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Relatedness Structure and Individual Identification in a Semi-fossorial Shrew (Soricidae: *Anourosorex squamipes*) — an Application of Microsatellite DNA

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Hon-Tsen Yu, Yu-Ying Liao and Chen-Hung Kao (2001) Relatedness structure and individual identification in a semi-fossorial shrew (Soricidae: *Anourosorex squamipes*) — an application of microsatellite DNA. *Zoological Studies* **40**(3): 226-232. We address the genetic relatedness of the mole shrew, *Anourosorex squamipes*, in Taiwan, employing 11 microsatellite DNA markers. These markers enable us to assign "bar codes" to each of the 36 mole shrews studied for individual recognition. We analyzed the relatedness among individuals sampled from populations at 3 levels of geographical scale, i.e., individuals sampled within a radius of 3, 15, and 100 km, respectively. The mean relatedness values were inversely proportional to the geographic realms of the populations: the mean relatedness value increased as the range decreased. However, even within the populations of a small range, variations of relatedness were no less than those of populations of larger ranges. Often local populations contained subgroups in terms of relatedness. http://www.sinica.edu.tw/zool/zoolstud/40.3/226.pdf

Key words: *Anourosorex*, Sociality, Relatedness, Shrew, Microsatellite DNA.

The mole shrew (*Anourosorex squamipes*) represents a unique radiation in lifestyle and adaptation among shrews of the family Soricidae (Hutterer 1985, Nowak 1991). *A. squamipes* is the sole extant species of the genus, and occurs in southwestern China, northern mountains of Burma and Thailand, and Taiwan. With a pointed nose, minute eyes, completely reduced pinnae, heavily built skull, short tail (< 10 mm), and short feet with long claws, the mole shrew looks more like a miniature mole (of the family Talpidae) than it does a shrew. Mole shrews are semi-fossorial, living underground and digging burrows, but also coming to the forest floor to search for food (Hutterer 1985, Yu 1993 1994). Yu (1994) suggested that several mole shrews might share the same burrow system, for 3-4 shrews were often caught successively by 1 trap placed at the same spot. Thus, the mole shrew may have peculiar social structure and behavior similar to those of some other subterranean mammals (Nevo 1979, Lacey et al. 1997, Lacey and Sherman 1997, Lacey 2000).

It is possible to infer several aspects of sociology, behavior, and spacing in animals from the genetic patterns uncovered by relevant markers. These aspects include the mating system (Zenuto et al. 1999), reproductive success (Stockley et al. 1994, Coltman et al. 1999), kinship (Queller and Goodnight 1989, Queller et al. 1993), dispersal (Favre et al. 1997, Mossman and Waser 1999), and individual identification (Roques et al. 1999). All these aspects depend largely on the capability to discern individual animals (i.e., individual recognition) and to infer the relatedness among a group of animals by appropriate genetic markers. The genetic approach can be particularly useful in studying animals that are difficult to observe directly, such as mole shrews.

Herein we report on the results of our investigation, using polymorphic microsatellite DNA markers, on the levels of relatedness among several groups of mole shrews across 3 geographic scales. Further-

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more, we demonstrate the potential of these microsatellite markers for individual recognition in future behavioral studies.

MATERIALS AND METHODS

Sample collection

A total of 36 mole shrews was collected from Taiwan for this study. Twenty-seven of the tissue (liver, kidney, and spleen) samples of the mole shrews were collected in 1989-1990 in an attempt to understand mammalian diversity in Taiwan (Yu 1993 1994): eighteen from several elevations along a river (Xia-Li-Xian-Shi) valley, five from Dwei-Guan, and 4 from Nan-Heng. All elevational sites of the Xia-Li-Xien-Shi lie within 15 km in horizontal distance. Four mole shrews were collected in 1995: two without information as to locality were donated by students of National Taiwan Univ.; the other 2 were collected from Wu-Ling. Tissues were preserved in liquid nitrogen following dissection soon after the animals were captured. In addition, five shrews were captured alive and brought back to the lab. in 1998 from a single locality (Chitou): two of these were used for genomic DNA extraction (from liver, spleen, kidney, etc.) and for constructing partial libraries (Yu and Liao 2000). The localities of the shrew specimens are shown in figure 1.

