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Evolutionarily Significant Units of Gray-Sided Vole (*Myodes rufocanus*) in Hokkaido, Japan

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The gray-sided vole, *Myodes rufocanus* (= *Clethrionomys rufocanus*) is a widespread species in Hokkaido, Japan. We applied an integrative approach to determine adaptive and neutral genetic variation in the gray-sided vole populations to identify full or partial Evolutionarily Significant Units. We surveyed 38 mainland populations and six island populations. The mitochondrial DNA control region was analyzed for neutral genetic variation and cranial measurements were taken as a proxy for adaptive variation. Data on neutral genetic variation indicate that most island populations are differentiated from the mainland populations. The islands of Teuri and Yagishiri, Rishiri and, Kunashiri can be regarded as partial ESUs based on their unique haplotypes. Partial ESUs based on morphological features can be identified in the eastern part of mainland with populations from Akkeshi and Shibecha. A full ESU designation can be given to Daikoku Island based on its unique haplotype and unique morphological feature.

Key Words: adaptive variation, ESU, mitochondrial DNA, morphometrics, neutral genetic variation, small mammal

INTRODUCTION

Variability in any trait is expected to be spatially structured even in widespread species of animals due to several physical and ecological barriers that isolate populations. Variation in these populations can be recognized based on neutral genetic variation (Moritz 1994a) and adaptive variation (Crandall et al. 2000). Neutral genetic variation is generated by genetic drift and/or founder effect owing to isolation while adaptive variation is caused by environmental conditions. These are the criteria used in the identification of a conservation unit, the Evolutionarily Significant Unit or ESU (Ryder 1986). However, full ESUs should be identified based on both adaptive and neutral genetic variation and populations showing only either of the components should only be designated as partial ESUs (de Guia and Saitoh 2007).

Historically isolated populations are likely to have a distinct evolutionary potential (Moritz 1994a), and may give rise to new species particularly endemic types which are especially likely to develop on islands because of their geographical isolation. Adaptive populations, on the other hand, possess the capability to adjust to an ever-changing environment. Using both neutral genetic variation and adaptive variation is essential because they provide a clear picture of the real and existing variation in populations. Populations may possess either adaptive variation or neutral genetic variation only, or they may vary from other populations in both aspects. However, because obtaining data for adaptive variation has been proven to be difficult, studies on spatial structure of populations have been greatly biased to neutral genetic variation.

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The gray-sided vole, *Myodes rufocanus* = *Clethrionomys rufocanus* (Sundevall 1846) is distributed in the northern Palearctic, from northern Fennoscandia extending eastern through Russia, Mongolia, China, Korea, and Japan. In Japan, it is found only in the northernmost island of Hokkaido where it is distributed throughout the mainland and in the northeastern islands of Rebun, Rishiri, Teuri, and Yagishiri, as well as in the eastern islands of Daikoku, Kunashiri and other islands in the southern Kurile region (Figure 1).

The *M. rufocanus* of Hokkaido has been described as undergoing further genetic differentiation from Russian continental populations through geographic isolation (Iwasa et al. 2000). If such processes are to be considered in the establishment of new species such as the red-backed vole *Myodes rex* Imaizumi, 1971 (Iwasa et al. 2000) which is confined to Hokkaido (Kaneko et al. 1998; Nakata 2000) and Sakhalin (Abramson et al. 2009), then it is possible that the Hokkaido *M. rufocanus* populations may give rise to new species, races, or subspecies in the future. This is not far-fetched since the *Myodes rufocanus* of Hokkaido has been originally identified as a distinct species, *Evotomys* (= previously *Clethrionomys, now Myodes*) *bedfordiae* (Thomas 1905) and presently regarded by some as a subspecies, *M. ruocanus bedfordiae*. Island populations have been taxonomically conflicting. For example, those in Daikoku Island and Rishiri Island have been classified as *Neoaschizomys sikotanensis* (Tokuda 1935) = *Clethrionomys sikotanensis, M. sikotanensis* (Imaizumi 1960) but which has recently been proven to be a junior synonym of *M. rufocanus* (de Guia et al. 2007; Motokawa 2008). At present, the species is considered a nuisance due to the damage it causes to forest plantations (Kaneko et al. 1998). However, detection of genetic and morphological variation across many populations in Hokkaido may help identify populations with distinct evolutionary potential or adaptive potential which are prioritized in terms of conservation.

