MEASURING SIMILARITY AMONG HISPID COTTON RATS 
(SIGMODON HISPIDUS) OF KNOWN RELATEDNESS WITH 
DNA FINGERPRINTING

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Determining mating strategies and social behavior of natural populations of mammals is 
equivocal when individuals can not be observed directly. DNA fingerprinting holds promise 
to elucidate relationships among individuals indirectly; however, few empirical data assess 
accuracy of DNA fingerprints in portraying relatedness in natural populations of mammals. 
Using the hispid cotton rat (Sigmodon hispidus) as a model, we determined accuracy of 
DNA fingerprinting for separating wild-caught individuals into categories according to their 
relatedness and ascertained repeatability of the pattern of DNA fingerprints. We used indi­ 
viduals from known breedings to compute similarity (S) in DNA fingerprints in pairwise matchings. 
Because relatedness of these individuals was known, we obtained the actual 
distribution of band-sharing scores and computed the known mean S for each relatedness 
category. We detected significant differences in S between primary, secondary, and unre­ 
lated categories but could not distinguish statistically among related groups within these 
categories. High within-gel repeatability in the DNA banding pattern indicated a robust 
technique, but variation in scoring among readers confirmed that a single individual should 
score gels. Our results demonstrated that DNA fingerprints can be used to determine re­ 
latedness categories and uncover behavioral-social interactions for species that can not be 
observed directly. Routine demographic information (e.g., body mass, spatial proximity) 
collected during field studies and used in conjunction with DNA fingerprinting can provide 
a finer level of distinction among related groups.

Key words: Sigmodon hispidus, hispid cotton rat, DNA fingerprinting, relatedness, social 
structure

Understanding social organization and mating systems of natural populations of 
mammals is important because these factors influence ecological parameters such as im­ 
migration and emigration (Brandt, 1992; 
Lidicker and Stenseth, 1992; Ribble, 1992), inbreeding and outbreeding (Lacy, 1992), 
and population regulation (Lidicker, 1988; Sheridan and Tamarin, 1988; Tamarin and 
Sheridan, 1987). Elucidating mating strategies and social behavior in such natural 
populations, however, is difficult when individuals can not be observed directly (e.g., 
ocurnal or in dense habitats), sexes are indistinguishable without close inspection, 
or mating behavior and fertilization are poorly correlated.

Progress toward understanding social or­
organization in populations of mammals with 
these characteristics has been achieved, but 
techniques used have strong limitations. For 
example, determining mating structure from 
radio telemetry or pairwise encounters in 
neutral arenas is problematic because of dif­
ficulty determining which individuals mate 
successfully. Similarly, using spatial prox­
imity of individuals to deduce, for example, 
that inclusion of home ranges of females in
the home range of a male reflects the male's reproductive success often is difficult to substantiate (Bondrup-Nielsen and Ims, 1986; Madison, 1985; Ostfeld, 1986; Sheridan and Tamarin, 1988).

Mating structure in mammals also has been studied by determining maternity by transfer of radionuclides (Pugh and Tamarin, 1988) or fluorescent powder (Kaufman, 1989) from labeled mothers to unlabeled offspring and then estimating paternity from protein electrophoresis (Pugh and Tamarin, 1988). Again, caution must be exercised when drawing conclusions. Fluorescent powder affects behavior of individuals, remains on animals for short periods (usually a matter of days), and may be picked up by unmarked individuals (Halfpenny, 1992; Kaufman, 1989; Mikesic and Drickamer, 1992). Protein electrophoresis usually involves destructive sampling to obtain sufficient amounts of tissue and is most useful when populations exhibit high levels of variation.

