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

T. R. Cary and C. D. Nickell

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 petiolule-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 single 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 cupped-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 trifoliate 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), 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 petiole length, but not as pronounced as the parental short petiole line. This suggested incomplete dominance for the gene controlling short petiole. 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-petiole 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 petiole 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 petiole length. Data from the F_{2:3} families confirm that the short petiole trait is controlled by a single incompletely dominant gene. When short and intermediate (heterozygous plants) petiole 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 petiole trait. We propose the genotype of short petiole plants for leaf type is {\textit{lclc}} and the genotype for the intermediate (heterozygous) individuals is {\textit{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

<table>
<thead>
<tr>
<th>Cross</th>
<th>Leaf type classification of 40 randomly selected F2 plants</th>
<th>Actual leaf type of F2:3 families derived from the 40 randomly selected F2 plants. F2:3 families classified as short or normal</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN89-3502TP × Thorne</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN89-3502TP × Hartwig</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN89-5699 × LN89-3502TP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ran × LN89-3502TP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorne × LN89-3502TP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
- Short (petiolule lengths less than 15 mm and cupped leaves).
- Intermediate (petiolule lengths between 16 and 29 mm and semicupped leaves).
- Normal (petiolule lengths greater than 30 mm and normal leaves).
- Leaf type classification of 40 randomly selected F2 plants. We are in the process of doing an allelism test with LN89-3502TP and an isolate having Pd1.
- Actual leaf type of F2:3 families derived from the 40 randomly selected F2 plants. F2:3 families classified as short or normal were derived from F2 plants classified as the same. Additional F2:3 families classified as short or normal were produced by misclassified intermediate F2 plants. F2:3 families classified as I were segregating for leaf type.
- One F2:3 row was lost due to planting error.

short, intermediate, and normal petiole lengths. All short petiole plants had fully cupped leaves, all intermediate plants had intermediate cupped leaves, and all normal petiole 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 F2 populations and F2:3 families. All plants classified as normal had normal pubescence. Trichrome counts of LN89-3502TP were not significantly different from counts of an isolate having the Pd1 gene for dense pubescence. This may indicate a close linkage between the genes lc for short petiole and Pd1 for dense pubescence. However, the expected recombination of a short petiole plant with normal pubescence was not observed in 5,000 plants observed in the F2 populations and F2:3 families. We are in the process of doing an allelism test with LN89-3502TP and an isolate having Pd1.

The results of this study demonstrate the short petiole 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 petiole trait is not fully understood and provides an area for further study.

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References

Received May 26, 1998
Accepted September 30, 1998
Corresponding Editor: Reid Palmer

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 homesteads 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 recognized 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 (Sclötterer 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 AACGC TTCCG-3′ (Refseth et al. 1997); linker oligo B (S62): 5′-GATCC GAAGCTT GGGTGCT CTGGCC-3′ (Refseth et al. 1997); vector oligo (S4): 5′-TATACGACTCATATA GGG-3′ (T7 promoter, Promega protocols); vector oligo (S15): 5′-TACACAGGAAAACAG CTATGAC-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 Sau3A 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 Sau3A (Biolabs) at 37°C for 3 h, followed by heat inactivation of the Sau3A 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 MagneticSphere® 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-
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).

