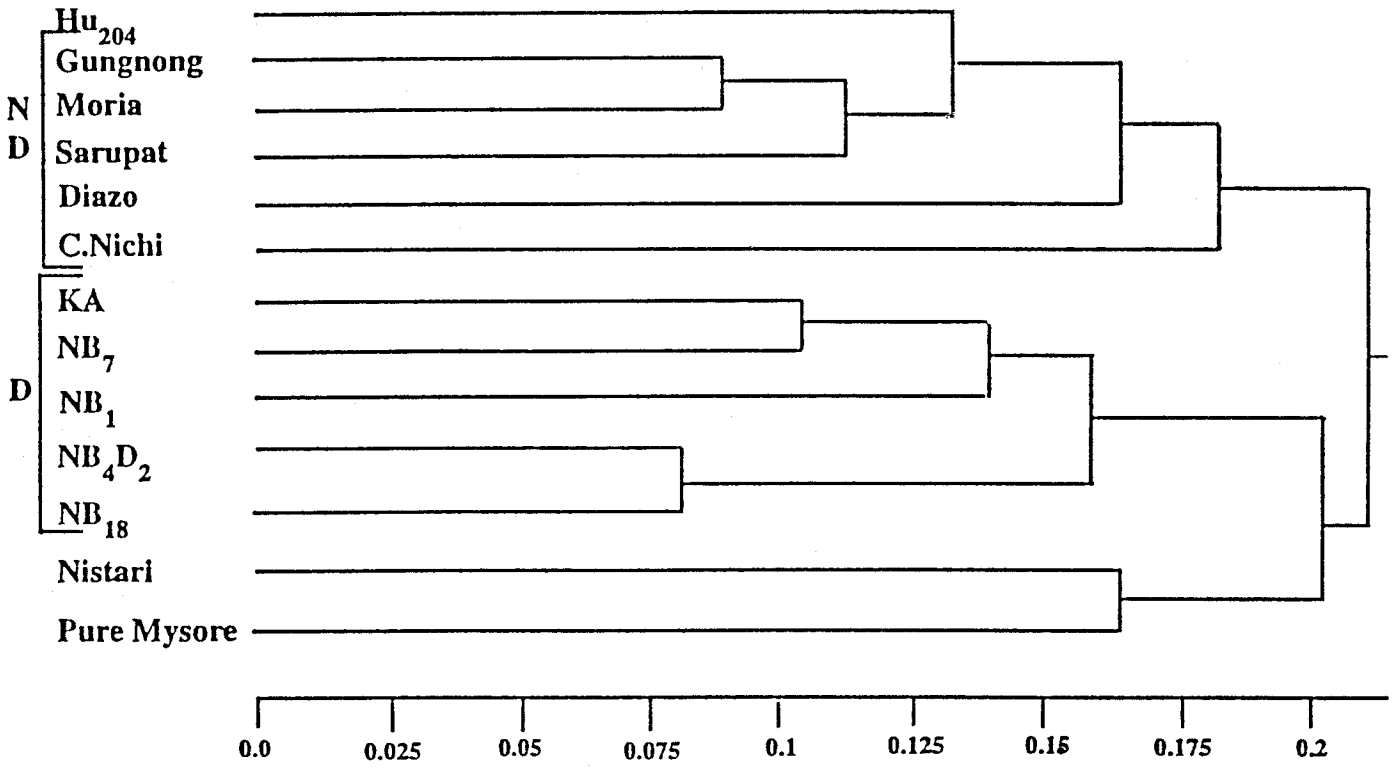

Figure 2. UPGMA phenogram showing relationships among various diapausing (D) and nondiapausing (ND) silkworm varieties. The phenogram is based on Bkm 2(8) fingerprinting of pooled DNA samples. Scale shows probable degree of divergence. See text for details.

taining 0.1% SDS for 15 min each, once at room temperature and twice at 60°C. The membranes were then exposed to x-ray films for 2–3 days.

Fingerprint Analysis

Autoradiographs were examined visually to score the number of hybridized bands. All bands showing similar molecular weights were considered to be identical. Each lane was scored for the presence or absence of a particular fragment. For all the DNA fingerprints analyzed, only distinguishable bands in the size range of 1.0–23 kb were scored. The similarity index (S) matrices were generated based on the number of shared fragments between each pair of fingerprints; $S = 2\ N_{AB}/(N_A + N_B)$ where N_{AB} is the number of bands shared by both lanes A and B, respectively, and N_A and N_B represented the total number of bands present in lanes A and B (Nei and Li 1979; Wetton et al. 1987). Mean and standard deviations were calculated (Blanchetot and Gooding 1994) using all possible pairwise combinations, that is, irrespective of sex and restriction enzyme as well as with respect to sex (male and female individuals), to restriction enzyme (*Bst*NI and *Hin*fI) and both. Using fingerprint data of pooled DNA samples digested with *Bst*NI and *Hin*fI, the genetic relatedness among the 13 silkworm varieties was estimated by calculating the difference value D in all possible pairwise combinations. The D between any two DNA fingerprint profiles was calculated as the number of bands that were different divided by the total number of fragments present in the two varieties (Gilbert et al. 1990). The D values were used to construct a phylogenetic tree using the UPGMA (unweighted pair group method with arithmetic means) option in the "neighbor" program (PHYLIP software, version 3.41; Felsenstein J, University of Washington, Seattle). Separate dendrograms were first constructed based on fingerprints obtained with the two restriction enzymes used, that is, *Bst* NI and *Hin*fI. As the two types of dendrograms were found to be similar, the fingerprint data resulting from the two enzymes were pooled together to construct the final dendrogram. The reliability of the dendrogram was also tested using other options (neighbor-joining and Fitsch–Margoliash) in the PHYLIP software. The UPGMA dendrogram was representative of all the dendrograms.

Results and Discussion

Genetic Variability Within Varieties

Genetic variability within silkworm varieties was analyzed based on DNA fingerprints using Bkm-2(8) derived probe on *Bst*NI- or *Hin*fI-digested DNA from male and female individuals of 13 silkworm varieties. A few representative examples of such profiles are shown in Figure 1A–D. DNA fingerprints of 104 male and 104 female individual silkworms revealed the hybridizable bands ranging from 1 to 23 kb. The DNA profile of individuals within a given variety showed a more or less identical pattern (Figure 1A–D, Table 1). Comparisons of the fingerprints were made irrespective of sex and restriction enzyme as well as with respect to sex and restriction enzyme (Table 1). No sex-specific DNA fingerprint pattern was observed (Figure 1). The similarity coefficient within a given variety did not show any variation with respect to sex or restriction enzyme (Table 1). In general, a high degree of similarity in Bkm DNA hybridization pattern of individuals within a variety was observed. These results on molecular similarity are highly valuable in view of the fact that in silkworm, only hybrids are reared for commercial silk production and high genetic similarity among individuals of each of the parental varieties involved in the hybrid is known to result in uniform, heterotic hybrids (Nagaraju et al. 1996).

Relationship Between Varieties

DNA fingerprinting with pooled DNA samples was carried out to study the genetic variation among the 13 silkworm varieties. Based on data from DNA profiles generated by Bkm 2(8)-derived probe, we constructed a dendrogram (Figure 2) that resolved the 13 silkworm varieties into two major clusters. These two clusters were comprised of five nondiapausing varieties and five diapausing varieties. The power of DNA fingerprinting in estimating the genetic relationship of populations in various species has been well demonstrated (Castagnone-Sereno et al. 1993; Meng et al. 1996; Nagaraja and Nagaraju 1995). The silkworm varieties (Moria and Sarupat) that shared the same geographical distribution are in the same cluster. Similarly the silkworm varieties (NB_4D_2 and NB_{18}) that are derived from the common pedigree are grouped in the same cluster. These studies clearly reveal the power of DNA fingerprinting in grouping silkworm varieties based on voltinism, geographical distribution, and pedigree relationships.

The results presented here demonstrate that DNA fingerprinting using multilocus Bkm 2(8)-derived probe offers a reliable and effective way of assessing genetic variability within and between the populations. However, what remains to be demonstrated is the association of such DNA profile-based genetic distance and the degree of heterosis and hybrid performance, which would provide a reliable avenue for crossbreeding programs in silkworm.

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References

Aggarwal RK, Majumdar KC, Lang JW, and Singh L, 1994. Generic affinities among Crocodilians as revealed by DNA fingerprinting with a Bkm derived probe. Proc Natl Acad Sci USA 91:10601–10605.

Blanchetot A and Gooding RH, 1994. Genetic variability and segregation analysis in *Glossina morsitans morsitans* (Diptera : Glossinidae) using DNA fingerprinting. Genome 37:289–295.