Microsatellite loci

Eleven polymorphic loci (H_e > 0.82) were selected to genotype the shrews. Cloning and characterization of these loci are described by Yu and Liao (2000). Primer sequences and the optimal annealing temperatures for each locus are given in Yu and Liao (2000). The sequences of the 11 loci are deposited in GenBank (accession numbers: AF261959-AF261969).

Genotyping

Individual genotypes were determined by polymerase chain reaction (PCR). The reactions were performed either with non-radioactive or radioactive primers. For non-radioactive PCR, 25 µl reactions were performed, containing 200 ng template DNA, 2.5 µl 10X buffer, 0.75 µl Mg^{+2} (25 mM), 1.5 µl dNTP (2.5 mM), 2.5 µl of forward and reverse primers (5 µM), 0.41 µl DNA *Taq* polymerase (5 U/µl, Promega), and 13.0 µl ddH_2O. Amplification was carried out with the following thermal profile: 95 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, the optimal annealing temperature (Yu and Liao 2000) for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. PCR products were run on 6% native polyacrylamide gel (15 x 17 x 0.2 cm), stained by Ethidium bromide and visualized on a UV light box. Non-radioactive PCR was used to screen for polymorphic loci and for the initial round of genotyping.

For radioactive PCR, one primer from each pair was 5'end-labeled with [γ^{32}P]-ATP (NEN) and T4 polynucleotide kinase (Promega), following the manufacturer's protocols. Each PCR reaction totaled 10 µl, containing 200 ng template DNA, 1 µl 10X buffer, 1 µl dNTP (2.5 mM), 1 µl unlabeled forward primer (2 µM), 1 µl unlabeled reverse primer (2 µM), 0.24 µl Mg^{+2} (25 mM), 0.05 µl DNA *Taq* polymerase (5 U/µl, Promega), 1 µl [γ^{32}P]-ATP-labeled primer and 2.71 µl ddH_2O. Amplification was carried out with the following thermal profile: 95 °C for 3 min, followed by 25 cycles of 95 °C for 15 s, the optimal annealing temperature (Yu and Liao 2000) for 2 min,

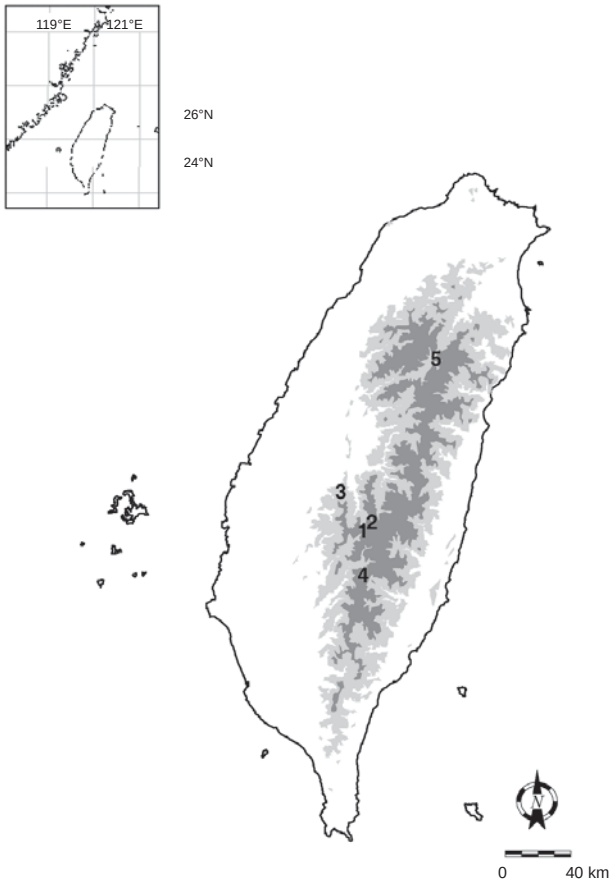

Fig. 1. Map of Taiwan showing the locations of sampling sites. 1, Xia-Li-Xian-Shi; 2, Dwei-Guan; 3, Chitou; 4, Nan-Heng; 5, Wu-Ling. Dark areas: elevations above 2000 m; gray areas: elevations between 1000 and 2000 m.