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

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Primer sequences (5’-3’)</th>
<th>GenBank numbers</th>
<th>(Repeat unit)n in clone</th>
<th>Number of allelesb</th>
<th>Ho (%)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EST1</td>
<td>GCACTCTGTATTTTAGTGGTTC GAACACACAGCAACGCTTCCA</td>
<td>AF960966</td>
<td>(AAAG)α10</td>
<td>7</td>
<td>80</td>
<td>234-266</td>
<td>55</td>
</tr>
<tr>
<td>EST2</td>
<td>CAGTGAACTTGAGTGAGAGC CTGGAATCATGCAAACTTCTT</td>
<td>AF960967</td>
<td>(AAAG)α9</td>
<td>9</td>
<td>80</td>
<td>205-257</td>
<td>52</td>
</tr>
<tr>
<td>EST3</td>
<td>CCACCTAAGGAACAAAGACCTG CTTTTACCATGCATGACCGG</td>
<td>AF960968</td>
<td>(GATG)α14 (AAAG)α7</td>
<td>10</td>
<td>90</td>
<td>280-332</td>
<td>60</td>
</tr>
<tr>
<td>EST4</td>
<td>ATCCAAATCTCCGCTTCTCTTA GCCAAAGGATTTACTCCTAGAG</td>
<td>AF960969</td>
<td>(AAAG)α8</td>
<td>11</td>
<td>90</td>
<td>146-190</td>
<td>52</td>
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<tr>
<td>EST5</td>
<td>GTTGTATCTCTAGCTGGACAG GCCCTCTGCCCTCTATCTCT</td>
<td>AF960970</td>
<td>(AAAG)α13</td>
<td>8</td>
<td>80</td>
<td>163-189</td>
<td>60</td>
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<tr>
<td>EST6</td>
<td>CAGTGAACTTGAGTGAGAGC CTGGAATCATGCAAACTTCTT</td>
<td>AF960971</td>
<td>(AAAG)α15</td>
<td>8</td>
<td>80</td>
<td>101-141</td>
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<td>EST7</td>
<td>TCTGGAATCTCTCATGCAAACTTCTT GCATGAAAACTGGTGACTGC</td>
<td>AF960972</td>
<td>(AAAG)α12</td>
<td>9</td>
<td>60</td>
<td>235-263</td>
<td>54</td>
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<tr>
<td>EST8</td>
<td>GCCCTCTGCCCTCTATCTCT CTGGCATTCTGACATAATCT</td>
<td>AF960973</td>
<td>(AAAG)α10</td>
<td>8</td>
<td>70</td>
<td>296-332</td>
<td>55</td>
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<tr>
<td>EST9</td>
<td>AAACAGCCCAGACACCATGAC ATGTATTTCCTCACCATGAC</td>
<td>AF960974</td>
<td>(AAAG)α3</td>
<td>7</td>
<td>0+</td>
<td>114-178</td>
<td>55</td>
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<tr>
<td>EST12</td>
<td>CATGGAATCTCTCATGCAAACTTCTT CTGGGATTCTGGATTG</td>
<td>AF960975</td>
<td>(AAAG)α17</td>
<td>2</td>
<td>10</td>
<td>129-141</td>
<td>55</td>
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<tr>
<td>EST14</td>
<td>ATGGGATTTTCCTCACCATGAC ATCTGATTTCAACACTGAC</td>
<td>AF960976</td>
<td>(AAAG)α10</td>
<td>5</td>
<td>40+</td>
<td>156-184</td>
<td>55</td>
</tr>
</tbody>
</table>

a May contain nonamplifying alleles.
b Number of alleles and heterozygosity (H₀) values were determined from 10 individuals.

**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)α6 primer (S6). The PCR reactions were performed using a hotstart approach in a total volume of 10 μl with 1× PCR buffer (AmpliTaq Gold buffer from Perkin Elmer), 4 mM MgCl₂, 0.2 mM of each dNTP, and 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 H₂O. 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 sequencer. 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-32P-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 tetrancleotide 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 sequence 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 directly be 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 8% and 90% (Table 1). Microsatellite alleles showed Mendelian patterns of segregation through one generation for 10 of these loci by analysis of 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 a related taxa. Of five loci trialed (EST2, EST3, EST4, EST8, EST9), four (except EST3) were successfully amplified in closely related species and genera, including Egerinia, Corucia, Cyclodomorphus, and Tiliqua (data not shown). Several other species of Egerinia 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.

References


Received January 20, 1998
Accepted June 30, 1998

Corresponding Editor: Stephen J. O’Brien

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

S. M. Shuster and L. Levy

Cuticular pigmentation is highly variable in Paracercis 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 heterogamy 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 Chariaux-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 heterogamy (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 heterogamy (ZW = females, ZZ = males) and that Str is W linked.

Materials and Methods
Field Collections
Isopods were collected from the spongozoa of the intertidal sponge Leucetta los- angelesensis 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 take 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 Str allel 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 and Tfr alleles do not interact, the use of AmsAmsAmsTfrTfr males 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 (Amphipora sp.) and brine shrimp flakes, with seawater changed every 4 days, as described in Shuster and Sassaman (1997).