Castagnone-Sereno P, Piotte C, Vijthof J, Arad P, Wajnberg E, Vanlerberghe Masutti F, Bongiovanni M, and Dalmasso A, 1993. Phylogenetic relationship between amphimictic and parthenogenetic nematodes of the genus *Meloidogyne* as inferred from repetitive DNA analysis. Heredity 70:195–204.

Dallas JF, 1988. Detection of cultivated rice by hybridization with a human minisatellite probe. Proc Natl Acad Sci USA 85:6831–6835.

Datta RK and Nagaraju J, 1993. Emerging applications of biotechnology in sericulture. In: Commercialisation of biotechnologies for agriculture and aquaculture (Srivastav UK and Chandrashekhar S, eds)., IBH Oxford; 201–210.

Gamo T, 1983. Biochemical genetics and its applications to the breeds of the silkworm. JARQ 16:264–272.

Geroges M, Lequarre AS, Castelli M, Hanset R, and Vassart G, 1988. DNA fingerprinting in domestic animals using four different minisatellite probes. Cytogent Cell Genet 47:127–131.

Gilbert DA, Lehman N, O'Brien SJ, and Wayne RK, 1990. Genetic fingerprints reflect population differentiation in the California Channel Island fox. Nature 244:764–766.

Goldsmith MR, 1991. Silkworm breeding for the '90s: new molecular and genetic tools to meet the challenge of the tropics. Sericologia 31:145–155.

Hu N and Messing J, 1982. The making of strand-specific M13 probes. Gene 17:271–277.

Jeffreys AJ, Wilson V, and Theim SL, 1985. Hypervariable minisatellite regions in human DNA. Nature 314: 67–73.

Lang JW, Aggarwal RK, Majumdar KC, and Singh L, 1993. Individualization and estimation of relatedness in Crocodilians by DNA fingerprinting with a Bkm-derived probe. Mol Gen Genet 238:49–58.

Meng A, Gong G, Chen D, Zhang H, Qi S, Tang H, and

Figure 1. Scanning electron micrograph of the distantennapedia mutation. Abnormal swelling of the third antennal segment is apparent, as are leglike distal segments (tarsal segments) and presence of the tarsal claws.

Gao Z, 1996. DNA fingerprint variability within and among parental lines and its correlation with performance of F_1 laying hens. Theor Appl Genet 92:769–776.

Nagaraja GM and Nagaraju J, 1995. Genome fingerprinting of the silkworm, *Bombyx mori* using random arbitrary primers. Electrophoresis 16:1633–1638.

Nagaraju J, Sharma A, Sethuraman BN, Rao GV, and Singh L, 1995. DNA fingerprinting in silkworm, *Bombyx mori* using banded krait minor satellite DNA-derived probe. Electrophoresis 16:1639–1642.

Nagaraju J, Urs R, and Datta RK, 1996. Cross breeding and heterosis in silkworm, *Bombyx mori,* a review. Sericologia 36:1–20.

Nei M and Li WH, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273.

Nybom H, 1991. Application of DNA fingerprinting in plant breeding. In: DNA fingerprinting: approaches and applications (Burke T, Dolf G, Jeffreys AJ, and Wolf R, eds). Basel, Switzerland: Birkhauser Verlag; 294–311.

Olszewska E and Jones KW, 1988. Vacuum blotting enhances nucleic acid transfer. Trends Genet 4:92–94.

Pramboon A, Shimada T, Fujiwara H, and Kobayshi M, 1995. Linkage map of random amplified polymorphic DNAs (RAPDs) of the silkworm, *Bombyx mori.* Genet Res Camb 66:1–7.

Shi J, Heckel DG and Goldsmith MR, 1995. A genetic linkage map for the domesticated silkworm, *Bombyx mori* based on restriction fragment length polymorphisms. Genet Res Camb 66:109–126.

Singh L, 1995. Biological significance of minisatellites. Electrophoresis 16:1586–1595.

Singh L and Jones KW, 1986. Bkm sequences are polymorphic in humans and are clustered in pericentric regions of various acrocentric chromosomes including the Y. Hum Genet 73:305–308.

Singh L, Philips C, and Jones KW, 1984. The conserved nucleotide sequences of Bkm which define sex in the mouse are transcribed. Cell 36:111–120.

Singh L, Purdom IF, and Jones KW, 1980. Sex chromosome associated satellite DNA: evolution and conservation. Chromosoma 79:137–157.

Singh L, Winking H, Jones KW, and Gropp A, 1988. Restriction fragment polymorphism in the sex-determining region of the Y chromosomal DNA of European wild mice. Mol Gen Genet 212:440–449.

Suzuki Y, Gage LP, and Brown DD, 1972. The genes for fibroin in *Bombyx mori.* J Mol Biol 70:637–649.

Wetton JH, Carter RE, Parking DT, and Wallen D, 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327:147–149.

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An *Antennapedia*-like Mutation in *Nasonia vitripennis*

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There is growing interest in the comparative genetics of development. This interest has been further stimulated by recent discoveries of homologous homeotic genes involved in the development of vertebrates and invertebrates (Bachiller et al. 1994; Lewis 1994). Homeotic genes are major regulators of basic body plan development in complex multicellular organisms (Gerhart and Kirschner 1997). Homeotic mutations were originally discovered in *Drosophila melanogaster* as mutations that transformed one body part into another. Examples include Antennapedia (''antenna-foot''), a dominant mutation that transforms the adult antenna into a leg (Ashburner 1989; Rogers and Kaufman 1996), and spineless-aristapedia, a mutation that transforms the arista, a distal section of the antenna, into distal leg segments (Duncan et al. 1998). The Antennapedia gene occurs in a cluster (ANT-C) with several other homeotic genes involved in head and thoracic development. Homologous clusters are found in several other invertebrates and in vertebrates, and are generically referred to as Hox or Hom clusters (Bachiller et al. 1994). Comparisons of homeotic genes in diverse organisms could provide important insights concerning the evolution of developmental patterns.

Nasonia vitripennis is genetically among the most characterized species of Hymenoptera (the bees, wasps, and ants). As in other hymenopterans, *Nasonia* has haplodiploid sex determination—males develop from unfertilized haploid eggs, whereas females develop from fertilized diploid eggs. Haplodiploidy facilitates the detection of recessive visible mutations. A number of visible mutations are available in *Nasonia* (Saul et al. 1965), and a 99 marker molecular map has recently been generated (Gadau et al., unpublished data). In addition, *Nasonia* has a short generation time (2 weeks), an advantage for genetic studies. Here we report the discovery of a homeotic mutation in the parasitic wasp *Nasonia vitripennis* that transforms the distal portions of the adult antennae into leg segments. We name this mutant distantennapedia (*dant*). Based on a phenotypic characterization, this mutant is similar to Antennapedia and spineless-aristapedia mutations in *Drosophila,* but its genetic homologies have not yet been determined.

The distantennapedia (*dant*) mutation was generated using ethyl methyl sulfonate (EMS) as part of studies attempting to generate mutations in the paternal sex ratio (PSR) chromosome of *Nasonia* (Werren 1991). For mutagenesis, adult males were fed a sucrose solution containing 0.005 M EMS and then mated to females. Virgin females were then given hosts (*Sarcophaga bullata* fly pupae) for oviposition and progeny production. Due to haplodiploid sex determination, virgin females produce ample numbers of only haploid male progeny. One F_1 female yielded sons showing the transformation of the antennae into apparent leg segments.

The antenna-leg transformation clearly shows distal leg segments, including the leg tarsi (Figure 1). The first two proximal segments appear to be normal antenna segments. The third and following distal segments appear to have been transformed into leg segments. The third segment (normally the first antenna segment following the scape) is abnormally swol-

len. Subsequent segments are clearly leglike in appearance, and tarsal claws are apparent on the distal region of the transformed leg.

The distantennapedia mutant is recessive. Males are fertile; however, they are generally less successful in courtship than wild-type males, possibly due to the absence of functional antennae. For example, 16 of 37 *dant* males (43%) failed to mate in pairings with heterozygous females, whereas wild-type mating frequencies are typically close to 100%. Both heterozygous and homozygous females are fertile. However, homozygous females sting hosts at a lower frequency than do heterozygous females (84%, $N = 37$ for *dant*/+ females versus 52%, for *dant*/*dant* females ($N = 31$); $P < .01$ chi-square). Interestingly among the females who did parasitize hosts, *dant*/*dant* virgin females produced significantly more progeny than did *dant*/+ virgin females (74.9 ± 31.0 SD versus 56.3 ± 22.9 SD; $P = .03$ Student's *t* test). Among the progeny of virgin heterozygous females, there is a nearly equal frequency of *dant* and + males (48.9% *dant,* $N = 1353$). Among the female progeny in crosses between *dant*/+ females and *dant* males, 49.3% *dantdant* are produced ($N = 969$). These results indicate that *dant* males and *dant*/*dant* females have egg to adult survival equal to that of + males and *dant*/+ females, respectively.