72 °C for 2 min, and a final extension step at 72 °C for 7 min. PCR products were run on regular denaturing 6% polyacrylamide sequencing gel. Sizes of alleles were estimated by using control DNA (PUC18) from a Thermo Sequenase Cycle Sequencing Kit (Amersham) as markers. Radioactive PCR was used for a 2nd round of screening: all alleles of different sizes detected in the 1st round of screening were run on comparison gels to accurately determine their sizes. Running radioactive PCR products on denaturing gels also helped reduce the confusion caused by heteroduplex bands that sometimes appeared in the 1st round of screening.

Data analysis

Heterozygosity. Genetic diversity of each locus was estimated by H_o, the proportion of the direct count of heterozygotic individuals in the population, and by H_e, the expected values calculated by a formula in Nei (1987).

Hardy-Weinberg equilibrium. The observed genotype frequencies were compared with Hardy-Weinberg expectations using Fisher's exact test (Guo and Thompson 1992). The comparison was done with GENEPOP (http://www.cefe.cnrs-mop.fr; Raymond and Rousset 1995).

Relatedness. To estimate genetic relatedness between individuals, we use the formula derived by Queller and Goodnight (1989):

$$R = \frac{\sum_x \sum_k \sum_l (P_y - P^*)}{\sum_x \sum_k \sum_l (P_x - P^*)}$$

where P_x is the frequency within the current individual x of the allele found at *x*'s locus *k* and allelic position *l*; P_y is the frequency of that same allele in the set of partners of *x* or the individual(s) to which one wants to measure *x*'s relatedness; and P^* is the frequency of the allele in the population at large, with the bias corrected by removing all putative relatives of *x*. Symmetrical *R* (also see Girman et al. 1997) was estimated by using the computer program RELATEDNESS (http://gsoft.smu.edu/GSoft.html). Here we let P^* equal 0, assuming that the individual under consideration is not related to any other member in the population. Consequently, *R* values ranged between 0 and 1.

We grouped the mole shrews into geographic regions scaled to their inclusive horizontal distance. Mole shrews from Chitou, Dwei-Guan, Nan-Heng, and Wu-Ling were treated as separate populations in the most-restricted scale (within a radius of 3 km). Second, all 18 mole shrews from a single watershed, Xia-Li-Xian-Shi (river), were grouped as a population in a "medium" radius of 12 km (see maps in Yu 1994). Finally, we grouped all 36 mole shrews from throughout Taiwan as the least-restricted population with a radius of ca. 150 km. The pairwise relatedness values were calculated for individuals within these geographic populations, respectively. Because of small sizes of the samples, we used bootstrap procedures (1000 replicates) to test the differences in relatedness (*R*) between any 2 geographic populations. Bootstrap procedures were performed for individuals and for loci. Since both procedures give congruent results (Liao 1999), we present the results of bootstrap by individuals. The bootstrap procedures are meant to obtain the most statistically sound conclusions possible, especially for localized populations with their small sample sizes.

F-statistics. F_{ST} and F_{IS} (Wright 1978) were calculated by the methods of Weir and Cockerham (1984). F_{ST} is used to test for population subdivision and F_{IS} for inbreeding. The calculations were done using GENEPOP (Raymond and Rousset 1995, http://wbiomed.curtin.edu.au/genepop).