Because of limitations with these techniques, use of DNA fingerprinting (Jeffreys et al., 1985) to investigate behavioral relationships has increased for many taxa including mammals (Amos et al., 1991; Cummings and Hallett, 1991; Harris et al., 1991; Hoagland et al., 1991; Pemberton et al., 1992; Reeve et al., 1990; Ribble, 1991; Tegelstrom et al., 1991). The hope of field biologists is that information on genetic relatedness can be extracted from similarities of banding patterns of DNA fingerprints and can be used to understand mating and social behavior. Although several investigators have tried various indirect methods to estimate relatedness (Lehman et al., 1992; Marinelli et al., 1992; Ribble, 1992), few empirical data exist to determine accuracy of DNA fingerprints in portraying relatedness in natural populations of mammals; for example, the relationship between values of band-sharing and actual relatedness is largely unknown. Without such data on background levels of similarity (S), estimates of relatedness (r) based on S of DNA fingerprints are subject to large sampling variances and, as a result, are of limited usefulness because distinguishing related from unrelated individuals becomes more difficult as r decreases (Lynch, 1988).

While difficult to determine for most species of mammals, actual relatedness for some species of birds has been determined from field observations (Haig et al., 1993; Piper and Rabenold, 1992; Reeve et al., 1992). Using such observations to establish lineages, Piper and Rabenold (1992) distinguished unrelated from first-order dyads with DNA fingerprints and, using mean band-sharing scores, separated first-order from second-order dyads for a population of stripe-backed wrens (Campylorhynchus nuchalis). Haig et al. (1993) also used field observations to determine kinship groups in red-cockaded woodpeckers (Picoides borealis) but were unable to distinguish kinship groups reliably with similarity alone because of high overlap in band-sharing.

Our objective was to assess the utility of DNA fingerprinting to determine relatedness among individuals from field populations of rodents for whom relatedness could not be determined by behavioral observations. As a model organism, we used hispid cotton rats (Sigmodon hispidus), a common inhabitant of prairies throughout the southeastern United States, Mexico, and northern South America (Cameron and McClure, 1988). We determined coefficients of S for primary and secondary categories of relatedness for cotton rats whose genealogies were known to evaluate the ability of DNA fingerprinting to separate wild-caught individuals into categories according to their relatedness. Because few published data assess repeatability of various aspects of the DNA-fingerprint technique for wild-caught species, we assessed repeatability of reading bands within gels.

MATERIALS AND METHODS

A breeding colony was founded from individual hispid cotton rats captured from a wild population at the University of Houston Coastal
Genomic DNA was obtained by cutting ca. 2 cm from the tip of the tail and immediately freezing and storing it at −80°C. DNA was extracted by powdering frozen tissue in liquid nitrogen and homogenizing it in 4 ml of homogenization buffer (0.1 M NaCl, 0.01 M Tris, pH 8.0, 0.025 M EDTA). The solution was transferred to serum-separator tubes (SST—Becton Dickinson, Rutherford, NJ) and was incubated for 4 h at 55°C with 5 units/ml of proteinase-K and 0.5% sodium-dodecyl-sulfate. DNA was precipitated with 2 volumes of 100% ethanol.

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DNA samples (8–10 µg) were digested with HinfI restriction endonuclease at 37°C for at least 3 h. The reaction was stopped by adding 1/6 volume 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 120 mM EDTA) and incubating at 65°C for 5 min. DNA restriction fragments were separated by electrophoresis on 0.8% agarose gels (18 cm by 25 cm by 0.7 cm) in 1x TBE (89 mM Tris, pH 8.0, 89 mM boric acid, 2 mM EDTA) buffer and run at 50 V for 48 h. A bacteriophage λ DNA-HindIII digest (New England Biolabs, Beverly, MA) was used as a size marker on all gels. Gels were prepared for Southern transfer by standard procedures (Sambrook et al., 1989), and DNA was transferred to nylon membranes (Hybond-N®, Amersham Corporation, Arlington Heights, IL) by vacuum blotting in 4x SSC (0.6 M NaCl, 0.06 M sodium citrate, pH 7.0) buffer for 40 min. DNA fragments were covalently bound to the membrane with ultraviolet light (12,000 J/l 15 s) using a Stratalinker® (Stratagene, La Jolla, CA).

Actual relationships among animals were unknown to the observer (M. Descalzi) doing DNA isolation and fingerprinting. Each sample was assigned a vial number for identification and was coded further by using lane number of electrophoretic gels. Only one investigator (G. Cameron) knew the correspondence between vial, lane, and rat number. Samples run on each gel consisted of an array of related and unrelated individuals, thus minimizing observer biases in scoring and analyzing DNA fragments.