Homozygous *dant*/*dant* females do not mate. This has been documented repeatedly during laboratory maintenance. This failure probably reflects the role of the antennae in female mate recognition. Due to inability of homozygous females to mate, the trait is typically maintained by mating *dant* males to *dant*/+ females. We have mapped the *dant* mutant to linkage group II, approximately 14 map units from the reddish eye color mutation *rdh5* (Saul et al. 1965). This is based on a total of 459 F_2 progeny, with 65 recombinants between *rdh5* and *dant.* A similar recombination rate of 22% was detected by a M. Pultz (personal communication). We are currently placing this mutation on the molecular marker map of *Nasonia.*

This mutation could be useful in isolating *Nasonia* homologs of homeotic genes found in other species. Likely candidates for homology are the drosophilid genes Antennapedia or spineless-aristapedia. The *Nasonia* distantennapedia mutation is phenotypically more similar to the spineless-aristapedia than to the drosophilid Antennapedia. Spineless-aristapedia mutations are alleles of the spineless (*ss*) locus. Alleles causing the aristapedia phenotype are recessive and involve transformations of distal antenna segments into leg segments, starting at the third segment. Alleles vary in expression from only a swelling of the third antennal segment to nearly complete transformations of the arista into tarsal segments, including formation of tarsal claws. In contrast, drosophilid Antennapedia mutations are dominant and typically involve complete transformations of the leg from most proximal antennal segment. Like spineless-aristapedia, the *Nasonia* distantennapedia mutation is recessive and involves the third and distal segments only. However, *Nasonia* do not have arista (a distal antenna structure found in some fly species), complicating the comparison.

The drosophilid Antennapedia locus maps within one major Hox cluster (ANT-C) and the spineless locus maps near the Bithorax cluster (BX-C), a second major Hox cluster found in *Drosophila melanogaster.* Therefore fine-scale mapping and cloning of the distantennapedia gene may provide molecular access to homeotic gene complexes in *Nasonia.* However, the homologies of this mutation to homeotic mutations in other species remains to be determined.

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References

Bachiller D, Macias A, Duboule D, and Morata G, 1994. Conservation of a functional hierarchy between mammalian and insect Hox/HOM genes. EMBO J 13:1930–1941.

Duncan DM, Burgess EA, and Duncan I, 1998. Control of distal antennal identity and tarsal development in *Drosophila* by spineless-aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev 12:1290–1303.

Gerhart J and Kirschner M, 1997. Cells, embryos, and evolution. Malden, Massachusetts: Blackwell Science.

Lewis EB, 1994. Homeosis: the first 100 years. Trends Genet 10:341–343.

Rogers BT and Kaufman TC, 1996. Structure of the insect head as revealed by the EN protein pattern in developing embryos. Development 122:3419–3432.

Saul GB II, Whiting PW, Saul SW, and Heidner CA, 1965. Wild-type and mutant stocks of *Mormoniella.* Genetics 52:1317–1327.

Werren JH, 1991. The PSR (paternal sex ratio) chromosome. Am Nat 137:392–402.

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Transmission Distortion at a Minisatellite Locus in the Harlequin Beetle Riding Pseudoscorpion

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The evolutionary importance of selfish genetic elements such as meiotic drive alleles remains controversial, at least in part because allelic transmission in large pedigrees has been examined in relatively few species. In a study involving 17 dams, 26 sires, and 418 offspring, we used single-locus DNA profiling to investigate patterns of allelic inheritance at the hypervariable c*Csc*MS13 minisatellite locus in the pseudoscorpion, *Cordylochernes scorpioides.* We detected one case of extreme transmission distortion (17:1), involving paternal alleles. Even when corrected for multiple comparisons, this bias was highly statistically significant. Since neither mutation nor linkage to a lethal recessive allele present in both parents can account for such a bias, the transmission distortion reported here seems most likely to be the result of meiotic drive.

Violations of Mendel's first law have been characterized in detail in the *segregation distorter* system (*SD*) of *Drosophila melanogaster* (Lyttle 1991, 1993; Moschetti et al. 1996; Palopoli and Wu 1996) and the *t* complex in *Mus musculus* (Patel-King et al. 1997; Silver 1993). There are fundamental differences in the way *t* and *SD* distorter alleles act (Hurst 1993; Lyon 1992). However, in both these systems, segregation distortion at autosomal loci affects heterozygous males and results from meiotic drive, a process in which one allele, the driver, acts during gametogenesis via a closely linked responder locus to sabotage gametes carrying alternative alleles (Lyttle 1991). In heterozygous *D. melanogaster* males, a substance is produced which impedes chromosome condensation in sperm carrying the wild-type allele, thereby halving the number of viable sperm (Hartl et al. 1967). Drive alleles can act only in the presence of a "sensitive" responder locus consisting of repetitive, heterochromatic DNA, with the degree of sensitivity positively correlated with the number of repeats (Pimpinelli and Dimitri 1989). Recently a further autosomal meiotic drive system has been identified in *M. m. musculus* with the apparently unusual

property of affecting females rather than males. In females that are heterozygous for an aberrant form of chromosome 1 found in remote Siberian populations (Agulnik et al. 1993), segregation distortion appears to be caused primarily by preferential transmission of the aberrant chromosome to the egg rather than to the polar body during the second meiotic division (see Ruvinsky 1995).

The general evolutionary importance of selfish genetic elements such as meiotic drive alleles remains controversial (Charlesworth et al. 1993; Hurst and Pomiankowksi 1991). On the one hand, it has been argued that meiotic drive is likely to be a rare or short-lived phenomenon (Maynard Smith and Szathmáry 1995), not only as a consequence of the stringent conditions required for the spread of drive alleles (tight linkage between a drive allele and an insensitive allele at the responder locus), but also because selection should strongly favor the evolution of suppresser alleles at modifier loci (Crow 1991). By contrast, Hurst and Pomiankowski (1991) have argued that meiotic drive may be relatively widespread. As these authors point out, it may be no coincidence that the species in which segregation distorters have been found are also well-studied species for which large pedigrees and genetic markers are available.

The development of single-locus minisatellite probes for the harlequin beetle riding pseudoscorpion (*Cordylochernes scorpioides*) has provided us with the opportunity to investigate patterns of allelic transmission at hypervariable loci in a viviparous, polyandrous arthropod (Zeh et al. 1997) that produces relatively large broods of offspring. Elsewhere we have reported significant transmission distortion in *C. scorpioides* at the c*Csc*MS23 locus (Zeh et al. 1994). Here we report a case of extreme transmission distortion in paternal alleles at c*Csc*MS13, a second *C. scorpioides* hypervariable minisatellite locus.

Materials and Methods

The DNA profiles used in this study were obtained from pedigrees which, in each case, consisted of a female, the two or three males to whom she had been mated in the laboratory, and a large, random sample of the female's offspring (for mating and rearing methods, see Zeh and Zeh 1992). While the majority of females (N = 14) were mated to unrelated males, three replications involved females mated to two of their full-sib brothers. Broods of first-stage nymphs were collected from the brood nest after hatching from the mother's external brood sac, and nymphs were then reared to the adult stage in individual vials. To provide DNA in sufficient quantity for the hybridization probe method of DNA profiling used in this study, it was necessary to sacrifice individuals for genotyping. Unfortunately, therefore, this study generated a "once-only" set of pedigrees in which parents exhibiting unusual transmission patterns could not be retested after genotyping. All mothers, putative sires and offspring were frozen at −70°C, pending molecular analysis.

DNA Profiling

DNA profiling was carried out as described elsewhere (Zeh et al. 1994), using the hypervariable single-locus minisatellite probe (c*Csc*MS13) cloned from a genomic library of *C. scorpioides.* Briefly, genomic DNA was isolated by grinding whole adults in 400 ml of 2× CTAB buffer and performing two chloroform and three phenol/chloroform extractions (Zeh et al. 1992). For each sample, one-third of the extracted DNA was retained (see below). The remaining two-thirds (2.5 μg) was digested for 10 h with a four-fold excess of *Hae*III or *Mbo*I and run on a 1% agarose gel for 36 h at 36 V in circulating TBE buffer. Size-fractionated DNA was capillary blotted and fixed onto nylon membranes (Zetabind, Cuno Inc.) by baking for 3 h at 80°C. Hybridization was carried out using double-stranded, gel-isolated probe inserts, random prime-labeled with ^{32}P. Membranes were hybridized at 63°C in phosphate buffer (Westneat et al. 1988) and washed for 30 min in 2× SSC, 0.1% SDS at 25°C and again at 65°C.