RESULTS

Number of alleles and allele frequency distribution

The allele frequency distributions were plotted based on allele size (estimated in the denaturing sequencing gels) for the 11 loci (Fig. 2). The numbers of alleles ranged from 10 (locus *AS1*) to 20 (loci *AS3* and *AS10*), with an average of 15.5. There were no consistent modes of distribution. Three loci, *AS1*, *AS2*, and *AS4*, had a nearly normal distribution. The others contained 1 to 3 dominant alleles, whereas the remaining alleles all existed at low frequencies, giving a flattened distribution.

Hardy-Weinberg equilibrium

A test of combined loci for 36 mole shrews indicated deviation from Hardy-Weinberg expectations of genotypes ($p < 0.001$). That was also true for all tests for the 11 individual loci (all $p < 0.05$). The deviation was caused by a deficiency in heterozygotes at all loci as revealed by score tests at individual loci (Rousset and Raynond 1995).

Relatedness of populations

We present 2 types of data on relatedness (*R*),

the observed and bootstrap values (Table 1). Bootstrap procedures offer a more-robust way of statistical testing, since our sample sizes are small. However, both observed and bootstrap values gave comparable results.

R increased as the scale of the population realm decreased, as intuition would suggest (Table 1). For bootstrap values, the mean *R* values of the 3 levels (150, 15, and 3 km) significantly differed, as their 95% CIs do not overlap. No differences existed between the 3 populations at the scale of 3 km (Dwei-Guan, Chitou, and Nan-Heng). The trend was generally followed in observed values (Table 4), but there were overlaps of the 95% CIs between Xia-Li-Xien-Shi and 2 populations at 3 km (Dwei-Guan and Chitou).

It is noteworthy that although the mean *R* values were greater in populations of smaller scale (3 km) than those of the "medium" scale (12 km) population, variations of *R* could be just as high as those of the "medium" scale. Two (Dwei-Guan and Chitou) out of the 3 populations of 3 km had comparable coefficients of variation (CV), of 48 and 40, respectively, to that of the Xia-Li-Xien-Shi, of 41 (Table 1). The situation with Chitou's population is particularly interesting since these individuals were captured from a bamboo grove of roughly less than 1 x 1 km: one individual, field no. Yu 1785, was less genetically related to the other 4. *R* (mean ± SE) between Yu 1785 and the other 4 mole shrews (0.1806 ± 0.0067) was much lower than those among the 4 mole shrews (0.3823 ± 0.098). Likewise, one mole shrew, field no. Yu 570, from Dwei-Guan had lower relatedness (*R* = 0.1357 ± 0.0448) than that among the other 4 individuals (*R* = 0.3591 ± 0.0287). Nonetheless, these individuals were all captured within a radius of 3 km.

Applying microsatellite DNA for individual identification

With high polymorphic levels of microsatellite loci, we could create an identification "bar code" number for each individual by combining genotypes