Hokkaido's islands have been isolated during different times (Naitoh and Ohdachi 2006). Rebun and Rishiri Islands were separated from mainland Hokkaido 12,000 years before present (BP). Teuri and Yagishiri Islands were separated 11,000 years BP while Kunashiri Island 7,000 years BP. The youngest is Daikoku Island, separated from the mainland only 5,000 years BP. The mainland has a relatively rugged terrain. A mountain ridge runs through the center seemingly dividing it into eastern and western parts. There are also several mountainous regions in the eastern and western parts. This topography may provide conditions for isolating populations of the gray-sided vole. Aside from these physical barriers, female voles are known to be philopatric (disperse <50m from their natal site) and form female kin clusters (Ishibashi et al. 1998). For the island populations, aside from their isolation from the mainland source and the existing island conditions, the time of separation of the island may influence the degree of differentiation. Neutral genetic variation and/or adaptive variation are expected to be greater between islands and mainland than would be among mainland populations. Island populations generally have reduced genetic variability compared with continental forms of the same species (Frankham et al. 2002). Others exhibit morphological divergence from the mainland populations (Pergams and Ashley 1999; Pergams et al. 2000) without accompanying genetic divergence (Gill 1980), e.g., the deer mouse, *Peromyscus maniculatus*, in the Californian Channel Islands where there are nine subspecies. Other small mammals previously widespread in the continental mainland but which have been isolated on small islands, distinct island forms or relict species have undergone drift or selection. Thus, these have been regarded as full species, such as the rice rat, *Aegialomys galapagoensis* = *Oryzomys bauri*, in the Galapagos Island (Berry 1998). Thus, although the gray-sided vole is distributed across mainland Hokkaido, populations may be differentiated and the species may exhibit genetic and morphological spatial structure over its geographic range.

In this study we used the mitochondrial DNA (mtDNA) control region, a highly variable region, as our genetic marker to trace the neutral genetic variation (Avise 1994). in the matrilineage of the gray-sided voles. This marker has proven to be an ideal marker in studies related to phylogeography (Moritz et al. 1987; Moritz 1994b; Ballard and Rand 2005) due to its high rate of sequence evolution, uniparental inheritance, and lack of recombination (Lowe et al. 2004) but is however limited to the study of female lines. We also measured 18 cranial characters as a proxy to examine the adaptive variation among the mainland and island populations of the gray-sided vole. Cranial variation in mammals has been proven (Hanken and Hall 1993; Marroig and Chevrud 2001; Viguier 2002; Cardini et al. 2007) to be driven by ecological or adaptive forces.

This study aimed to determine the genetic spatial structure of the gray-sided vole in Hokkaido, Japan and determine whether the island and mainland populations of the gray-sided vole in Hokkaido, Japan are morphologically differentiated so that full or partial Evolutionarily Significant Units (ESUs) can be identified.

MATERIALS AND METHODS

Sample collection

The samples (n=353) used in this study were collected from 44 local populations (Figure 1) from 2004 to 2006. The Hokkaido Regional Office, Forestry Agency of Japan

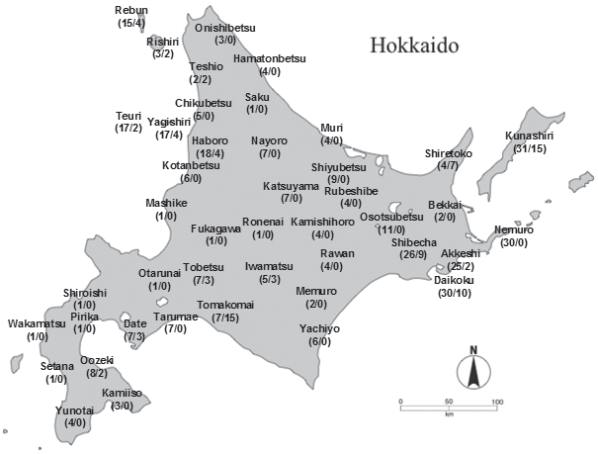

Figure 1. Map showing the 44 sampling areas in Hokkaido. Numbers in parentheses indicate the number of individuals examined (genetic/morphological).

collected most of the samples. Personal sampling was only done in Akkeshi, Daikoku Island, Teuri Island, Yagishiri Island, and Rishiri Island. Samples from Kunashiri Island, Nayoro, Nemuro, Shiretoko, and Tomakomai were provided by other researchers and students. Liver tissues were collected from the samples and preserved in 99% ethanol for DNA extraction. Skulls (n= 87) were collected, cleaned, and kept for the morphological analysis.