For each replication, DNA samples from the mother, the putative sires, and approximately 25 offspring were run on a single gel. For three-male replications, 10–15 additional offspring were run on a second gel with the remaining DNA of the parents. The highly variable nature of these minisatellite loci (heterozygosities ≥ 0.95; see Zeh et al. 1994) made it possible to assign paternity by simple visual comparison of offspring bands with putative paternal bands (Figure 1).

Analyzing Patterns of Allelic Transmission

A total of 43 heterozygous parents were examined for allelic transmission. Within each set of parents and offspring, parental alleles were assigned a letter alphabetically from highest to lowest molecular weight and the offspring genotyped accordingly. The extent of transmission ratio distortion (TRD) for each dam and sire was quantified by calculating a χ^2 goodness-of-fit statistic based on 1:1 Mendelian expectations and a P value determined from the cumulative binomial distribution.

Results

Our DNA profiling study of allelic transmission in the 17 dams and 26 sires detected one case of extreme transmission distortion. In one replication, in which a female was mated to three unrelated males and produced a mixed-paternity brood, the offspring sired by male A exhibited a 17:1 bias in transmission of paternal alleles (χ^2_1 = 14.22; see Figure 1). Assuming a pattern of Mendelian inheritance in which both alleles are transmitted with equal probability, it is extremely unlikely that such a bias, taken as an isolated case, could occur by chance (P = .000145 from binomial expectations). Moreover, the 17:1 bias remained highly significant, even when the significance level was adjusted for multiple tests using the sequential Bonferroni method (Rice 1989; sequential Bonferroni P = .05/43 = .001163).

A second case of an apparently significant deviation from the expected 1:1 ratio involved a 17:6 bias in the transmission of alleles of a female mated to two of her brothers. However, this distortion proved to be not significant when corrected for multiple comparisons (χ^2_1 = 5.261; $P >$ sequential Bonferroni value of .05/42).

Discussion

In our investigation of allele transmission at the c*Csc*MS13 hypervariable minisatellite locus in *C. scorpioides,* we found one instance of extreme transmission distortion in which 17 of a male's 18 offspring inherited his lower molecular weight allele. Elsewhere we have reported a similar but less extreme distortion (19:5) in paternal allele transmission at a second hypervariable minisatellite locus (c*Csc*MS23) in this pseudoscorpion (Zeh et al. 1994). Such deviations from 1:1 could result from meiotic drive acting during spermatogenesis to render sperm carrying nondrive alleles incapable of fertilization. Alternatively, transmission distortion in *C. scorpioides* might be caused by the differential mortality of genotypes, if for example the minisatellite locus were linked to a second locus with a lethal recessive allele occurring in the heterozygous state in the two par-

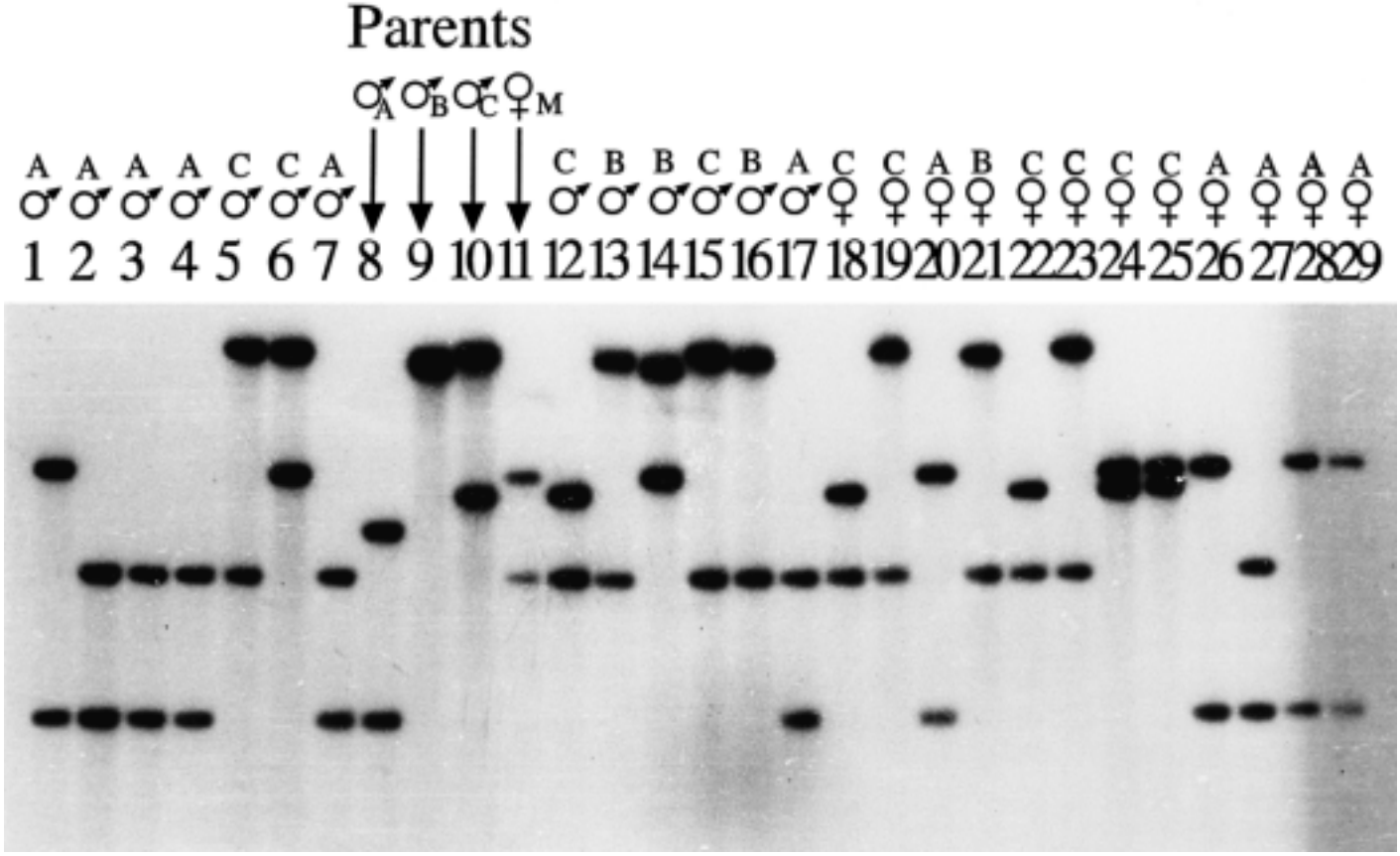

Figure 1. An apparent case of meiotic drive in *C. scorpioides.* A gel probed with c*Csc*MS13 demonstrates mixed paternity in the offspring of a female (M) mated to three males (A, B, and C). Above each offspring's lane we indicate the sex of the offspring and the identity of the sire. In this set of 25 progeny, male A sired 11 offspring, all carrying his lower molecular weight allele. A further 19 offspring were run on a second gel (not shown), of which male A sired seven. One offspring in this second group inherited male A's higher molecular weight allele, giving an overall 17:1 bias.

ents. Because of homozygote mortality, such linkage should result in a 2:1 transmission bias in both the sire and the dam. However, the pattern of paternal allelic transmission at the c*Csc*MS13 locus reported here differs significantly from the 2:1 bias predicted to result from such a lethal recessive linkage mechanism (χ^2_1 = 6.25, P = .014). In addition, transmission of maternal alleles to the offspring of the male in question showed no evidence of any bias (9:9). Mutation-based explanations for the 17:1 bias also seem extremely unlikely, since mutation rate at the c-*Csc*MS13 locus has been estimated to be only 0.006 (Zeh et al. 1997).

The pattern of high versus low molecular weight allelic transmission associated with cases of extreme transmission distortion in *C. scorpioides* is consistent with meiotic drive being the underlying mechanism. In the case of sabotage-based meiotic drive systems such as the *SD* locus in *Drosophila,* sensitivity to meiotic drive is positively correlated with the number of heterochromatic repeats possessed by alleles (and hence with their molecular weight) at a closely linked responder locus (Pimpinelli and Dimitri 1989). Thus c*Csc*MS13 might be, or might be closely linked to, a responder locus rather than being a drive locus itself. In this situation, one would expect to see the higher molecular weight allele being transmitted at a lower frequency, as was the case for both the 17:1 bias reported here and the 19:5 bias detected at the c*Csc*MS23 locus (Zeh et al. 1994).

Although meiotic drive appears to be the most likely explanation for transmission distortion in *C. scorpioides,* clearly more research is needed to unambiguously determine the mechanism responsible for biased allelic inheritance in this pseudoscorpion. One promising approach lies in the development of primers for PCR amplification of alleles at the c*Csc*MS13 locus. Amplification from minute quantities of DNA, for example, from parental hemolymph samples, would obviate the need to sacrifice individuals for genotyping, thereby enabling identification and propagation of distorter alleles in laboratory stocks.