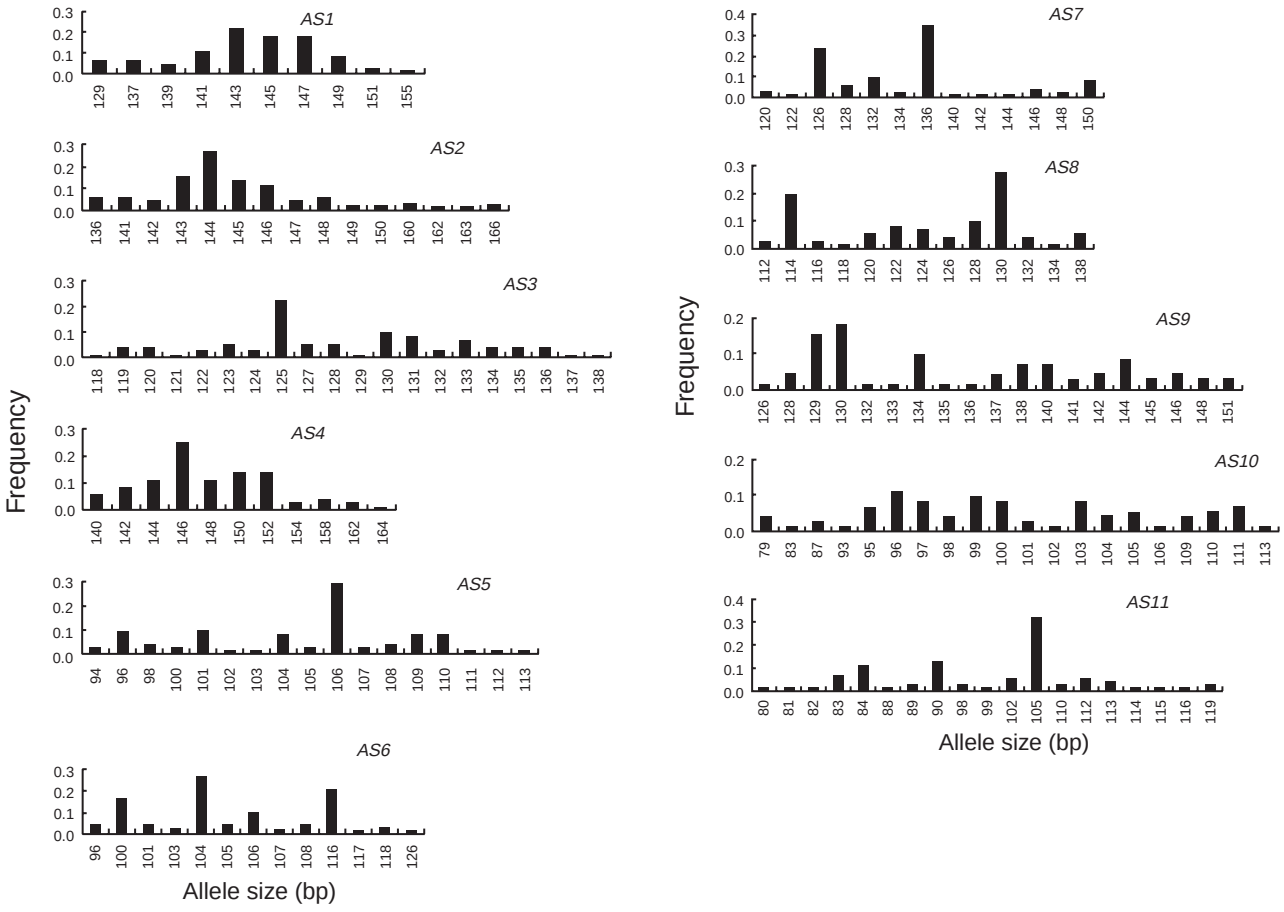

Fig. 2. Allele sizes (bp) and distribution of alleles at 11 polymorphic microsatellite loci in mole shrews (*Anourosorex squamipes*).

at the 11 loci. In fact, we could assign each of the 36 mole shrews a unique "bar code" number using just 2 loci: e.g., *AS10* and *AS3*; *AS10* and *AS4*; *AS10* and *AS9*; *AS10* and *AS5*. However, a future behavioral genetics study in 1 locality may require the application of additional loci, when individuals of close kinship are involved. The markers can also be used for pedigree inferences with capture-recapture data on the shrew, which is currently being planned.

DISCUSSION

Combined with preliminary observations on natural history, molecular genetic markers can be used to infer behavioral and ecological interactions among individuals in animal societies, including genetic relatedness, patterns of paternity, and individual reproductive success (Hughes 1998, Lacey 2000). We demonstrated that the 11 polymorphic microsatellite markers employed herein are suitable for studying these characteristics in mole shrew (*Anourosorex squamipes*) populations. Below, we discuss aspects regarding (1) the application of these markers and (2) the relatedness patterns uncovered with the current data.