Procedures

Neutral Genetic Variation

Genomic DNA was extracted from individual voles using a modified Chelex protocol (Walsh et al. 1991). The mtDNA control region and flanking regions were amplified. For most of the samples (except Akkeshi, Nemuro, Kunashiri Island and Daikoku Island), the primers Lpro (5' – TCAGCACCCAAAGCTGATATTCTACTT – 3') and Hphe (5' – ATCTAAGGCATTTTCAGTGCTTTGCTT – 3'), which were designed based on a *M. rufocanus* mtDNA sequence, were used to amplify the target sequence. PCR amplification was carried out using a Robocycler (Stratagene) in 20 µL reaction mixtures containing 0.2 mM dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% (w/v) gelatin, 0.25 µM of each primer, and 0.5 unit of AmpliTaq Gold (Applied Biosystems). About 0.5 µL of genomic DNA (10 - 100 ng) was used for each reaction. After incubation at 95° C for 10 minutes, cycling was performed for 43 cycles of 20s at 93°C, 20s at 50°C and 60s at 72°C, with a postcycling extension at 72°C for seven minutes. After removing excess primers and dNTP using Exo-SAP-IT (Amersham Biosciences), the partial sequence of mtDNA was determined using the sequencing primer Lpro and were analyzed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 3100 Avant Genetic Analyzer. Samples from Akkeshi, Nemuro, Kunashiri Island, and Daikoku Island were amplified using a universal PCR primer pair; L15926 and H00651 (Kocher et al. 1989). The partial sequence of mtDNA was determined using L15926 and internal primers HIP2 (5' – CCAAATTTGCCTATCCTTGC – 3'), HIP8 (5' – TCGTCCATACGTTCCCCTTA – 3') and LIP2 (5' – CTTGGGGGTGACTAACCTGA – 3') by using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) on an ABI Prism 310 Genetic Analyzer.

The DNA sequences were aligned using the program Clustal X 1.83 with further correction by eye. Using the software package Arlequin version 2.000 (Schneider et al. 2000), several genetic diversity indices including gene diversity (H), number of segregating sites (S), mean number of pairwise nucleotide differences and nucleotide diversity (π) were estimated for the populations. A median-joining (MJ) network (Bandelt et al. 1999) was also constructed to infer the direction and number of nucleotide substitutions among haplotypes using the software, Network 4.200 (Fluxus–engineering.com).

Cranial Traits

Sample size for this analysis is smaller since only skulls from adult individuals were included in the study. We measured 18 cranial characters (Appendix A) as described in Abe (1973) and illustrated in Abe (2000) and de Guia et al. (2007) using a digimatic caliper from six island populations: four northwestern populations (Rebun, Rishiri, Teuri and Yagishiri) and two northeastern island populations (Kunashiri and Daikoku). Ten mainland populations were analyzed: two in the northwestern region (Teshio and Haboro), three from the northeastern area (Shiretoko, Shibecha, and Akkeshi), one central population (Iwamatsu), three southwestern populations (Tobetsu, Tomakomai, and Date) and, one southwestern population (Oozeki).

These cranial measurements were analyzed using the program JMP version 6 (SAS Institute Inc.). A Hierarchical Cluster Analysis using the ward method of all the cranial characters was used to determine whether the cranial characters from the different populations were variable. We then performed the Principal Component Analysis (PCA) to characterize the morphological clusters and determine which measurements contribute in distinguishing the skull features for each cluster. Differences between clusters were analyzed by the Kruskal-Wallis test or by Wilcoxon signed-rank test for paired cranial characters as appropriate. The Canonical Discriminant Analysis was also performed to group individuals among populations. Differences between the means of the identified groups were tested using Wilk's Lambda . While PCA maximizes the total variation explained by each principal component,

discriminant function analysis focuses on the extent to which that variation is partitioned among groups, thereby maximizing the separation of the groups.

RESULTS

Neutral genetic variation

Characterization of sequence variation

The 745-bp partial mtDNA control region of 44 local populations of the vole was sequenced. The base substitutions at 91 variable positions defined 146 distinct haplotypes. No deletions or insertions were observed. Most substitutions were transitions. Transversions were observed only at 15 positions and multiple substitutions were observed at four positions (#s 144, 236, 587 and 589).

Genetic Diversity Indices

Since the island populations of Daikoku, Teuri, and Yagishiri possessed a single haplotype all the values for the genetic indices generated were zero. This was also the case for the mainland populations of Wakamatsu, Ronenai, Saku, Pirika, Shiroishi, Fukagawa, Otarunai, and Mashike wherein a single individual was sampled. All the other populations exhibited high diversities (Refer to Table 1).

Genealogical relationships among haplotypes

Among the 146 haplotypes, 24 (16%) were shared (see Figure 2: nodes with more than one color) between and among populations sampled. The maximum number of populations which shared a single haplotype was eight and the minimum was two. The populations in the eastern and northwestern regions shared haplotypes with near neighbors as well as distant populations. On the other hand, populations in the southwestern region did not share haplotypes with near neighbors but only with distant populations. There was a single case of island-mainland haplotype sharing between Rebun Island and two mainland populations (Kamishihoro and Onishibetsu).