In pilot studies, DNA fingerprints were produced using the minisatellite-core sequence from bacteriophage M13 (Vassart et al., 1987) as a hybridization probe. However, M13 produced <20 hybridizing restriction fragments per cotton rat, and intensity of the hybridization signal was not reliable. Consequently, we used a minisatellite probe, D4.2, which was isolated from the Drosophila genome using the M13 minisatellite core sequence as a hybridization probe (Jacobson et al., 1992). D4.2 produced a stronger, more reliable hybridization signal than M13, resulting in >30 scorable bands on autoradiographs. In preliminary studies, estimates of similarity based on D4.2 hybridization patterns were comparable to those produced by M13. Probe DNA was labeled with 32P-α-dCTP (3,000 Ci/mM) by the random–hexamer method (Feinberg and Vogelstein, 1983) using a commercial labeling kit (Amersham Corporation). Prehybridization and hybridization were carried out in a solution consisting of 0.5% N-lauroylsarcosine, 0.5% powdered non-fat milk, 0.5 M NaCl, 0.1 M Na2HPO4, and 0.005 M Na2EDTA at 60°C. Post-hybridization washes consisted of one wash in a solution of 1% powdered non-fat milk, 1 mM Tris, pH 8.0, and 1% N-lauroylsarcosine solution for 20 min, followed by four 5-min washes in 1xM Tris, pH 8.0. All washes were done at room temperature (ca. 21°C). Filters were briefly blotted to remove excess moisture, wrapped in Saran Wrap®, and exposed to Kodak® XAR film for 72 h at room temperature without intensifying screens.

Four gels were run, each containing 26 samples and λ-HindIII size markers in the outer lanes. We assumed that bands assorted independently as established by Bruford et al. (1992) for birds. A standard window that included all 28 lanes was created on the autoradiographs, starting from the sample wells and extending downward ca. 14 cm. Actual size of the window was adjusted from gel to gel to ensure that restriction fragments from 23 to 4 kilobases (kb, as estimated by comparison to size markers) were included. Each autoradiograph was photographed for a permanent record and to facilitate band scoring and comparisons.

Center, 56 km southeast of Houston, Texas. All crosses began with unrelated individuals; 87 individuals from 17 different families were used.
FIG. 1.—Sample autoradiograph (Gel A) of DNA fingerprint of *Sigmodon hispidus*. Each lane contained genomic DNA from a single individual. DNA was digested with the restriction endonuclease Hinfl, separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with radioactively labeled minisatellite D4.2 DNA. Exposure time was 72 h at room temperature without an intensifying screen. Restriction fragment sizes ranged from ca. 23 kb to 3.0 kb. Position of a common restriction fragment used for lane matching is evident at ca. 8 kb. Lanes 19 and 22 contained DNA samples from the same individual (rat 3792).

After all bands were counted in each lane on photographs, adjacent lanes were compared to identify shared bands by position and intensity. Then, adjacent pairs of lanes were cut from photographs and compared to the rest of the lanes. This was done sequentially starting with lanes 1 and 2, followed by the next pair, and so on. A monomorphic band ca. 8 kb in size found in most individuals (Fig. 1) was used as a common marker to aid in alignment when comparing non-adjacent lanes. Lanes containing a number of bands that were 3 SD below the average number for the entire gel were judged to contain insufficient DNA and were excluded from analyses. This procedure eliminated declines in band-sharing values and consistency of scoring that occurred with the distance that lanes were separated on gels (Piper and Rabenold, 1992). We tested for an effect of imprecise technique by analyzing data with and without lanes containing low amounts of DNA and peripheral lanes that could have migrated at different rates than central lanes. Neither amount of DNA in a lane nor differential migration (i.e., edge effect) affected our results (Descalzi, 1993).

