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Estimating the relatedness in a population of grey squirrels *Sciurus carolinensis*, using DNA fingerprinting

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Studies on the behaviour of grey squirrels *Sciurus carolinensis* Gmelin, 1788 and other species belonging to the same genus, suggest they exhibit dominance hierarchies among both males and females, and their mating strategy is promiscuous or polygynous. However, there is little information available on how dominance translates into reproductive success or on the levels of genetic variation or genetic substructuring within a population. To address these questions, we used multilocus DNA fingerprinting to quantify genetic diversity and estimate relatedness amongst individuals in a free-ranging population of grey squirrels. Background band-sharing values revealed a high degree of genetic diversity existed. The frequency distribution of band-sharing values between known first order relatives and presumed unrelated animals was significantly different. Estimates of relatedness between individuals were therefore based on threshold levels of genetic similarity and used as a tool to infer close kinship. Possible kinship groups were identified using the spatial distribution of the home ranges of adults and juveniles. Levels of relatedness between neighbouring adults was low with five family units identified comprised of putative parents and their offspring. Several juveniles from the single cohort revealed levels of relatedness intermediate between first order relatives and unrelated animals, suggesting possible second order relationships (eg half siblings) existed. The implications of these relationships are discussed.

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Introduction

Grey squirrels and other species within the tree squirrel genus *Sciurus* (F. Sciuridae), do not defend exclusive territories but have overlapping home range systems, with the exception of lactating females (Bakken 1959, Taylor 1969, Hampshire 1985). Behavioural observations have shown that a linear dominance hierarchy exists within populations of *Sciurus* squirrels and that social rank is related to weight, sex and age (Taylor 1966, Pack *et al.* 1967, Thompson 1977,

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Wauters and Dhondt 1985, 1989, Allen and Aspey 1986). However, the spatial extent over which one particular hierarchy operates within a forest has not been determined.

Field studies indicate that the mating system of tree squirrels is best described as a dominance polygyny. Oestrus occurs on one day only and is asynchronous among the females in a population (Webley and Johnson 1983, Gurnell 1987). This creates a temporally and spatially limited resource that is contested by males, and provides the potential for dominant males to monopolise the matings within local populations. Prior to mating, squirrels engage in a mating chase with dominant males at the head of the chase, taking up position immediately behind the female (Pack *et al.* 1967, Thompson 1977, Wauters *et al.* 1990, Koprowski 1993a). Gaining close proximity to the female is critical because the male at the head of the chase is the one that usually mates with the female (Pack *et al.* 1967, Thompson 1977, Wauters *et al.* 1990, Koprowski 1993a, b).

However, the pattern of mating is complicated by the existence of alternative reproductive tactics. For example, young satellite males obtain 'sneaky' matings and females actively remove copulatory plugs to facilitate multiple matings (Wauters *et al.* 1990, Koprowski 1992, 1993a). These tactics confound any predictions of reproductive success in the *Sciuridae* based solely on field observations, and there is little information available concerning how dominance translates into reproductive success or on the normal levels of genetic variation or genetic substructuring within grey squirrel populations. To address this, we have used DNA fingerprinting techniques (Jeffreys 1985a, b) to determine the local kinship structure within a wild population of grey squirrels by quantifying genetic diversity and estimating levels of relatedness amongst individuals. In common with other studies of this nature (Westneat 1990, Jeffreys *et al.* 1991, Hunter *et al.* 1992) we have used DNA fingerprinting data as a tool to infer close kinship, eg parent--offspring and sibling-sibling, based on empirical distributions of band-sharing values amongst known relatives and non-relatives. Band-sharing values of known first order relatives are expected to be significantly higher than those of individuals that are unrelated. Capture-mark-recapture techniques and the spatial distribution of the home ranges of adults and juveniles were used as an aid to identifying putative kinship groups in the wild population.

Methods

Sample methods

The study area was a 9 ha mature oak (*Quercus robur*) woodland site situated in Alice Holt Forest on the borders of Hampshire and Surrey in Southern England. Squirrels were trapped throughout one breeding season from March to July 1993. There were 14 trapping points throughout the compartment. Standard live trapping methods were used (see Gurnell 1996). Each captured animal was weighed, sexed and given a unique toe clip mark (under Home Office licence PIL 80/02140). The toe clippings were immediately placed in liquid nitrogen and then stored –80°C for subsequent extraction of DNA.

In addition, the breeding status of each animal was recorded (pregnancy or lactation in females and testes size in males).