The MJ network of the 146 haplotypes from the 44 populations showed indefinite phylogeographic partitioning. Figure 2 shows that the nodes (haplotypes) of the same color (belonging to the same population) are generally not linked or related to each other. In the mainland, neighboring populations were generally not related with each other and there were shared haplotypes between and among distantly located populations. However, most island populations showed some common features indicating differentiation from the mainland populations. Most of the island populations like Daikoku, Rishiri, and Kunashiri possessed unique haplotypes. Teuri and Yagishiri Islands which are adjacent in the northwest part shared a single unique haplotype.

Cranial Traits

Cluster Analysis

The Hierarchical Cluster Analysis using the ward method revealed five distinct clusters (1-5). Cluster 1 formed the cluster consisting of the most number of individuals (38%) from various populations stretching from the southwestern region (Date, Tomakomai, and Tobetsu), the northwestern islands (Rebun and Yagishiri), central area of Iwamatsu and in the northeastern region of Shiretoko, and the island of Kunashiri. Cluster 2 was formed primarily by the northwestern populations of Rishiri Island, Teshio, Haboro and Teuri Island and some individuals from Kunashiri Island. Except for Kunashiri Island, individual samples of this cluster were restricted to the northern portion of Hokkaido. Cluster 3 was formed by fewer individuals but included more populations than Cluster 1. This Cluster included distant populations such as a southwestern population (Oozeki) and northwestern island population (Rishiri). All of the individuals from Daikoku Island formed Cluster 4. Cluster 5 was formed by populations from Akkeshi and Shibecha, restricted to the eastern part of mainland Hokkaido.

Morphologically, the populations sampled can be categorized as having common, localized or exclusive cranial characters. Populations with common cranial characters share morphological features with most of the populations. These are those that belong to clusters 1, 2, and 3. There is no apparent morphological spatial structure for these populations. Populations with localized cranial characters share traits with neighboring population/s, such as Akkeshi and Shibecha. Populations with exclusive cranial characters exhibit unique morphological features not shared with any other populations. This is the case for the Daikoku Island.

Principal Component Analysis (PCA)

The first two principal components accounted for 63% of the total variation present in the original variables. The first principal component explained 51% of the variances and the second principal component explained 12%. The first principal component was sufficiently explained by five (Condylobasal length, CBL; Palatal length, PL; Length of braincase, LB; Zygomatic width, ZW and Diastema, D) of the 18 cranial measurements. The second principal component was sufficiently explained by three (Posterior length of nasals, PLN; Nasal width, NW and Interorbital width, IW) of the 18 cranial measurements (see Table 3). The first principal component characterizes the skull as long and wide due to the longer CBL and wider

Table 1. Genetic diversity indices of the gray-sided vole populations based on the partial mtDNA sequences consisting of the 5' sequence of the control region (745 bp). *n*: number of individuals, *k*: number of haplotypes, *H*: gene diversity ± variance, *S*: number of segregating sites (transition, transversion), *d*: mean number of pairwise nucleotide differences ± variance, π: nucleotide diversity ± variance (multiplied by 100). Columns are also included for the number of samples analyzed for the cranial traits and morphological cluster assignment. Color code refers to color node in Figure 2.