An index of similarity ($S$) was used to estimate the proportion of bands shared by two individuals:

$$
S = \frac{2N_{AB}}{N_A + N_B}
$$

where $N_{AB}$ = number of bands shared between individuals A and B, $N_A$ = total number of bands scored for individual A, and $N_B$ = total number of bands scored for individual B (Wetton et al., 1987). All pairwise comparisons of $S$ were computed for each gel without prior knowledge of actual relationships. When all indices had been computed, genealogies were consulted so that relationships could be assigned to each pairwise
comparison of individuals: parent and offspring (P-O); full-siblings and full-siblings (SIBS); half-siblings and half-siblings (H-SIBS); aunt-uncle and niece-nephew (AU-NN); cousin and cousin (Cous); grandparent and grandchild (GRAND), or unrelated/unrelated (UNREL). When two individuals could be assigned to more than one type of relationship (e.g., a female was both aunt and grandmother to another), the closer relationship was used (i.e., aunt in this example). In this analysis, an individual was used in more than one pairwise comparison so that data used to compute S were not independent.

To test this potential source of bias, we computed S on a subset of the data that used individual animals in only one pairwise comparison (Piper and Rabenold, 1992). Because S for each relatedness category did not differ between this subset and the full data set, we present analyses for the full data set. Variation in S was analyzed by using means and standard deviations of band-sharing scores to compute two-tailed 95% CI for all relatedness categories (Piper and Rabenold, 1992).

We used two measures to evaluate repeatability of gel reading. Within-gel repeatability was assessed with S to determine band sharing between lanes on a gel containing DNA samples from the same individual. Variation in scoring bands among observers was assessed by having five persons experienced in interpretation of genomic Southern blot autoradiographs, although not necessarily DNA fingerprints, score the number of bands per lane on gel A (Fig. 1). Because distribution of the number of bands is about normal (Lynch, 1991), mean number of bands per gel counted by various readers was analyzed by one-way analyses of variance (ANOVA). We did not specifically test for among-gel variation in bands of the same individual because we restricted among-individual comparisons to the same gel. However, we did run eight individuals on multiple gels (range = 2–4 gels/individual) and computed number of bands on each gel for each individual to assess among-gel variation. Unless indicated otherwise, data below are reported as $\bar{X} \pm 1 SE$.

**RESULTS**

To address the question if relationships could be distinguished based upon S, indices of similarity were categorized into relatedness groups based on pedigrees. Primary, secondary, and unrelated categories could be distinguished with 95% CI. However, because of broadly overlapping CIs, there was no significant difference among related groups within the primary (e.g., P-O did not differ significantly from SIBS) or secondary (e.g., H-SIBS, AU-NN, Cous, and GRAND did not differ significantly from each other) category (Table 1).

Expected relatedness for each category was computed from standard Mendelian inheritance probabilities. There was a significant positive correlation between relatedness (range = 0–0.5) and mean S. A linear regression explained 91% of the variation in these data ($Y = 0.258 + 0.363X$, $t = 7.21, P < 0.001$). For unrelated animals (assumed relatedness = 0), $S$ was 0.259.

For one test of within-gel repeatability, rat 3792 had 37 bands scored in lane 19 and 39 bands scored in lane 22 (Fig. 1)—with 37 shared bands, $S = 0.97$. For the other test of within-gel repeatability, three individual rats were run on a single gel. Individual 1 had 15, 13, 14, and 15 total bands in four lanes, respectively, and $S = 0.89$ ($n = 6$ pairwise comparisons); individual 2 had 12 bands in each of two lanes, and $S = 1.0$ ($n = 1$ comparison); and individual 3 had 24 bands in each of three lanes, and $S = 1.0$ ($n = 3$ comparisons). Mean within-gel similarity for these three individuals (0.96 ± 0.04) was consistent with the value for rat 3792.

Number of bands scored for gel A (Fig.
1) differed among the five observers (mean number of bands for each reader = 23.4, 30.0, 30.3, 31.3, and 34.5—\( F = 38.9; \text{ d.f.} = 4.125, P \ll 0.001 \)). Lowest and highest scores differed significantly from each other and from the other three readings, but the intermediate three readings did not differ significantly from each other. Bands for the same individual that were run on different gels were repeatable (\( X = 30.6 \pm 3.3 \text{ bands/individual}, CV = 16.5; \text{ averaged over 8 individuals} \)).