One of the inherent drawbacks of using microsatellite markers is the presence of null alleles (Callen et al. 1993, Paetkau and Strobeck 1995, Pemberton et al. 1995, Ishibashi et al. 1996, Jarne and Lagoda 1996, Treuren 1998). In the presence of null alleles, a population would tend to deviate from Hardy-Weinberg equilibrium (Treuren 1998), showing a heterozygote deficiency. Although the deviation from Hardy-Weinberg equilibrium and deficiencies of heterozygotes in our results could be attributed to null alleles, there can be other causes. At least, either inbreeding or Wahlund effects can also result in heterozygote deficiencies. The overall inbreeding coefficient F_{IS} (Wright 1978) was estimated to be 0.257, yet it is not statistically significant to reject the hypothesis of F_{IS} equal to 0. Therefore, inbreeding appears unlikely to be a strong cause of the deficiency. However, there is significant substructure in the sample, because 8 of the 11 loci have F_{ST} (Wright 1978) values that are high and significantly greater than 0 (all $p < 0.001$). Combining these subpopulations is likely to cause a heterozygote deficiency. Moreover, we detected no null homozygotes or false heterozygotes, even though we employed 2 independent methods to screen the alleles. It seems, therefore, that the heterozygote deficiency can not be attributed to the presence of null alleles.

We found a noteworthy pattern of relatedness of mole shrews examined here (Table 1). More-localized populations tend to have individuals with higher mean relatedness, which is not at all unexpected. At the level of an inclusive range of 3 km, three populations (Dwei-Guan, Chitou, and Nan-Heng) consistently contained individuals more closely related to one another than what occurred in the population of 12 km (Xia-Li-Xien-Shi). Yet the variations in relatedness of 2 of the 3 localized populations (Dwei-Guan and Chitou) are no less than that of Xia-Li-Xien-Shi. Both populations of Dwei-Guan and Chitou include individuals with quite different relatedness values. This implies that, somehow, local mole shrews had permitted recruitment of members from outside their kinship. It would be interesting to further investigate the microgeographic spacing of the separate "kinship" lineages to reveal the social interactions of the mole shrews.

We conclude that microsatellite markers are suitable for behavioral genetic, sociobiological, and biogeogaphical studies in mole shrews in the future. It is particularly useful when the capture-recapture method is employed to reveal the spatial association of individual mole shrews in a local population.

Acknowledgments: Chu-Fong Lo and members of

Table 1. Mean relatedness values, *R*, for mole shrew populations of different geographic realms. Both observed and bootstrap estimates are included. CV, coefficient of variation; CI, confidence interval

Population	Observed		Bootstrap	
(sample range; sample size)	*R* (CV)	95% CI	*R*	95% CI
Total (150 km; 36)	0.1666 (69)	0.1290-0.2042	0.1842	0.1741-0.1944
Xia-Li-Xian-Shi (12 km; 18)	0.2601 (41)	0.2107-0.3095	0.2975	0.2859-0.3091
Dwei-Guan (3 km; 5)	0.2697 (48)	0.1559-0.3835	0.4215	0.4019-0.4411
Chitou (3 km; 5)	0.3016 (40)	0.1958-0.4075	0.4466	0.4279-0.4652
Nan-Heng (3 km; 4)	0.3995 (14)	0.3466-0.4524	0.5497	0.5331-0.5664
Wu-Ling (3 km; 2)	0.4667	–	–	–

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半穴居性短尾鼩的親屬關係結構和個體辨識 — 微隨體 DNA 的一個應用

于宏燦[1] 廖郁英[1] 高振宏[2]

我們以 11 個微隨體 DNA 基因座遺傳標誌，探討臺灣短尾鼩(*Anourosorex aquamipes*)的親屬關係。利用這 11 個遺傳標誌，我們可以設定辨識身分的「遺傳條碼」給 36 隻短尾鼩。我們研究三個地理範圍的短尾鼩族群，分別是 3 公里、15 公里和 100 公里的直徑範圍，估算每個族群內個體間的親屬關係值。族群的平均親屬關係值和直徑範圍大小成反比：即平均親屬關係值隨範圍縮小而漸增。然而即使在小範圍內，個體間親屬關係值的變異並不亞於大範圍的變異，而且在小範圍內亦有親屬關係值不同的亞群存在。

關鍵詞：*Anourosorex*，社會性，親屬關係，鼩鼱，微隨體 DNA。

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