Population	*n*	*k*	Color code	Genetic diversity indices				*n* analyzed for cranial traits	Morphological cluster
				H	*S*	*d*	π		
Daikoku	30	1		0.000 ± 0.0000	0 (0, 0)	0.000 ± 0.000	0.000 ± 0.000	10	4
Akkeshi	25	10		0.883 ± 0.0354	29 (26, 3)	6.687 ± 3.264	0.897 ± 0.488	2	5
Shibecha	26	18		0.966 ± 0.0196	39 (33,6)	6.230 ± 3.057	0.836 ± 0.457	9	5
Nemuro	30	8		0.798 ± 0.055	22 (21,1)	5.294 ± 2.630	0.711 ± 0.393	-	-
Kunashiri	31	12		0.918 ± 0.025	24 (22,2)	4.404 ± 2.234	0.591 ± 0.334	15	1, 2, 3
Rebun	15	5		0.781 ± 0.064	10 (9,1)	4.876 ± 2.518	0.654 ± 0.379	4	1, 3
Bekkai	2	2		1.000 ± 0.500	8 (8,0)	8.000 ± 6.000	1.073 ± 1.139	-	-
Oozeki	8	8		1.000 ± 0.062	17 (13,4)	5.750 ± 3.079	0.771± 0.471	2	3
Teshio	2	2		1.000 ± 0.500	18 (10,8)	5.000 ± 3.873	0.671 ± 0.735	2	2
Yunotai	4	3		0.833 ± 0.222	9 (8,1)	4.666 ± 2.885	0.626 ± 0.462	-	-
Hamatonbetsu	4	4		1.000 ± 0.177	13 (12,1)	6.833 ± 4.075	0.917 ± 0.653	-	-
Nayoro	7	6		0.952 ± 0.095	16 (13,3)	6.381± 3.442	0.856 ± 0.529	-	-
Onishibetsu	3	3		1.000 ± 0.272	9 (9,0)	6.000 ± 3.928	0.805 ± 0.658	-	-
Tarumae	7	3		0.667± 0.160	8 (8,0)	3.238 ± 1.895	0.435 ± 0.291	-	-
Tobetsu	7	5		0.904 ± 0.103	11 (10,1)	5.238 ± 2.881	0.703 ± 0.443	3	1, 3
Kamishihoro	4	4		1.000 ± 0.177	8 (7,1)	4.166 ± 2.609	0.559 ± 0.418	-	-
Osotsubetsu	11	11		1.000 ± 0.039	16 (16,0)	4.618 ± 2.454	0.620 ± 0.372	-	-
Tomakomai	7	7		1.000 ± 0.076	21 (20,1)	7.238 ± 3.862	0.972 ± 0.594	15	1, 3
Teuri	17	1		0.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	2	2
Yagishiri	17	1		0.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	4	1, 3
Iwamatsu	5	5		1.000 ± 0.126	20 (19,1)	8.400 ± 4.690	1.127 ± 0.736	3	1, 3
Katsuyama	7	7		1.000 ± 0.076	18 (15,3)	7.619 ± 4.048	1.024 ± 0.623	-	-
Chikubetsu	5	5		1.000 ± 0.126	5 (5,0)	2.800 ± 1.768	0.376 ± 0.277	-	-
Shiyubetsu	9	9		1.000 ± 0.052	25 (23,2)	6.805 ± 3.540	0.913 ± 0.539	-	-
Shiretoko	4	4		1.000 ± 0.177	5 (4,1)	2.666 ± 1.778	0.358 ± 0.285	7	1, 3
Yachiyo	6	6		1.000 ± 0.096	17 (15,2)	7.067 ± 3.867	0.948 ± 0.599	-	-
Kotanbetsu	6	3		0.600 ± 0.215	8 (8,0)	3.867 ± 2.256	0.519 ± 0.350	-	-
Rubeshibe	4	4		1.000 ± 0.177	15 (14,1)	8.667 ± 5.080	1.163 ± 0.814	-	-
Haboro	18	13		0.948 ± 0.039	25 (23,2)	7.497 ± 3.673	1.006 ± 0.551	4	2
Setana	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Wakamatsu	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Ronenai	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Saku	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Pirika	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Shiroishi	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Muri	4	4		1.000 ± 0.177	13 (12,1)	7.000 ± 4.167	0.940 ± 0.668	-	-
Date	7	7		1.000 ± 0.076	17 (17,0)	5.904 ± 3.208	0.793 ± 0.493	3	1, 3
Rawan	4	4		1.000 ± 0.177	10 (8, 2)	5.167 ± 3.160	0.693 ± 0.507	-	-
Fukagawa	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Otarunai	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Mashike	1	1		1.000 ± 0.0000	0 (0, 0)	0.000 ± 0.0000	0.000 ± 0.0000	-	-
Kamiiso	3	2		0.667 ± 0.314	5 (5,0)	3.333 ± 2.323	0.447 ± 0.389	-	-
Memuro	2	2		1.000 ± 0.500	4 (4,0)	4.000 ± 3.162	0.537 ± 0.600	-	-
Rishiri	3	3		1.000 ± 0.272	4 (3,1)	2.667 ±1.919	0.358 ± 0.321	2	2, 3