**DISCUSSION**

While some studies used DNA fingerprinting to determine paternity in mammals (Cummings and Hallett, 1991; Marinelli et al., 1992; Ribble, 1991; Tegelstrom et al., 1991) and Piper and Rabenold (1992) assessed usefulness of DNA fingerprinting in determining relatedness in striped-backed wrens, our study was unique in exploring the feasibility of using DNA fingerprints to determine parentage in secretive mammals (Table 1). We distinguished among primary, secondary, and unrelated categories of relatedness of *S. hispidus*, using a single enzyme, a single probe, and a single scorer. In a similar fashion, Piper and Rabenold (1992) separated primary, secondary, and unrelated categories for striped-backed wrens but could not obtain a level of sensitivity similar to ours unless a mean score was computed over two enzymes, two probes, and two scorers and only adjacent lanes on a gel were used. Haig et al. (1993) discovered that high background S compromised their ability to distinguish related groups for red-cockaded woodpeckers. While we would expect similarities for the primary category of relatedness to exceed the theoretical value of 0.5, especially when considering that background relatedness rarely is zero in field populations, several studies, including ours, report similarity values <0.5 (Haig et al., 1993; Piper and Rabenold, 1992; Ribble, 1991). There is no clear explanation for this deviation, but it has not been significant when tested (Ribble, 1991).

Our results are notable because conditions in our study (i.e., having no information with which to group animals to be compared on a single gel) represented a worst-case scenario. In a typical field study, relatedness categories that could not be distinguished by S alone could be separated by also using field information collected when trapping animals. For example, P-O and SIBS could be distinguished with S alone (Table 1). However, after distinguished from secondary and unrelated groups by using S, parents can be separated from offspring using body mass because they belong to different age (size) cohorts. To be sure, separation by body mass requires sampling the population at critical times, for example, after weaning when lower-mass SIBS just enter the population. Similarly, GRAND could not be separated from SIBS using S alone, but again body mass could be used. However, separating these groups is not as critical as separating P-O from SIBS, because it is unlikely that GRAND and SIBS will co-occur in a rodent population where probability of survival to old age is low. On the other hand, SIBS, H-SIBS and COUS, and AU-NN and P-O cannot be distinguished from each other by age (i.e., body mass) but can be separated by S into primary and secondary categories. Within the secondary category, AU-NN and GRAND can be distinguished from COUS and H-SIBS on the basis of body mass, but COUS cannot be distinguished from H-SIBS by either S or age. In addition, sex or spatial proximity of trapped individuals also can be used in conjunction with S and age (size) to unravel relatedness.

Our data and those of others (Haig et al., 1993; Jones et al., 1991; Mannen et al., 1993) substantiated the expectation that S was correlated positively with relatedness (Table 1; Lynch, 1988). However, there is evidence for non-linear relationships in populations of lions (*Panthera leo*) from the Serengeti (Packer et al., 1991) and when
relatedness is >0.5 (Haig et al., 1993; Jones et al., 1991). Lynch (1988) expressed concern that detecting differences among relatedness categories would become more difficult when relatedness decreased. On the other hand, Jones et al. (1991) contended that they could distinguish among individuals with relatednesses of 0.0, 0.25, and 0.50, because there was little overlap in the distribution of bands shared. In a like fashion, we distinguished among primary and secondary relatedness categories with S alone despite of a background S >0 (Table 1).

Background band-sharing for a population is the mean probability that a band in an individual’s fingerprint also is present in another randomly chosen individual. Accurate assessment of background band-sharing is important because it affects the probability of misassigning parentage or misidentifying the relatedness category of two individuals (Haig et al., 1993). Relatively high similarity among unrelated individuals is reported herein and is predicted to occur because of large sampling variance associated with similarities in DNA fingerprints (Lynch, 1988). Ideally, background band-sharing is determined by knowing the pedigree of a population as we did for S. hispidus (S = 0.259, Table 1). Estimates from other pedigrees showed that background band-sharing for field populations was similar (0.16–0.22, Jones et al., 1991; 0.27–0.31, Keane et al., 1991; 0.21–0.41, Piper and Rabenold, 1992) or higher (0.7–1.0, Gilbert et al., 1990; 0.55, Haig et al., 1993; 0.8, Reeve et al., 1990).