Multilocus DNA fingerprinting

Genomic DNA was extracted from toe clips by grinding in liquid nitrogen before adding to an extraction buffer (0.025M EDTA, 1.5M NaCl). Proteinase K (400 μg/ml) and 10% SDS, were added and incubated overnight at 50°C. DNA was extracted and purified with phenol (pH 8.0) once, then an equal volume of phenol and chloroform-isoamyl alcohol (24:1) twice and then chloroform-isoamyl alcohol (24:1) once. DNA was precipitated with 5M NaCl (final conc.0.4M) and two volumes of ethanol. The DNA pellet was resuspended in 500 μl of sterile water. 10 μg of DNA was digested with *Hinf* I (Pharmacia) in the presence of 10mM spermidine (Sigma) and incubated at 37°C overnight. Electrophoresis was carried out using 0.8% agarose (Sigma) in 1× TBE buffer at 45 volts for 48 hours. DNA was transferred to Hybond N (Amersham) by Southern blotting (Southern 1975) and bound to the filter by UV crosslinking. Prior to loading samples, 10ng of an internal marker, λ DNA digested with *Bst*YI (New England Biolabs) were added, allowing for the accurate calculation of DNA fingerprint fragment sizes after reprobing the filters with [α^{32}P]dCTP (6000 μCi/mmol, Amersham) labelled λ DNA. 20ng of the human minisatellite DNA probe, 33.15 (Jeffreys 1985a) was [α^{32}P]dCTP labelled using the random priming method (Feinburg and Vogelstein 1983). Filters were prehybridised overnight at 65°C in 0.25M phosphate buffer (7% SDS, 0.1mM EDTA, 1% BSA, Church and Gilbert 1984). Filters were hybridised overnight at 62°C, washed at 62°C once for 15 minutes in 0.25M phosphate buffer, 1% SDS, then twice for 20 minutes in 2 × SSC, 0.1% SDS, and exposed to β-Max Hyperfilm (Amersham) for 3-7 days with intensifying screens at −80°C.

Band scoring and band-sharing analysis

All minisatellite fragments between 2–23kb were scored. The internal λ markers were used as a control for differential mobility amongst samples (Galbraith *et al.* 1991) and therefore scoring was not restricted to adjacent lanes, allowing pairwise comparisons to be made amongst all relevant individuals on a single gel. For each pairwise comparison, bands of similar intensity and migration distance (within approximately 0.5mm of each other using the nearest internal marker fragment for reference), were declared as similar. Bands that differed by greater than 1mm were considered separate fragments (David-Gray 1995). To determine the degree of genetic similarity between DNA fingerprints from two individuals, the band-sharing coefficient (x) was calculated, $x = [(Nab / Na) + Nab / Nb)] / 2$, where Nab - number of bands of similar intensity and electrophoretic mobility in individuals a and b, Na – total number of bands in a which could be scored if present in b, and Nb – total number of bands in b which could be scored if present in a (Jeffreys *et al.* 1985b, Burke and Bruford 1987). This provides an index which ranges from 0, no bands are shared, to 1, all bands are shared. Because the number of alleles at any given locus is finite, unrelated individuals will share a proportion of their DNA fingerprint bands called background band-sharing (Lynch 1988). Background band-sharing was estimated by comparing DNA fingerprints from 15 adult squirrels presumed to be unrelated by randomly selecting samples from individuals trapped in different parts of the study area and whose observed home ranges did not overlap. For comparison, band-sharing values between known first order relatives were determined by obtaining tissue samples from five wild caught females and their fetuses (n = 17) from Alice Holt Forest. These DNA samples were used to plot the frequency distribution of band-sharing values amongst first order relatives and between first order relatives and unrelated individuals.

Testing for significance of band-sharing coefficients

We adopted the method of Quinn *et al.* (1994) which adjusts the calculation of expected frequencies in ch-squared contingency analysis to take into account the band-sharing between unrelated individuals in the study population. Any individuals in pairwise comparisons which gave high band-sharing coefficients, were assumed to be related if the band sharing coefficient was found to be statistically significantly higher than expected by chance in the study population.