The discovery of extreme bias in allelic inheritance in *C. scorpioides* is of general interest because it provides support for the view that departures from Mendelian inheritance may be more widespread in nature than is currently appreciated. The evolutionary significance of meiotic drive and other forms of transmission distortion remains uncertain (Hurst and Pomiankowski 1991; Zeh and Zeh 1996), largely because transmission distortion is difficult to detect in most organisms. Minisatellite markers have already proved to be extremely powerful tools for both forensics (Jeffreys et al. 1985) and paternity assignment (Burke 1989), as well as for fine-scale phylogenetic reconstruction (Armour et al. 1996; Gilbert et al. 1990). As the results of our study demonstrate, development of minisatellite markers for invertebrate species that produce large numbers of offspring also has the potential to provide a window on the still largely unexplored role of selfish genetic elements in determining patterns of allelic inheritance.

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References

Agulnik SI, Sabantsev ID, Orlova GV, and Ruvinski AO, 1993. Meiotic drive on aberrant chromosome 1 in the mouse is determined by a linked distorter. Genet Res 61:91–96.

Armour JA, Anttinen T, May CA, Vega EE, Sajantila A, Kidd JR, Kidd KK, Bertranpetit J, Paabo S, and Jeffreys AJ, 1996. Minisatellite diversity supports African origin for modern humans. Nat Genet 13:154–160.

Burke T, 1989. DNA fingerprinting and other methods for the study of mating success. Trends Ecol Evol 4: 139–144.

Charlesworth B, Coyne JA, and Barton NB, 1993. Meiotic drive and unisexual hybrid sterility: a comment. Genetics 133:421–424.

Crow JF, 1991. Why is Mendelian segregation so exact? BioEssays 13:305–312.

Gilbert DA, Lehman N, O'Brien SJ, and Wayne RK, 1990. Genetic fingerprinting reflects population differentiation in the California Channel Island fox. Nature 344: 764–767.

Hartl DL, Hiraizumi W, and Crow JF, 1967. Evidence for sperm dysfunction as the mechanism of segregation distortion in *Drosophila melanogaster.* Proc Natl Acad Sci USA 58:2240–2245.

Hurst LD, 1993. A model for the mechanism of transmission ratio distortion and for *t*-associated hybrid sterility. Proc R Soc Lond B 253:83–91.

Hurst LD and Pomiankowski A, 1991. Causes of sex ratio bias may account for unisexual sterility in hybrids: a new explanation of Haldane's rule and related phenomena. Genetics 128:841–858.

Jeffreys AJ, Wilson V, and Thein SL, 1985. Individual-specific "fingerprints" of human DNA. Nature 316:76–79.

Lyon MF, 1992. Deletion of mouse *t-complex distorter-1* produces an effect like that of the *t*-form of the distorter. Genet Res 59:27–33.

Lyttle TW, 1991. Segregation distorters. Annu Rev Genet 25:511–557.

Lyttle TW, 1993. Cheaters sometimes prosper: distortion of Mendelian segregation by meiotic drive. Trends Genet 9:205–210.

Maynard Smith J and Szathmáry E, 1995. The major transitions in evolution. Oxford: W.H. Freeman.

Moschetti R, Caizzi R, and Pimpinelli S, 1996. Segregation distortion in *Drosophila melanogaster*: genomic organization of responder sequences. Genetics 144:1665–1671.

Palopoli MF and Wu C-I, 1996. Rapid evolution of a coadapted gene complex: evidence from the *segregation distorter* (*SD*) system of meiotic drive in *Drosophila melanogaster.* Genetics 143:1675–1688.

Patel-King RS, Benashski SE, Harrison A, and King SM, 1997. A *Chlamydomonas* homologue of the putative murine *t* complex distorter Tctex-2 is an outer arm dynein light chain. J Cell Biol 137:1081–1090.

Pimpinelli S and Dimitri P, 1989. Cytogenetic analysis of segregation distortion in *Drosophila melanogaster*: the cytological organization of the responder (Rsp) locus. Genetics 121:765–772.

Rice WR, 1989. Analyzing tables of statistical tests. Evolution 43:223–225.

Ruvinsky A, 1995. Meiotic drive in female mice: an essay. Mamm Genome 6:315–320.

Silver IM, 1993. The peculiar journey of a selfish chromosome: mouse *t* haplotypes and meiotic drive. Trends Genet 9:250–254.

Westneat DF, Noon WA, Hudson KR, and Aquadro CF, 1988. Improved hybridization conditions for DNA "fingerprints" probed with M13. Nucleic Acids Res 16:4161.

Zeh DW and Zeh JA, 1992. Dispersal-generated sexual selection in a beetle-riding pseudoscorpion. Behav Ecol Sociobiol 30:135–142.

Zeh DW, Zeh JA, and Bermingham E, 1997. Polyandrous sperm-storing females: carriers of male genotypes through episodes of adverse selection. Proc R Soc Lond B 264:119–125.

Zeh DW, Zeh JA, Coffroth M-A, and Bermingham E, 1992. Population-specific DNA fingerprints in a neotropical pseudoscorpion (*Cordylochernes scorpioides*). Heredity 69:201–208.

Zeh DW, Zeh JA, and May CA, 1994. Charomid cloning vectors meet the pedipalpal chelae: single-locus minisatellite DNA probes for paternity assignment in the harlequin beetle-riding pseudoscorpion. Mol Ecol 3: 517–522.

Zeh JA and Zeh DW, 1996. The evolution of polyandry I: intragenomic conflict and genetic incompatibility. Proc R Soc Lond B 263:1711–1717.

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Microsatellite Variation Reveals Low Genetic Subdivision in a Chromosome Race of *Sorex araneus* (Mammalia, Insectivora)

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Two hundred and forty-five individuals of the common shrew (*Sorex araneus,* Insectivora, Mammalia) from 24 sampling localities situated in four different valleys of the western European Alps were genotyped for six microsatellite loci. Allelic variability ranged from 3 to 32 different alleles at a single locus and the average gene diversity over all loci was 0.69. An analysis for *F* and *R* statistics revealed weak genetic population subdivision (F_{st} = 0.032; R_{st} = 0.016). This suggests considerable gene flow and little phylogeographic structure within and between valleys. We tested whether a stepwise mutation model (SMM) better explained variation at the microsatellite loci than an infinite allele model (IAM). No trend in favor of either model was detected.

The common shrew (*Sorex araneus*) offers an opportunity to study patterns of chromosome evolution because more than 50 distinct chromosomal races have been described in this species (Zima et al. 1996). Many interracial hybrid zones were studied with a variety of genetic markers to understand the role of chromosomes in racial and species evolution (Brünner and Hausser 1996; Frykman et al. 1983; Fedyk et al. 1993; Lugon Moulin et al. 1996; Lukacova et al. 1993; Narain and Fredga 1996; Searle 1993; Szalaj et al. 1996). The main type of mutation responsible for chromosomal variation in the common shrew is centromeric (Robertsonian) fusion (Volobouev 1989). Fusion events between different acrocentrics occurred during isolation in the last ice ages of Europe (Searle 1984b) leading to population isolates exhibiting unequal chromosome combinations (monobrachial homologies). Metacentric chromosomes tended to spread subsequently through populations and became largely fixed (Taberlet et al. 1994). Forty of 60 possible types of metacentrics have been recorded. In 28 cases the metacentric is found in a fixed state in at least one chromosome race.

Several cytological and histological studies on male and female common shrews have shown that these centric fusions have little effect on fertility (Garagna et al. 1989; Searle 1984a; Wallace and Searle 1994). The effects of geographic subdivision and gene flow patterns are likely mechanisms responsible for the rapid fixation of centric fusions in different populations of the common shrew. Slatkin (1981) showed that geographic subdivision of populations and migration rates between these units strongly affects the fixation probabilities and fixation times of alleles of different types. Stochastic processes (genetic drift) are also thought to have a strong impact on the spread and fixation of a new chromosomal rearrangement (e.g., Chesser and Baker 1986; Hedrick 1981).

Therefore studies on gene flow and genetic structure of common shrew populations are a prerequisite to the understanding of their chromosomal evolution. Such studies were missing to date, due to the lack of polymorphic markers. Between karyotypically differentiated races, allozyme variation is low, and at the population level, informative polymorphic loci are absent (Frykman et al. 1983; Hausser 1991; Searle 1985). The recent isolation and characterization of polymorphic microsatellites for the common shrew (Balloux et al. 1998; Wyttenbach and Hausser 1996; Wyttenbach et al. 1997) allow us to

examine here patterns of gene flow and genetic structure of populations of *Sorex araneus* on a geographically small scale, within a chromosome race.