Sampling tail tissue minimized harmful effects on individuals, a requirement of long-term studies of natural populations and provided DNA for several gel runs per individual. The small amounts of DNA obtained from many sampling methods would suffice if few individuals (i.e., <20) that could be run on a single gel were being studied. Enough DNA would be collected by these methods to rerun a gel if technical problems occurred. However, for population-level questions where it might be necessary to run individuals multiple times for comparison with other individuals, larger quantities of DNA, as we obtained from tail tissue, are necessary. Use of D4.2 as a hybridization probe resulted in 31.4 ± 0.48 (range = 16–41; Fig. 1) readable bands compared to 14–24 bands obtained from other probes for other mammalian species (e.g., Cummings and Hallett, 1991; Hoagland et al., 1991; Marinelli et al., 1991; Ribble, 1991).

We demonstrated that scoring DNA fingerprints within gels was highly repeatable (S = 0.96–0.97). However, we found significant variation among observers in number of bands read per sample on the same gel even when each observer followed the same rules for detecting band separation. This result reinforced a common standard that one trained individual should score all gel autoradiographs to provide a consistent interpretation of band number and identity. However, our finding is important, because some recent studies continue to use multiple observers to determine banding patterns (e.g., Haig et al., 1993; Keane et al., 1991; Piper and Rabenold, 1992).

We support the rule-of-thumb that band numbers or S should be compared only within gels, but it may be difficult to avoid among-gel comparisons in studies such as ours that use large numbers of animals that must be run on different gels. Among-gel variation was low in our study, although high among-gel variation for a few gels was used to eliminate them from the final analyses, thereby avoiding biases caused by variation in characteristics of gels (Descalzi, 1993). The number of among-gel comparisons could be minimized by grouping those animals to be compared on the same gel (e.g., animals from the same family or animals from the same location or habitat). Arranging such a grouping is straightforward when relationships are known from breeding in the laboratory (Cummings and Hallett, 1991; Hoagland et al., 1991; Marinelli et al., 1992). For field populations,
however, developing criteria for grouping individuals becomes problematic because relationships among individuals are not always certain. For birds and other organisms that are easily seen, field observations could be used to assign relatedness (Haig et al., 1993; Piper and Rabenold, 1992), and closely related animals could be grouped on a gel. For more secretive species, basic field data including sex, body mass, and spatial proximity among individuals would provide information on which to base groupings. Because our study was a blind test, no information was available to form a basis for groupings. In fact, we purposefully mixed related and unrelated individuals to minimize any biases that might have occurred in reading gels, and, thus, our results illustrated a worst-case scenario.

Multilocus DNA fingerprints may not have the discriminatory power that single locus and other molecular approaches have in individual and group identification (Bruford et al., 1992; Lynch, 1988); however, facilities, expertise, and expense associated with isolation and development of single-locus probes may preclude their use by investigators addressing ecological questions (Burke et al., 1991). In addition, Brookfield and Parkin (1993) argued that an unreasonably large number of loci were needed to distinguish relationships other than primary with single-locus probes. Because it can be applied with relatively minor technical adjustments to a wide diversity of taxa, multilocus DNA fingerprinting will remain an important tool to address questions involving social and behavioral interactions in natural populations, particularly when used in conjunction with demographic information. Multilocus DNA fingerprinting can elucidate paternal relations when maternal relationships have been determined by some other technique, but importantly, we demonstrated that analyses of DNA fingerprints can be used alone or in conjunction with other field information to determine parental relationships, distinguish unrelated from related individuals, and distinguish among primary and secondary relatedness categories. With this technical ability, questions concerning mating and social structure of cryptic mammals can be addressed.

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