Home range analysis

The field methods were not designed to measure home range size and indeed the number of captures for each individual was small (mean = 2.31, SD = 1.101, n = 87). However, we have termed the Minimum Convex Polygons that were estimated using the Ranges IV computer programme (Biotrack, Wareham, Dorset) as 'capture areas' for each squirrel and have estimated the relative position of each individuals' capture area by determining % overlap during the breeding season. Because the total number of squirrels captured was large (87 individuals were caught and marked during the trapping season from Dec to Jul, 1993) it was not possible to carry out pairwise band-sharing comparisons between each individual and every overlapping individual in the population. Therefore, we focused on assessing the potential levels of relatedness between individuals which were found in close spatial association with newly weaned juveniles (eg juveniles born during the 1993 winter breeding season). Individuals with capture areas which overlapped each juvenile by > 5% were selected for DNA fingerprinting analysis. Any juveniles that overlapped other juveniles were considered potential siblings and were also included in the analysis.

Results

Genetic diversity and band-sharing values

The grey squirrel DNA fingerprints revealed complex individual specific banding patterns indicating that high levels of genetic variation existed within the Alice Holt population. The average number of scorable bands per DNA fingerprint for presumed unrelated individuals was 22.9 (n = 14, SD = 3.90) and the mean background band-sharing coefficient (x) value was 0.22 (n = 91 pairwise comparisons, SD = 0.094, range 0.08 to 0.51). The average number of scorable bands per DNA fingerprint for individuals with known first order relationships (eg parent-offspring; siblings) was 20.4 (n = 18, SD = 2.70) and the mean x-value was 0.71 (n = 22 pairwise comparisons, SD = 0.058, range 0.60–0.84). All pairwise comparisons between closely related individuals were statistically significantly higher than the background band-sharing coefficient ($p < 0.01$, Quinn *et al.* 1994). The band-sharing values fall into two statistically distinct groups with no overlap in their frequency distribution (t-test, $p < 0.0001$, Fig. 1). Fingerprint bands, therefore, were assumed to be shared due to common descent rather than to high levels of background band-sharing, a prerequisite for using DNA fingerprinting to answer questions regarding relatedness and kinship structure in wild populations.

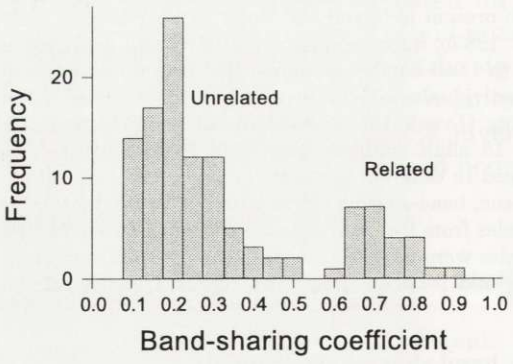

Fig. 1. Frequency distribution of DNA fingerprint band-sharing values for first order relatives and unrelated grey squirrels from Alice Holt Forest. Band-sharing was calculated according to Jeffreys *et al.* 1985b and Burke and Bruford 1987.

For the purposes of identifying relatedness between wild caught individuals, we assumed that all animals

with x-values that were comparable to the range found for first order relationships (x-values of 0.60 or above) and were significantly different from the mean background band-sharing when $p < 0.01$, shared close kinship and therefore were putative relatives.

Levels of relatedness and social structure

In all, nine out of 13 juveniles were overlapped by first order relatives (mean x-value = 0.68, SD = 0.062, n = 16). Five distinct kinship groups were identified. Each cluster was highly suggestive of family units comprised of either putative mothers or fathers in association with their newly weaned offspring. No nuclear families were identified and, because DNA fingerprint data were not available from the absent parent, it was not possible to fully establish parentage or sibships with certainty.

The putative parentage and sibships determined for group 1 was one adult male, one adult female and two juveniles. Exact parentage could not be determined here as several fingerprint bands in each juvenile could not be traced back to either male or female. This suggests group 1 consisted of either a putative mother, her two offspring and a close male relative, or a putative father, his two offspring and a close female relative. The x-value between the adult male and female (0.59) fell just below that adopted as indicating a first order relationship, although this clearly suggests a close genetic relationship. Groups 2, 3 and 4 comprised of one adult female and two juveniles, one adult female and three juveniles and one adult female and a single juvenile, respectively. Group 5 comprised of an adult male and a single juvenile (Table 1).