Materials and Methods

Characterization of Biological Material and Sampling Localities

A total of 245 individuals of the common shrew were trapped at 24 different localities in four distinct valleys in the Swiss and French Alps (Figure 1). The sample size at each locality consisted of 5 to 19 individuals. The habitat can be characterized as a subalpine area of the European Alps where pastures alternate with forests. Mountain ridges between the valleys are expected to function as barriers to migration, but alpine passes connecting the valleys might allow migration (see Figure 1). DNA samples of individuals were obtained by noninvasive sampling (toe clipping) and stored in 70% alcohol. All individuals were sampled within the chromosome race Bretolet. This race exhibits a variable karyotype and several chromosome combinations are found in homozygous or heterozygous states (Hausser 1991).

Genetic Analysis

Total genomic DNA was extracted by digestion with proteinase K overnight at 37°C and purified by extracting twice with phenol/chloroform and once with chloroform. The samples were then desalted and concentrated by ethanol precipitation. Six microsatellite loci (dinucleotides showing an AC-repeat motif, but L57 exhibiting a compound sequence with a CCA-trinucleotide) were used as genetic markers (Wyttenbach et al. 1997). Microsatellite polymorphism was analyzed using the polymerase chain reaction (PCR). The 10 μl reaction mixture contained 50–100 ng DNA template, 0.5 μM of each oligodeoxinucleotide primer, 100 μM dCTP, dGTP, and dTTP, 10 μM dATP, 0.02 μl ^{33}P-dATP at 1000 Ci/mmol, 1.5–3.0 mM $MgCl_2$, 1× ExtraPol II reaction buffer, and 0.5 U ExtraPol II *Taq* polymerase (Chemie Brunschwig AG, Switzerland). After an initial denaturing step of 3 min at 94°C, samples were processed through 32 cycles consisting of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C. After amplification, aliquots of the reaction mixtures were mixed with 0.5 volume of formamide loading buffer, denatured 2 min at 90°C, and electrophoresed on standard DNA sequencing gels (6% acrylamide, 8 M urea). Fixation, drying, and autoradiography followed standard procedures. A sequencing reaction was used as a size marker.

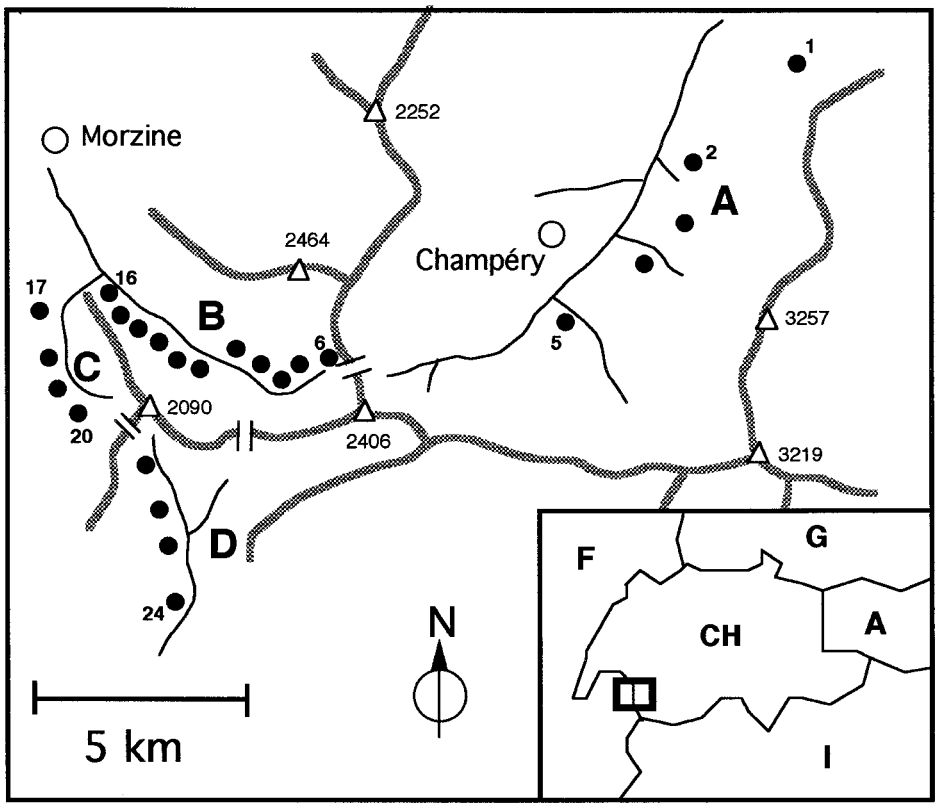

Figure 1. Geographic map showing the localities (full circles, $n = 24$) where individuals of the common shrew were lived trapped ($n = 245$); four distinct valleys (A, B, C, and D) separated by mountain ridges (thick lines) are indicated; thin lines correspond to rivers; valleys are interconnected by alpine passes; triangles represent tops of mountains (meters); insert (below right) indicates the geographic position of the trapping area on a larger scale (A, Austria; CH, Switzerland; G, Germany; I, Italy; F, France).

Statistical Analysis

Microsatellite Mutation Models

The mutational mechanisms associated with microsatellite evolution are debated (for a review see Goldstein and Pollock 1997). Therefore, when analyzing population genetic data using microsatellites, one should select an appropriate mutation model (Jarne and Lagoda 1996). Classically two extreme mutation models have been considered for microsatellite loci: the stepwise mutation model (SMM), originally developed for allozyme data (Ohta and Kimura 1973), and the infinite allele model (IAM), where each mutation event is supposed to create a new allele in the population (Kimura and Weiss 1964). To evaluate the adequacy of the IAM and SMM we computed the expected number of alleles (k_e) given the observed heterozygosity (H_o) for each locus and population under both mutation models using the method of Estoup et al. (1995a) and refined by Cornuet and Luikard (1997).

Genetic Polymorphism and Linkage Disequilibrium

Allele frequencies were obtained by counting band polymorphism after gel electrophoresis. Genetic polymorphism was estimated using Nei's unbiased gene diversity (Nei 1987). Allelic frequencies are available upon request (from A.W.). Exact tests for genotypic linkage disequilibrium were computed using GENEPOP 1.2 (Raymond and Rousset 1995). For all cases, the Markov chain was set to 100,000. The overall significance of multiple tests was estimated by Fisher's combined probability test.

Genetic Subdivision

F statistics were computed according to Weir and Cockerham (1984) using the software FSTAT 1.2 (Goudet 1995). Jackknifing over loci was performed. One-tailed tests of the significance of F_{is} and F_{st} were obtained from 5000 permutations of alleles within samples for F_{is} and 5000 permutations of genotypes among samples for F_{st}. In this way the distribution of the null hy-

Table 1. Observed (k_o) and expected (k_e) values of the number of alleles and observed heterozygosity (H_o) at six microsatellite loci under the infinitive allele size model (IAM) and stepwise mutation model (SMM)

	Locus	n	k_o	H_o	k_e (IAM)	P (IAM)	k_e (SMM)	P (SMM)
A	L9	102	17	0.89	19.5	0.29	11.5	0.03*
A	L16	102	3	0.44	3.7	0.36	3.0	0.67
A	L45	102	5	0.50	4.3	0.51	3.4	0.19
A	L57	100	12	0.67	6.9	0.02*	4.5	0.00*
A	L62	102	14	0.91	21.8	0.01*	13.2	0.44
A	L67	102	11	0.80	11.4	0.51	6.5	0.02*
B	L9	204	28	0.92	29.8	0.39	15.3	0.00*
B	L16	206	3	0.36	3.3	0.44	2.8	0.54
B	L45	206	8	0.38	3.5	0.01*	2.9	0.00*
B	L57	206	20	0.68	8.4	0.00*	4.9	0.00*
B	L62	206	14	0.90	25.1	0.00*	12.3	0.32
B	L67	206	9	0.78	2.7	0.13	6.4	0.13
C	L9	124	21	0.89	19.9	0.42	11.0	0.00*
C	L16	126	3	0.49	4.3	0.23	3.3	0.58
C	L45	126	4	0.19	1.9	0.15	1.9	0.06
C	L57	126	13	0.73	9.0	0.09	5.3	0.00*
C	L62	126	15	0.91	24.7	0.00*	14.3	0.46
C	L67	126	9	0.78	11.3	0.26	6.2	0.11
D	L9	88	20	0.94	27.1	0.04*	18.8	0.40
D	L16	88	3	0.52	4.3	0.23	3.4	0.56
D	L45	86	6	0.44	3.5	0.14	3.0	0.03*
D	L57	86	14	0.68	6.9	0.00*	4.6	0.00*
D	L62	88	12	0.84	13.6	0.35	7.9	0.06
D	L67	88	10	0.85	14.2	0.10	8.3	0.27

A, B, C, and D indicate the respective valley in which individuals were sampled (see Figure 1); IAM = infinite allele size model; SMM = stepwise mutation model; n = number of analyzed chromosomes; P (IAM) and P (SMM) are the probabilities that the number of alleles is as or more different from the theoretical value under both mutation models; *$P < .05$.

pothesis ($F_{is}/_{st} = 0$) is obtained and tested against the alternative hypothesis ($F_{is}/_{st} > 0$) (Excoffier et al. 1992; Goudet 1995). F_{st} is commonly used to estimate the number of migrants between subpopulations, under the assumptions of an island model at equilibrium between random genetic drift and migration (Slatkin 1987). Cockerham and Weir (1993) showed that the estimator θ of F_{st} can be related to the number of migrants N_m by $\theta = 1/(1 + 4N_m)$, providing that mutation rate is small. R_{st} is an F_{st}-like estimator of population differentiation derived by Slatkin (1995) that allows a better estimation of the number of migrants when the mutation pattern follows an SMM. We estimated R_{st} following Michalakis and Excoffier (1996) and Rousset (1996) who derived an R_{st}-like statistic obtained from the variance components of a nested ANOVA.