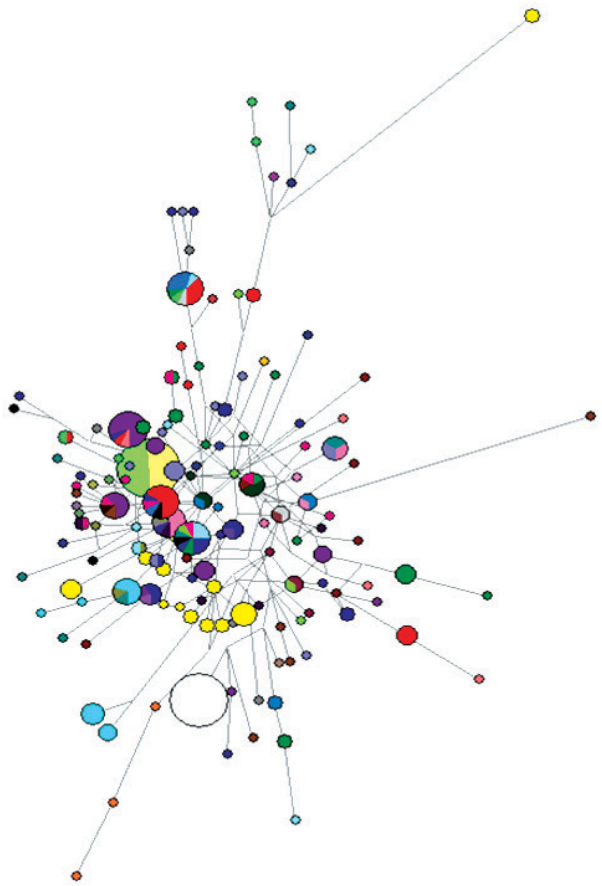

Figure 2. Median-joining network (Bandelt et al. 1999) of the 146 observed mtDNA control region sequences. Diameters of circles are proportional to the frequencies of the respective haplotypes. The colors indicate the local population where the haplotype was observed.

ZW. The second principal component, on the other hand, characterizes the skull as having wider space between eyes and a wider rostrum due to the wider nasals.

Figure 3 shows the relationship of the five clusters. Among these clusters, there was an overlap between Clusters 1 and 3 while Clusters 2, 4 and 5 formed distinct groups. Generally, the cranial character that contributed to the second principal component (IW) can differentiate Clusters 1, 2 and 3 from Clusters 4 and 5. This character was wider for those belonging to clusters 1, 2 and 3 than for those in clusters 4 and 5 (Figure 3; Wilcoxon test, $Z = 6.87$, $P < .001$). On the other hand, the cranial character that contributed to the first principal component (CBL) can differentiate Clusters 1 and 3 from Cluster 2, which had longer CBL than the other two. Among the three, Cluster 3 was shortest relative to the two other clusters mentioned (Figure 3; Kruskal-Wallis test, $^2 = 49.30$, $P < .0001$). Therefore, the CBL of Cluster 2 was significantly longer than that of Cluster 3 (Figure 3B; Wilcoxon test, $Z = 3.84$, $P < .0001$). Cluster 4 had longer skull than those of Cluster 5.

Table 2. Summary of the Principal Component Analysis of the 87 *Myodes rufocanus* adult individuals from 16 populations using the 18 cranial measurements. The highlighted variables contributed to the 1st and 2nd principal components.

Cranial Variables	Principal Component	
	1	2
CBL	0.31340	-
PL	0.29523	-
DB	-	-
LB	0.28449	-
LR	-	-
NL	-	-
WR	-	-
PLN	-	0.41559
ZW	0.29368	-
MW	-	-
NW	-	0.49634
PF	-	-
D	0.28935	-
WT	-	-
IW	-	0.61316
M	-	-
UT	-	-
LT	-	-

Table 3. Summary of the Canonical Discrminant Analysis of the 87 *Myodes rufocanus* adult individuals from 16 populations using the 18 cranial measurements. The highlighted variables contributed to the 1st and 2nd canonical discriminant function.

Cranial Variables	Canonical Discriminant Function	
	1	2
CBL	-	-
PL	-	1.701
DB	-	-
LB	-	1.081
LR	-	-
NL	-	-
WR	-2.291	-
PLN	-	-
ZW	-	-
MW	-	-
NW	-	-
PF	-	-
D	-	-
WT	-	1.125
IW	4.812	-
M	-	-
UT	-	-
LT	-	-

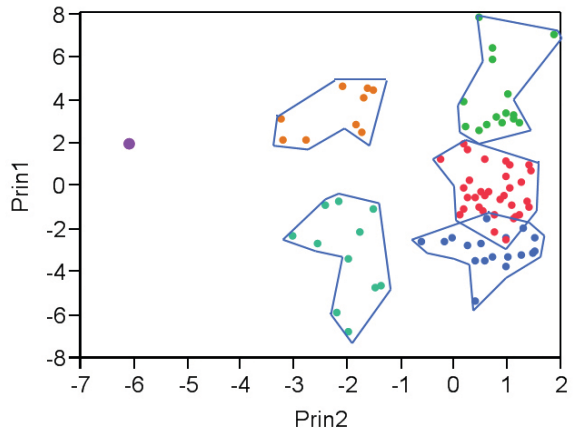

Figure 3. Relationship of the five distinct clusters based on the first and second principal components. Red= Cluster 1; Dark Green= Cluster 2, Blue=Cluster 3; Orange= Cluster 4; Light Green =Cluster 5. *Purple dot indicates sample from Daikoku Island with smaller IW than the rest of the samples in that cluster.