Juvenile relationships

In addition, we carried out pairwise comparisons amongst all 13 juveniles to determine the real levels of relatedness between the cohort from one breeding season. This provides further information on the social structure and possible mating system that existed within the population. No definitive first order relationships were identified, although an x-value of 0.59 between two juveniles strongly suggested that they were full sibs. Interestingly, several juveniles (n = 8) were found to have x-values amongst themselves which fell between the range of the two distributions (0.50–0.60). Furthermore, these x-values were found to be significantly different from the mean unrelated x-value ($p < 0.01$). No half sibling juveniles which shared the same parent were identified,

Table 1. Kinship groups identified from DNA fingerprint analysis of grey squirrels from Alice Holt forest. Results show within group mean band-sharing found between putative first order relatives. * – groups 4 and 5 consisted of 1 pairwise comparison, SD was not applicable.

Group	n	Mean x-value	SD
1	4	0.67	0.044
2	3	0.72	0.095
3	4	0.68	0.071
4	2	0.60	*
5	2	0.69	*

and thus we did not find any evidence of multiple paternity within the family units in this study.

Discussion

The results from our DNA fingerprinting analysis indicate that the study population was highly genetically diverse. Further, the distribution frequencies of band-sharing values for unrelated animals and animals with known first order relationships has shown that high levels of band-sharing are not due to chance but are the result of common descent (Quinn *et al.* 1994), thus permitting the estimation of relatedness between individuals. Other studies (eg Westneat 1990, Pemberton *et al.* 1992, Hunter *et al.* 1992, Quinn *et al.* 1994, Watt and Fenton 1995) have used a band-sharing analysis based on the empirical distribution of band--sharing values between relatives and non-relatives in the absence of segregation data. In common with our results, these studies found that the frequency distribution of band-sharing values for first order relatives and unrelated individuals fell into two distinct peaks with no overlap occurring between the two groups. However, we are aware there are statistical difficulties associated with relatedness based on band-sharing alone (Lynch 1988, 1990, Brock and White 1991, Burke *et al.* 1991, Hanotte *et al.* 1992).

Our results show first order relationships were established only between a small subset of closely associated adults and juveniles. Neighbouring adult animals showed similar levels of band-sharing between them as individuals which were considered unrelated. The social structure of the population appears therefore to consist largely of unrelated adult animals and close knit kinship groups composed of mothers or fathers and their putative offspring.

Capture area overlap was a relatively informative but restricted approach for identifying relatives. The method proved most successful at identifying mothers, with nine of the 13 juveniles being assigned putative maternity based on overlap data. Females have relatively small stable home ranges particularly during the breeding season (eg Kenward 1985, Wauters and Dhondt 1992, Wauters *et al.* 1994) and juvenile squirrels are known to forage in their mothers home ranges for several weeks between weaning and dispersal (Gull 1977, Larsen and Boutin 1994, Wauters *et al.* 1994). Few adult males with first order relationships were identified. However, trapping was not conducted during the period when females were in oestrus and, in contrast to females, males are highly mobile and range widely in search of oestrus females during the breeding season and particularly dominant males who are known to gain more matings than subordinates (eg Thompson 1977, 1978, Farentinos 1979, Benson 1980, Kenward 1985, Wauters and Dhondt 1992).

Although only one further first order relationship was identified from the non-overlapping intra-juvenile analysis, many juveniles shared large numbers of bands significantly higher than the mean background band-sharing value for unrelated individuals ($p < 0.01$), although not within the range estimated for first

order relationships. This level of band-sharing may represent second order relationships, ie half siblings. On a cautionary note, however, Westneat (1990) found the distribution of band-sharing values between full sibs and half sibs was less exact because there was a degree of over lap between the tail ends of the two groups. Thus, assumptions based on second order relationships are difficult to substantiate and we would need to determine the true levels of band-sharing amongst second order relatives within the squirrel population to draw firm conclusions from these data. However, juveniles appear, on average, to share higher levels of bands than adults animals which do not overlap, which suggests some degree of relatedness. We would expect a large number of half sibling relationships in a polygynous mating system in which access to oestrus females is restricted to a small subset of dominant males. Such a non-random mating system will affect the genetic substructuring of the population and consequently influence how genetic variation is distributed amongst its members.

In conclusion, although the use of multilocus DNA fingerprinting in this study has provided valuable information on the levels of genetic variability and levels of relatedness amongst individuals, the technique is clearly limited when applied to strongly polygynous or promiscuous systems particularly when supporting behavioural observation data is lacking. To overcome these problems, the development of species specific microsatellite markers (Litt and Luty 1989) which provide a more reliable and unambigious method for band-sharing analysis, would be the next step in this research to address future questions regarding mating strategy and reproductive success in this species.

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