Isolation by Distance

Because F_{st} can be considered as a genetic distance (Reynolds et al. 1983), estimates among pairs of populations can be obtained to produce an F_{st} matrix using FSTAT. This matrix can be used to estimate whether the association to a matrix of geographical distances among the same pairs of populations is stronger than expected from chance employing Mantel tests (Manly 1991; Mantel 1967). We considered the shortest dispersal distance between localities to construct the geographic distance matrix. To test whether the association was statistically significant we carried out randomization tests using permutation procedures (5000). If the correlation between the two matrices was significantly positive, then genetic distances would increase with geographical distances, as is expected under isolation by distance (Slatkin 1993). The first distance matrix tested includes all localities (1–24). Valley D can be accessed not only through the alpine pass from locality 20 in valley C, but also through the alpine pass from locality eight in valley B (see Figure 1). Therefore a second distance matrix was tested in which localities 9–20 were omitted.

Table 2. Number of alleles, gene diversity, inbreeding coefficient (F_{is}), and fixation index (F_{st} and R_{st}) with their respective standard errors for each microsatellite locus

Locus	Number of alleles	Gene diversity	F_{is} (f)	SE (F_{is})	F_{st} (θ)	SE (F_{st})	R_{st}	$R_{st} < F_{st}$
L9	32	0.92	0.035	0.020	0.025***	0.008	0.007	Yes
L16	3	0.46	0.086	0.070	0.065***	0.038	−0.0019	Yes
L45	8	0.37	0.011	0.042	0.036**	0.026	−0.004	Yes
L57	24	0.69	0.066*	0.031	0.030***	0.014	0.019	Yes
L62	17	0.88	0.025	0.031	0.026***	0.008	0.044	No
L67	11	0.80	−0.003	0.038	0.028***	0.012	0.069	No
All loci	95	0.69	0.027*	0.017	0.032***	0.005	0.016	

Unbiased gene diversity was calculated after Nei (1987) where $H_e = 1—\Sigma(p_i)^2$, F_{is} (f), and F_{st} (θ) are given in jackknife values, confidence intervals within (F_{is}) and among (F_{st}) localities were obtained using 5000 permutations, R_{st} was calculated as described in the text.

*$P < .05$, **$P < .01$, ***$P < .001$.

Results

Microsatellite Mutation Models

The computations were applied individually to each of the four alpine valley samples. Table 1 provides observed and expected values with their respective probabilities of the number of alleles at each locus for each valley. There seems to be no general trend in favor of one mutation model or the other, the six loci showing a large array of possible situations. For two loci (L16 and L67), observed values are compatible with both mutation models in all four samples. For three other loci (L9, L45, and L62), different results are obtained in different samples. Note that for L9 and L62 the same model (SMM for L9, IAM for L62) is rejected in the same three samples (A, B, and C) whereas it is not in the last sample (D). For the last locus (L57), the observed number of alleles largely (and significantly for three samples out of four) exceeds expected values under both models.

Polymorphism, Heterozygosity, and Linkage Disequilibrium

Polymorphism at the six microsatellite loci was high, ranging from 3 to 32 alleles depending on the locus (Table 2). Gene diversity varied between 0.37 and 0.92. Average gene diversity over all marker loci was 0.69. Analysis for linkage disequilibrium resulted in nonsignificant values, suggesting no genetic linkage between the loci (data not shown).

Gene Flow and Population Structure

Over all loci, both F_{st} and F_{is} were significantly greater than zero, indicating a small, but significant heterozygote deficiency between and within localities (Table 2). F_{st} appeared concordant across marker loci and localities. The overall F_{st} of 0.032 is equivalent to about 8 "island

model immigrants" arriving at each locality per generation. Genetic differentiation between valleys was insignificant (data not shown). Values for F_{is} were not concordant across marker loci and localities. Locus 16 and locus 57 showed relatively high F_{is} values. With the exception of locus 57, all loci had F_{is} values that were not significantly greater than zero. Overall, R_{st} is 0.016 (Table 2), which is smaller in magnitude than F_{st} (0.032). Per locus estimates ranged from −0.019 to 0.069, showing much more variation across loci than F_{st}. The largest F_{st} estimate (0.065) was for locus L16, which had the lowest R_{st} estimate. Out of the six loci, four had lower R_{st} than F_{st}. The overall R_{st} provided an estimated number of "island model immigrants" of 15 individuals per generation.

Testing the geographic distance matrix against the pairwise F_{st} matrix, we found a significant positive correlation between the geographical and genetic distance in both cases ($r = 0.251$, $P < .025$ and $r = 0.275$, $P < .027$ omitting localities 9–20).

Discussion

Conventional F statistic analysis assumes that the genetic markers follow an infinite allele model (IAM) (Jarne and Lagoda 1996). Studies on the mutational mechanisms at microsatellite loci have shown that these markers might follow a stepwise mutation model (SMM) (e.g., Valdes et al. 1993), a two-phase mutation model (TPM) (DiRienzo et al. 1994) or an IAM (e.g., Estoup et al. 1995b). Our results do not show a trend in favor of either model. This supports the use of F and R statistics and indicates that there is no defined mutation model that universally explains the process of mutation at microsatellite loci (Feldmann et al. 1997). It is likely that the precise mutation process at a locus depends on the internal sequence of the microsatellite (perfect or imperfect sequence), the size of the repeat motif, the presence or absence of compound sequences (e.g., microsatellites consisting of a di- and trinucleotide), and the flanking sequences of the loci.

Genetic variability at allozyme loci in the common shrew is very low and the observed heterozygosity at more than 30 loci ranged from 0.03 to 0.07 (e.g., Frykman et al. 1983; Wojcik and Wojcik 1994). The six microsatellite markers used in the present study show a 10- to 20-fold increase in gene diversity (average H_e among six loci is 0.69). The absence of genetic linkage, the presumed neutrality of microsatellites, and the high allelic variability at the population level make these markers an excellent tool to study genetic population structure and patterns of gene flow on a small geographic scale.

The small, but significant heterozygote deficiency between localities ($F_{st} = 0.032$, $P < .0002$) indicates a slight geographic subdivision at the geographic level sampled. This means that the total sample (including the four valleys) cannot be regarded as collected from a panmictic population. Low geographic subdivision is also suggested by the overall R_{st} value (0.016). These results suggest an N_m of 8 to 15 (immigrants arriving at each locality in each generation) and therefore considerable gene flow among localities. Such a level of gene flow (indirect method) is in agreement with direct observations showing that these shrews are able to disperse several kilometers (Tegelström and Hansson 1987). Our results also support the view of Bengtsson and Frykman (1990). These authors found high levels of gene flow across hybrid zones between different chromosome races. However, their results were based on only one informative allozyme locus. As shown by the Mantel tests, spatial distance is associated with genetic distance. This shows that geographically closer populations are also closer genetically, provided that these populations had differentiated in situ (Sokal and Rohlf 1995).

The overall F_{is} value of 0.027 is small, but significantly greater than zero. Values for F_{is} are not concordant across marker loci and localities. Only locus 57 is significantly different from zero. A closer inspection of the F_{is} values across localities revealed that high F_{is} values were clustered in valley D. Omitting the four localities of valley D and reanalyzing the sample retaining the 20 localities from valleys A, B, and C resulted in nonsignificant values ($F_{is} = 0.019$, $P = .111$). This finding suggests that within the majority of localities (20 of 24), individuals mate randomly and therefore no inbreeding is expected. This is in accordance with the observation that young shrews disperse when they leave their mothers, and they do not live in social groups (Croin-Michielsen 1966). Our results, together with data on allozyme variation following Hardy–Weinberg proportions (Frykman et al. 1983; Searle 1985), are good evidence that the common shrew is an outbreeding species.