Five of the F1 females were crossed to unmarked α-1-1 sires, and the F2 generation was reared to maturity as described above. Five F1 females were crossed to unmarked α-1-1 sires, three F1 males were crossed to unmarked α-1-1 females, and the F2 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: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 < 0.001, 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).
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 \( \Sigma G_{(d=11)} = 8.93, P > .50; G_{\text{pooled}(d=1)} = 1.26, P > .20; G_{\text{heterogeneity}(d=10)} = 7.67, P > .50; \) Table 1). Among adults, both Str and family sex ratio showed Mendelian inheritance within and among all families \( \Sigma G_{(d=11)} = 7.75, P > .70; G_{\text{pooled}(d=1)} = 0.79, P > .50; G_{\text{heterogeneity}(d=10)} = 6.96 \) 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 = \( 2.55 \times 10^{-10}, N = 103 \)). Unmarked \( F_3 \) 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 \( \alpha \) sires from families with unbiased sex ratios (lineage \( \alpha-1 \)) 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 \( \alpha \) males, resembles Str but involves a somewhat different distribution of cuticular pigmentation (i.e., a longitudinal band of dark pigment on the \( \text{distal} \) 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 \( Paracerceis sculpta \) \((F_1-F_3)\)

<table>
<thead>
<tr>
<th>Generation</th>
<th>At birth*</th>
<th>At maturity</th>
<th></th>
<th>Sex ratios/#/Str</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Str</td>
<td>+</td>
<td>Str</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>( G_{(d=1)} )</td>
<td>( G_{(d=10)} )</td>
<td>( G_{(d=1)} )</td>
<td>( G_{(d=10)} )</td>
</tr>
<tr>
<td>( F_1 )</td>
<td>34</td>
<td>38</td>
<td>72</td>
<td>0.22</td>
</tr>
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<td></td>
<td>13</td>
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<td>22</td>
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<td>6</td>
<td>8</td>
<td>14</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>20</td>
<td>29</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>21</td>
<td>38</td>
<td>0.42</td>
</tr>
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<td></td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>1.36</td>
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<tr>
<td>( F_2 )</td>
<td>18</td>
<td>24</td>
<td>42</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>21</td>
<td>48</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>18</td>
<td>36</td>
<td>0.00</td>
</tr>
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<td></td>
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<td>28</td>
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<td></td>
<td>21</td>
<td>22</td>
<td>43</td>
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<tr>
<td>Total</td>
<td>181</td>
<td>203</td>
<td>384</td>
<td>8.93</td>
</tr>
</tbody>
</table>

* \( G_{(d=1)} = 1.26, P > .10; G_{(d=10)} = 7.67, P > .50. \)

* \( G_{(d=1)} = 0.79, P > .10; G_{(d=10)} = 6.96, P > .10. \)

* \( G_{(d=1)} = 1.18, P > .01; G_{(d=10)} = 6.57, P > .50. \)

* \( P < .05. \)
High Levels of Conservation at Microsatellite Loci Among Ictalurid Catfishes

Z. Liu, G. Tan, H. Kucuktas, P. Li, A. Karsi, D. R. Yant, and R. A. Dunham

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 ca-
thus) and flathead catfish (Pylodictus olivari-
as) loci were also amplified. The micro-
satellite markers are highly polymorphic for all catfish species tested and are herited 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 mark-
ers allowing generation of maximum geneti-
ic 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 (Ictalurus punctatus) × blue catfish (I. furca-
tus) 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 estab-
lished (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 tetracnucleotide 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 EcoRV, Rsal, and HaeIII. 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, La Jolla, California). Plasmid vector was digested by restriction endonuclease EcoRV and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer 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 titrating the efficiency of 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)n, (GA)n, (AAT)n, (CCAT)n, (CAG)n, and (CAA)n. 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-32P 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 MgCl2, 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-
ion 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 repeat sequence (Table 1). Eight were composite microsatellites containing two or more types of repeat sequences. Most microsatellite clones contained high A/T-rich microsatellite Repeat. 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.
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 genera. Thirteen of 14 pairs 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 Wintere 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 Wintere 1995; Menotti-Raymond and O’Brien 1995). Recently Zardoya et al.
(1996) tested conservation of MFRs of six microsatellite loci, and demonstrated that most primers were able to amplify from 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 primers 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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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., Ip359). 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)n, as probes. Their locus numbers are Ip30, Ip41, Ip44, Ip401, Ip438, and Ip443, same as the middle row.

References


The Yellow Color Inheritance in Rainbow Trout

S. Dobosz, K. Goryczko, K. Kohlmann, and M. Korwin-Kossakowski

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 the 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.