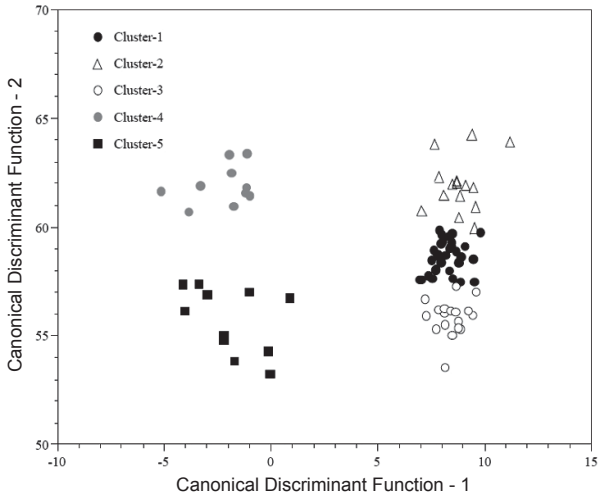

Figure 4. Plot of the first and the second canonical discriminant function.

Canonical Discriminant Analysis

The succeeding Canonical Discriminant Analysis made it possible to select the features that most visibly express the distinctness of the cranial traits in the five clusters. Table 3 shows the contribution of the five characters to the discrimination of the clusters. Canonical Function 1 explained 75.5% of the variation while Canonical Function 2 explained 21.6% of the variation (Wilks' $\lambda = 0.0032$, $P<.0001$). The standard canonical coefficient for the first canonical function is largest in IW (4.812) and WR (-2.291). For the second canonical function, the standard canonical coefficient is largest in PL (1.701), then in WT (1.125) and then LB (1.081). Figure 4 shows that canonical function 1 discriminates between Clusters 4 and 5 from Clusters 1, 2 and 3. The latter have wider interorbital widths and wider rostrums. Canonical function 2 discriminates Cluster 4 which has longer braincase, longer palatines and wider tympanum than those in Cluster 5.

DISCUSSION

Neutral genetic variation

Our results suggest that overall the mainland populations of *M. rufocanus* in Hokkaido are not genetically isolated from each other and that gene flow between and among populations have been taking place. A plausible explanation for the apparent lack of spatial structure in the mainland is that the population is relatively young and that the mtDNA has not diverged long enough to be distinct among regions. The presumably highly variable ancestral types from continental Asia which were brought to Hokkaido via Sakhalin may have been maintained and may mask the true genetic spatial structure of the gray-sided voles in Hokkaido. Our results are consistent with other studies (Wakana et al. 1996; Frisman et al. 2000), although the methods and sample size varied. Wakana et al. (1996) proposed that following glaciation, *M. rufocanus* moved to Hokkaido from the Asian continent and that during the last period when Hokkaido was connected to continental Asia by a land bridge, the species maintained gene flow with populations from mainland Hokkaido and its island and with the continent. Dobson (1994) described that during this period, Hokkaido and Sakhalin were continuously connected from 60,000 years BP until about 8,000 BP. This theory, however, is in contrast with the findings of Iwasa et al. (2000) which have described Hokkaido as genetically differentiated from the northeast Asian populations. In the future, our samples could be compared with those from the northeastern Asian continent to determine degree of variation.

The island populations, on the other hand, such as Daikoku, Rishiri and Kunashiri possess unique haplotypes. Teuri and Yagishiri which are neighboring islands shared a single unique haplotype. This suggests that these two islands were separated from each other relatively recently than the other islands and hence could be genetically considered as one unique gene pool. It is suggested that they have undergone founder events due to a small number of founding individuals and the small island size. This is also the case for Daikoku island which possesses a single unique haplotype. In the case of Rebun Island, it is suggested that either the population has maintained a haplotype shared with mainland populations or that there is actual gene flow between this island and the mainland.

The islands of Daikoku, Teuri and Yagishiri conform with the generalization that small mammals in islands are almost always less genetically variable than mainland populations (Berry 1996). However, this is not true for the island populations of Kunashiri, Rishiri, and Rebun which all had relatively high genetic diversity indices (Table 1). All island populations therefore do not necessarily undergo founder effects and random genetic drift. Our results suggest that for the islands in Hokkaido, island size influences the degree of genetic variability since Diakoku, Teuri, and Yagishiri are the three islands with the smallest land area. A microsatellite analysis of the same island populations conforms with our results and revealed that genetic diversity of these islands in terms of allelic richness positively correlated with island sizes but not with isolation period (Saitoh et al. 2008).