Summarizing, the genetic population structure of the common shrew can be described as consisting of continuous populations showing little phylogeographic structure with individuals diffusing to fairly large distances, even among valleys separated by alpine passes. An isolation by distance model seems to be adequate to explain the genetic structure in this species. If we want to get a clear picture of the chromosome evolution in the common shrew, it is important to understand which mechanisms contribute to the rapid spread and fixation of metacentric chromosomes. The high migration rate of the common shrew, as shown in the present study, might enable a newly arisen centric fusion to spread quickly through a population. Stochastic processes such as drift are influenced by the genetic population structure of a species. Therefore microsatellites will be useful to study the role of genetic structure in relation to fixation processes of chromosomal rearrangements in the common shrew.

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References

Balloux F, Ecoffey E, Fumagalli L, Goudet J, Wyttenbach A, and Hausser J, 1998. Microsatellite conservation, polymorphism, and GC content in shrews of the genus *Sorex* (Insectivora, Mammalia). Mol Biol Evol 15:473–475.

Bengtsson BO and Frykman I, 1990. Karyotype evolution: evidence from the common shrew (*Sorex araneus* L.). J Evol Biol 3:85–99.

Brünner H and Hausser J, 1996. Genetic and karyotypic structure of a hybrid zone between the chromosomal races Cordon and Valais in the common shrew, *Sorex araneus.* Hereditas 125:147–158.

Chesser RK and Baker RJ, 1986. On factors affecting the fixation of chromosomal rearrangements and neutral genes: computer simulations. Evolution 40:625–632.

Cockerham CC and Weir BS, 1993. Estimation of gene flow from F-statistics. Evolution 47:855–863.

Cornuet JM and Luikart G, 1997. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144: 2001–2014.

Croin-Michielsen N, 1966. Intraspecific and interspecific competition in the shrews *Sorex araneus* L. and *Sorex minutus* L. Arch Néerland Zool 17:73–174.

DiRienzo AA, Peterson C, Garza JC, Valdes AM, Slatkin M, and Freimer NB, 1994. Mutational processes of simple-sequence repeat loci in human populations. Proc Natl Acad Sci USA 91:3166–3170.

Estoup A, Garnery L, Solignac M, and Cornuet JM,

1995a. Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 140:679–695.

Estoup A, Tailliez C, Cornuet JM, and Solignac M, 1995b. Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apis mellifera* and *Bombus terrestris* (Apidae). Mol Biol Evol 12: 1074–1084.

Excoffier L, Smouse PE, and Quattro JM, 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Fedyk S, Zajkowska M, and Chetnicki W, 1993. Study of a contact between two chromosomally monomorphic races of *Sorex araneus* L. (common shrew). Heredity 71: 221–226.

Feldman MW, Bergman A, Pollock DD, and Goldstein DB, 1997. Microsatellite genetic distances with range constraints: analytic description and problems of estimation. Genetics 145:207–216.

Frykman I, Simonsen V, and Bengtsson BO, 1983. Genetic differentiation in *Sorex* I. Electrophoretic analysis of the karyotypic races of *Sorex araneus* in Sweden. Hereditas 99:279–292.

Garagna S, Zuccotti M, Searle JB, Redi CA, and Wilkinson PJ, 1989. Spermatogenesis in heterozygotes for Robertsonian chromosomal rearrangements from natural populations of the common shrew, *Sorex araneus.* J Reprod Fert 42:167–171.

Goldstein DB and Pollock DD, 1997. Launching microsatellites: a review of mutation processes and methods of phylogenetic inference. J Hered 88:335–342.

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

Hausser J, 1991. The cytogenetics of the *Sorex araneus* group and related topics. Proceedings of the ISACC Second International Meeting, Lausanne and Arzier, Switzerland. Mem Soc Vaud Sci Nat 19:1–151.

Hedrick PW, 1981. The establishment of chromosomal variants. Evolution 35:322–332.

Jarne P and Lagoda PJL, 1996. Microsatellites: from molecules to populations and back. Trends Ecol Evol 11:424–429.

Kimura M and Weiss GH, 1964. The stepping stone model of population structure and the decrease of the genetic correlation with distance. Genetics 49:561–576.

Lugon Moulin N, Wyttenbach A, Brünner H, Goudet J, and Hausser J, 1996. Study of gene flow through a hybrid zone in the common shrew (*Sorex araneus*) using microsatellites. Hereditas 125:159–168.

Lukacova L, Pialek J, and Zima J, 1993. A hybrid zone between the Ulm and Drnholnec karyotypic races of *Sorex araneus* in the Czech Republic. Fol Zool 43(suppl 1):53–61.

Manly BJF, 1991. Randomization and Monte Carlo methods in biology. London: Chapman & Hall.

Mantel N, 1967. The detection of disease clustering and generalized regression approach. Cancer Res 27:209–220.

Michalakis Y and Excoffier L, 1996. A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics 142:1061–1064.

Narain Y and Fredga K, 1996. A hybrid zone between the Hällefors and Uppsala chromosome races of *Sorex araneus* in central Sweden. Hereditas 125:137–145.

Nei M, 1987. Molecular evolutionary genetics. New York: Columbia University Press.

Ohta T and Kimura M, 1973. The model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a genetic population. Genet Res 22:201–204.

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

Reynolds J, Weir BS, and Cockerham CC, 1983. Estimation of the coancestry coefficient: basis for a short-term genetic distance. Genetics 105:767–779.

Rousset F, 1996. Equilibrium values of measures of population subdivision for stepwise mutation processes. Genetics 142:1357–1362.

Searle JB, 1984a. Nondisjunction frequency in Robertsonian heterozygotes from natural populations of the common shrew, *Sorex araneus* L. Cytogenet Cell Genet 38:265–271.

Searle JB, 1984b. Three new karyotypic races of the common shrew *Sorex araneus* (Mammalia: Insectivora) and a phylogeny. Syst Zool 33:194–194.

Searle JB, 1985. Isoenzyme variation in the common shrew (*Sorex araneus*) in Britain, in relation to karyotype. Heredity 55:175–180.

Searle JB, 1993 Chromosomal hybrid zones in eutherian mammals. In: Hybrid zones and the evolutionary process (Harrison RG, ed). New York: Oxford University Press; 309–353.

Slatkin M, 1981. Fixation probabilities and fixation times in a subdivided population. Evolution 35:477–488.

Slatkin M, 1987. Gene flow and the geographic structure of natural populations. Science 236:787–792.

Slatkin M, 1993. Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47:264–279.

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

Sokal RR and Rohlf FJ, 1995. Biometry. New York: W.H. Freeman.

Szalaj KA, Fedyk S, Banaszek A, Chetnicki E, and Ratkiewicz M, 1996. A hybrid zone between two chromosome races of the common shrew, *Sorex araneus,* in eastern Poland: preliminary results. Hereditas 125:169–176.

Taberlet P, Fumagalli L, and Hausser J, 1994. Chromosomal versus mitochondrial DNA evolution: tracking the evolutionary history of the southwestern European populations of the *Sorex araneus* group (Mammalia, Insectivora). Evolution 48:623–636.

Tegelström H and Hansson L, 1987. Evidence of long distance dispersal in the common shrew (*Sorex araneus*). Z Säugetier 52:52–54.

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

Volobouev VT, 1989. Phylogenetic relationships of the *Sorex araneus-arcticus* species complex (Insectivora, Soricidae), based on high-resolution chromosome analysis. J Hered 80:284–290.

Wallace BMN and Searle JB, 1994. Oogenesis in homozygotes and heterozygotes for Robertsonian chromosomal rearrangements from natural populations of the common shrew, *Sorex araneus.* J Reprod Fertil 100:231–237.

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

Wojcik JM and Wojcik AM, 1994. Protein variation in the common shrew (*Sorex araneus* L.) in Poland, in relation to karyotype. Fol Zool 43(suppl 1):53–61.

Wyttenbach A and Hausser J, 1996. Microsatellites in *Sorex araneus* (Insectivora): highly polymorphic markers for population genetic studies within and between chromosomal races. Hereditas 125:209–217.

Wyttenbach A, Favre L, and Hausser J, 1997. Characterisation of simple sequence repeats in the genome of the common shrew. Mol Ecol 6:797–800.

Zima J, Fedyk S, Fredga K, Hausser J, Mishta A, Searle JB, Volobouev VT, and Wojcik JM, 1996. The list of the chromosome races of the common shrew (*Sorex araneus*). Hereditas 125:97–107.

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