Publications concerning yellow coloration in rainbow trout (Onchorhyncus 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 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 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 albinism.

Step 1

Experiments and Results

Step 1

Experiments and Results

Step 2

Experiments and Results

Step 3

Experiments and Results

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Experiments and Results

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Experiments and Results

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

<table>
<thead>
<tr>
<th>Group (pooled families)</th>
<th>Female parent</th>
<th>Male parent</th>
<th>Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>2</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>3</td>
<td>Wild</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>4</td>
<td>Yellow- meiotic gynogenesis</td>
<td>1 wild:1 yellow</td>
<td>All yellow uniform tint</td>
</tr>
</tbody>
</table>

The Journal of Heredity 1999:90(2)
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—aabbb) 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 males (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)

<table>
<thead>
<tr>
<th>Family</th>
<th>Putative parents genotypes (females × males)</th>
<th>Wild</th>
<th>Palomino</th>
<th>Albino</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>aabB × AaBb</td>
<td>131</td>
<td>73 (90)</td>
<td>37 (30)</td>
<td>5.74</td>
</tr>
<tr>
<td>2</td>
<td>aabB × AaBb</td>
<td>50</td>
<td>32 (39)</td>
<td>22 (15)</td>
<td>7.56*</td>
</tr>
<tr>
<td>3</td>
<td>aabB × AaBb</td>
<td>201</td>
<td>127 (137)</td>
<td>38 (46)</td>
<td>3.85</td>
</tr>
<tr>
<td>4</td>
<td>AaBb × aabB</td>
<td>104</td>
<td>78 (79)</td>
<td>30 (27)</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>AaBb × aabB</td>
<td>136</td>
<td>58 (79)</td>
<td>18 (27)</td>
<td>17.03**</td>
</tr>
<tr>
<td>6</td>
<td>AaBb × aabB</td>
<td>155</td>
<td>96 (109)</td>
<td>39 (36)</td>
<td>2.39</td>
</tr>
</tbody>
</table>

χ² with 1 df, *P < .05, **P < .01.
( ) = expected numbers.

Table 3. Segregation of alleles

<table>
<thead>
<tr>
<th>F₀</th>
<th>Parents phenotype: Wild × Palomino or Palomino × Wild</th>
<th>Parental genotype: AaBb × aabB or aabB × AaBb</th>
<th>F₁</th>
<th>Progeny: Segregation of alleles</th>
<th>Ratio</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild female (AaBB) × Albino male (aabb)</td>
<td></td>
<td>1</td>
<td>AaBb, AaBb</td>
<td>1</td>
<td>Wild</td>
</tr>
<tr>
<td>2</td>
<td>aabB, aabB</td>
<td></td>
<td>2</td>
<td>Wild</td>
<td>4</td>
<td>Wild</td>
</tr>
<tr>
<td>3</td>
<td>Palomino or Palomino</td>
<td></td>
<td>3</td>
<td>Palomino</td>
<td>3</td>
<td>Palomino</td>
</tr>
<tr>
<td>4</td>
<td>Wild or Wild</td>
<td></td>
<td>4</td>
<td>Albino</td>
<td>1</td>
<td>Albino</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Lot</th>
<th>Male color (putative genotype)</th>
<th>Number of eyed eggs</th>
<th>Survival (%)</th>
<th>Number of embryo with black eyes</th>
<th>Number of embryo with red eyes</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild (Aa × b)</td>
<td>1084</td>
<td>84.2</td>
<td>535</td>
<td>549</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>Wild (Aa × b)</td>
<td>577</td>
<td>45.3</td>
<td>288</td>
<td>289</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>Wild (Aa × b)</td>
<td>355</td>
<td>28.0</td>
<td>187</td>
<td>168</td>
<td>1.02</td>
</tr>
<tr>
<td>4</td>
<td>Wild (Aa × b)</td>
<td>248</td>
<td>19.9</td>
<td>464</td>
<td>484</td>
<td>0.42</td>
</tr>
<tr>
<td>5</td>
<td>Wild (Aa × b)</td>
<td>921</td>
<td>78.0</td>
<td>445</td>
<td>476</td>
<td>1.04</td>
</tr>
<tr>
<td>6</td>
<td>1 Palomino (aaBb)</td>
<td>1051</td>
<td>83.4</td>
<td>1051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2 Palomino (aaBb)</td>
<td>955</td>
<td>77.8</td>
<td></td>
<td>955</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3 Palomino (aaBb)</td>
<td>1018</td>
<td>81.4</td>
<td>1018</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1 Albino (aabb)</td>
<td>439</td>
<td>34.1</td>
<td>439</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chi-square χ² = 3.84. *Testing the ratio: wild (embryo with black eyes) to yellow (embryo with red eyes) as 1:1.