Cranial Traits

The present results indicate that in mainland Hokkaido, clear morphological patterns are only in the eastern populations, Akkeshi and Shibecha. Our results suggest that selection pressure in the mainland may vary very little to effect spatial morphological changes among the mainland populations. But with the relatively small sample sizes from each population and fewer populations represented compared with the genetic variation, our study emphasizes the difficulty of obtaining data for adaptive variation and which are possible reasons for the biases in classifying ESUs based only on genetic data (de Guia and Saitoh 2007). Thus, even though our study was able to detect morphological variation in the mainland, more populations should have been represented.

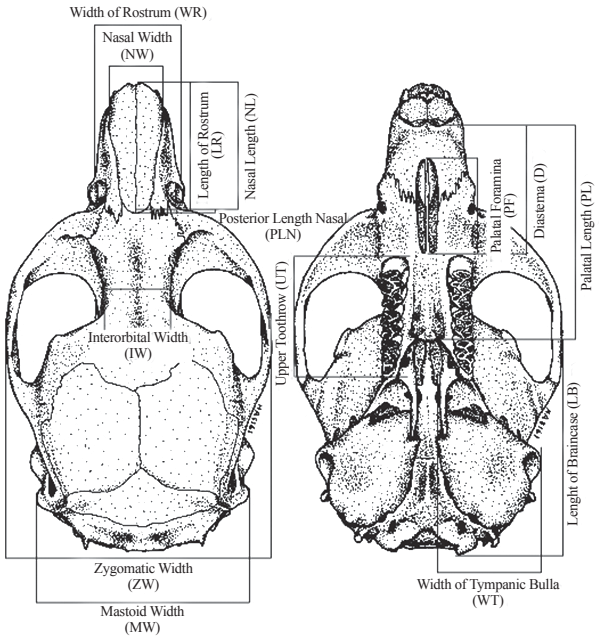

Figure 5. Dorsal and ventral views of the cranial characters measured in this study (after Abe 1973). Original drawings were published by Abe (2000)

Morphological changes of small mammals in islands have been well-documented (Lomolino 1985; Palkovacs 2003; Lomolino 2008) and have excited many evolutionary biologists because of their apparent deviation from mainland forms. This has brought forth the Island Rule (Foster 1964) which states the tendency for insular populations of rodents to be larger than their mainland counterparts but which has been shown not to be true for all. Among the island populations in Hokkaido, only Daikoku Island showed morphological variation among other populations. This adaptive shift in certain cranial characters of the Daikoku vole has been documented before (Abe 1973; de Guia et al. 2007) and has been attributed to the absence of predators and competitors in the island. Island size may also play a role in the morphological divergence of island forms (Pergams and Ashley 2001).

Identification of ESUs

ESUs are ideal conservation units for populations showing intra-specific variation (Moritz 1994). They allow identification of priority populations for conservation of a particular species with several populations. ESUs are preferred over subspecies since most subspecies are merely geographic variants of a species (Ryder 1986; Zink 2004) which do not reflect actual genetic and adaptive variation. It has only been successfully adapted under the U.S. Endangered Species Act in application to the Pacific salmon (Waples 1991). While the ESU concept remains a debated topic among conservation biologists and its actual application inconsistent (see de Guia and Saitoh 2007), identification of ESUs is important because it allows us to recognize important populations even before they become threatened with extinction and enables us to identify populations in the process of diversification. A concern though for the successful application of the ESU is its limitation only to countries or areas with the resources necessary to define this unit (DeWeerdt 2002). Identification of Full and partial ESUs (de Guia and Saitoh 2007) may be used instead in areas with limited time, resources and capabilities.

For the Hokkaido *M. rufocanus* populations, Akkeshi and Shibecha in the northeast region of mainland Hokkaido is a partial ESU exhibiting adaptive variation while the islands of Daikoku, Rishiri and, Kunashiri are partial ESUs possessing neutral genetic variation. These areas possess one component of variation that may lead to further differentiation from the other populations. Daikoku Island is a Full ESU that exhibits a distinct evolutionary potential and adaptive variation not found in any other population in Hokkaido. This population has both components of variation necessary to give rise to new forms or even species in the future.

CONCLUSION

From the genetic data, we were able to determine that most of the island populations were differentiated from the mainland populations. Therefore, the Islands of Kunashiri, Rishiri, Teuri and Yagishiri can be considered as partial ESUs based only on their unique genetic feature. From the morphological data, we were able to determine that the cranial characters were localized in the eastern populations of Akkeshi and Shibecha. On the other hand, these two populations can be considered as a partial ESU due to their distinct morphological feature. The gray-sided vole population in Daikoku Island can be designated as a Full ESU based on its distinct morphological and genetic characters. Based on our data, the *M. rufocanus* population in Daikoku exhibits a distinct evolutionary potential and adaptive variation not found in any other population in Hokkaido. This population should be managed as a separate population.

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