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

<table>
<thead>
<tr>
<th>Lot</th>
<th>Male color (known genotype)</th>
<th>Number of alevins</th>
<th>Survival (%)</th>
<th>Number of wild alevins</th>
<th>Number of alevins with black eyes</th>
<th>Number of alevins with red eyes</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild (Aaab)</td>
<td>924</td>
<td>85.2</td>
<td>462</td>
<td>462</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Wild (Aaab)</td>
<td>518</td>
<td>89.8</td>
<td>262</td>
<td>256</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Wild (Aaab)</td>
<td>309</td>
<td>87.0</td>
<td>167</td>
<td>142</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Wild (Aaab)</td>
<td>840</td>
<td>88.6</td>
<td>412</td>
<td>219</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Wild (Aaab)</td>
<td>781</td>
<td>84.8</td>
<td>390</td>
<td>391</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Palomino (aaBb)</td>
<td>948</td>
<td>90.2</td>
<td>455</td>
<td>493</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Palomino (aaBb)</td>
<td>843</td>
<td>88.3</td>
<td>427</td>
<td>416</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Palomino (aaBb)</td>
<td>915</td>
<td>89.9</td>
<td>450</td>
<td>465</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Albino (aabb)</td>
<td>408</td>
<td>92.9</td>
<td>408</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chi-square χ² = 3.84, 5.99 (1 or 2 df, respectively).

Table 6. Survival from eyed eggs to the alevins stage and the alevins body and eyes’ color in experimental lots

<table>
<thead>
<tr>
<th>Lot</th>
<th>Male color (putative genotype)</th>
<th>Number of alevins</th>
<th>Survival (%)</th>
<th>Number of wild alevins</th>
<th>Number of alevins with black eyes</th>
<th>Number of alevins with red eyes</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild (Aaab)</td>
<td>924</td>
<td>85.2</td>
<td>462</td>
<td>462</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Wild (Aaab)</td>
<td>518</td>
<td>89.8</td>
<td>262</td>
<td>256</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Wild (Aaab)</td>
<td>309</td>
<td>87.0</td>
<td>167</td>
<td>142</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Wild (Aaab)</td>
<td>840</td>
<td>88.6</td>
<td>412</td>
<td>219</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Wild (Aaab)</td>
<td>781</td>
<td>84.8</td>
<td>390</td>
<td>391</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Palomino (aaBb)</td>
<td>948</td>
<td>90.2</td>
<td>455</td>
<td>493</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Palomino (aaBb)</td>
<td>843</td>
<td>88.3</td>
<td>427</td>
<td>416</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Palomino (aaBb)</td>
<td>915</td>
<td>89.9</td>
<td>450</td>
<td>465</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Albino (aabb)</td>
<td>408</td>
<td>92.9</td>
<td>408</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chi-square χ² = 3.84, 5.99 (1 or 2 df, respectively).

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

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

A. Sharma, M. P. Niphadkar, P. Kathirvel, J. Nagaraju, and L. Singh

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). 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 pedigrees 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)]; Aggarwal et al. 1994; Lang et al. 1993; Singh 1995; Singh and Jones 1986; Singh et al. 1980, 1984, 1988] in generating DNA fingerprint 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 differed 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 (Hu204, Ka, NB1, NB7, NB18, and NB2) and seven nondiapausing (C. nichi, Gungnong, Moria, Nistari, Pure Mysores, Diazos, 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 (Su- zuki 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 RNa se 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.

Accepted September 30, 1998

Corresponding Editor: Bernie May
Figure 1. Bkm 2/8 hybridization pattern of DNA from (A) Pure Mysore, (B) Nistari, (C) NB4D2, and (D) NB18 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 Hinf I (A, B, and D) and BstNI (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)

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Restriction enzyme</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BstNI</td>
<td>0.970 ± 0.02</td>
<td>0.968 ± 0.02</td>
<td>0.875 ± 0.05</td>
<td>0.866 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Hindl</td>
<td>0.923 ± 0.06</td>
<td>0.917 ± 0.07</td>
<td>0.966 ± 0.02</td>
<td>0.870 ± 0.07</td>
</tr>
</tbody>
</table>

DNA Fingerprinting
For each gel lane, 8–10 μg of BstNI- or Hindl-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-base, 5 mm thick, 0.8% agarose gels at 60°C). Samples were electrophoresed in 30 cm×0.8% (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 BstNI or Hindl, were run in a single gel. Similarly, pooled DNA samples digested with either BstNI or Hindl were run in a single gel to estimate among variety differences. Gel fractionated DNA samples were transferred onto Hybond-N membrane (Amerham, UK) using a vacuum blotting assembly by 30 mm Hg (Olszewskia 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 ^32P-labeled probe was prepared with respect to sex as well as restriction enzyme, sex, restriction enzyme, and irrespective of sex and restriction enzyme (mean).

![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.](image-url)
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; \( S = 2N_{ab} / (N_a + N_b) \) 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; Wettton 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 or \( Hin \)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 nondiapauing varieties and five diapauing 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; Nagaraju and Nagaraju 1995). The silkworm varieties (Moria and Sarupat) that shared the same geographical distribution are in the same cluster. Similarly the silkworm varieties (\( NB \)D1 and \( NB \)D2) 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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 Meng A, Gong G, Chen D, Zhang H, Qi S, Tang H, and
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 generally 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 (Gadou 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 F1 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-
Subsequent segments are clearly leg-like 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% dant/dant 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 F2 progeny, with 65 recombinants between rdh5 and dant. A similar recombination rate of 22% was detected by 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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Received June 29, 1998
Accepted September 30, 1998
Corresponding Editor: Ross MacIntyre

The evolutionarily important 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 CScsMS13 minisatellite locus in the pseudoscorpion, Cardiorychernes scorpoides. 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 distortion system (SD) of Drosophila melanogaster (Lyttle 1991, 1993; Moschetta 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 Pomiankowski 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 suppressor 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. 1994). Here we report a case of extreme transmission distortion in paternal alleles at a second loci (cCscMS23) in C. scorpioides, 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 (cCscMS13) 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 Haell or Mbol 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 32P. 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 χ² 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 (χ² = 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 (χ² = 5.261; P > sequential Bonferroni value of .05/42).

Discussion

In our investigation of allelic transmission at the cCscMS13 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 (cCscMS23) 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 was 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 cCscMS13 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.

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 cCscMS13 locus reported here differs significantly from the 2:1 bias predicted to result from such a lethal recessive linkage mechanism ($\chi^2 = 6.25, P = .014$). In addition, transmission of paternal 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 cCscMS13 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 cCscMS13 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 cCscMS23 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 cCscMS13 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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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 (Fst = 0.032; Rs = 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 chromosomal evolution because more than 50 distinct chromosomal races have been described in this species (Zima et al. 1996). Many interfiracial hybrid zones were studied with a variety of genetic markers to understand the role of chromosomes in racial and species evolution (Brüner and Hausser 1996; Frykman et al. 1993; 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 (Volo-bouev 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). Meta-centric 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 meta-centric 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 (Bailly 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 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 oligodeoxynucleotide primer, 100 μM dCTP, dGTP, and dTTP, 10 μM dATP, 0.02 μl 32P-dATP at 1000 Ci/mmol, 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.

**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_e) 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 FST and FST were obtained from 5000 permutations of alleles within samples for FST and 5000 permutations of genotypes among samples for FST. In this way the distribution of the null hy-

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**Figure 1.** Geographic map showing the localities (full circles, n = 24) where individuals of the common shrew were 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).
isolation by distance (Slatkin 1997) that allows a better estimator of population differentiation derived by Slatkin (1995) which localities 9±20 were omitted.

**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 $F_{st}$ 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 0.89). 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 \( N_e \) 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 \( F_{ST} \) value of 0.027 is small, but significantly greater than zero. Values for \( F_{ST} \) are not concordant across marker loci and localities. Only locus 57 is significantly different from zero. A closer inspection of the \( F_{ST} \) values across localities revealed that high \( F_{ST} \) 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 (\( F_{ST} = 0.019 \), \( P = .111 \)). 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; Seare 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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Received December 27, 1997
Accepted September 30, 1998

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Corresponding Editor: Rodney Honeycutt