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Phylogenetic Relationships among Seven Taxa of the Japanese Microtine Voles Revealed by Karyological and Biochemical Techniques

IKUYA YOSHIDA[1], YOSHITAKA OBARA[2] and NORIMASA MATSUOKA

Department of Biology, Faculty of Science,
Hirosaki University, Hirosaki 036, Japan

ABSTRACT—Chromosomes and 15 different protein systems of seven taxa of the Japanese microtine voles were examined to establish their phylogenetic relationships, making use of differential staining and protein electrophoretic techniques. While interspecific variations in the size of C-bands were observed only in the Y chromosomes, a highly homologous G-banding pattern as well as the karyotypic similarity were found among the microtine taxa examined, indicating that all of the microtine taxa here dealt with are regarded to be closely related to each other. The biochemical dendrogram for the seven taxa constructed from the Nei's genetic distances between taxa by using the UPGMA clustering method showed that the seven microtine taxa could be classified into three groups, though the differences of the D values were small in general, and this classification was well consistent with the karyological evidence of the interspecific Y-chromosome variations and their geographic distribution pattern. Furthermore, the electrophoretic results indicated that the *andersoni* complex should be classified not as a member of the genus *Aschizomys* or *Eothenomys,* but as that of the genus *Clethrionomys,* and that both *andersoni* and *niigatae* may still be at the subspecies level from their high genetic similarity. Phylogenetic relationships among the Japanese microtine voles are discussed in some detail from karyological and biochemical viewpoints.

INTRODUCTION

Ever since the first description of *Evotomys* (= *Clethrionomys*) *smithii, E. bedfordiae, E. andersoni* and *E. mikado* [1, 2], taxonomy of the Japanese microtine voles has been studied by many workers mainly from a morphological standpoint [3–9]. However, in spite of the extensive examination on the phylogeny of these microtine voles, their phylogenetic relationships are still in a great controversy.

While the voles of Hokkaido and its adjacent islands have been classified into either two species [9–11], three species [12–14] or five species [15, 16], the voles of Honshu, Shikoku and Kyushu into either two species [9, 14, 17] or five species [5, 6, 15, 18] excluding *Microtus montebelli.* On the other hand, four different views on the generic allocation of *andersoni* have been proposed on the basis of the traditional taxonomic criteria such as dental and skull systems: *andersoni* should be included in either the genus *Clethrionomys* [14, 17], the genus *Aschizomys* [5], the subgenus *Aschizomys* of the genus *Clethrionomys* [6], or the genus *Eothenomys* [9]. The specific (and even generic) allocation of *andersoni* has still remained indistinct and unresolved.

In the last decade, chromosome banding and protein electrophoretic techniques have provided much relevant, in some cases critical, information on the phylogenetic relationships in various groups of mammals [19–22]. As to the Japanese microtine species, no systematic investigation on the chromosome banding pattern as well as on the electrophoretic analysis of proteins has been made so far, except for a couple of case reports on the chromosome banding analysis [23–25]. In this study, we have attempted to establish the phy-

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[1] Present address: Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo 060, Japan.

[2] To whom reprint requests should be addressed.

logenetic relationships among the Japanese microtine species by using chromosome banding and protein electrophoretic techniques.

MATERIALS AND METHODS

Animals

A total of 72 specimens from seven microtine taxa were examined in this study. They were all trapped alive at various localities of Honshu, Hokkaido and Rishiri Island. For nomenclature of the voles studied here the taxonomic system of Corbet [14] was adopted. Triplet and doublet of alphabets in parentheses show abbreviations for their scientific names.

Clethrionomys rutilus mikado (Crm): Mt. Petegari (1050 m), Hokkaido (♂0, ♀2); *C. rex* (Cre): Rishiri Shrine and Kanrosen, Rishiri Is. (♂4, ♀4); *C. rufocanus sikotanensis* (Crs): Rishiri Shrine, Rishiri Is. (♂2, ♀0); *C. rufocanus bedfordiae* (Crb): Mt. Petegari (1050 m), Shojiyama, Yunotai and Miyauta, Hokkaido (♂13, ♀11); *C. andersoni andersoni* (Caa): Mt. Iwaki, Zatōishi and Ainai, Aomori Pref., Nagabashiri, Akita Pref., Asahikousen, Yamagata Pref. (♂16, ♀9); *C. andersoni niigatae* (Can): Jumonjitoge (2050 m), Saitama Pref. (♂2, ♀2); *Eothenomys smithi* (Es): Nanzawakousen and Nezame, Nagano Pref., Eiheiji and Heisenji, Fukui Pref. (♂5, ♀2). All of the animals examined are preserved as standard museum specimens (skin with skull) or formalin-fixed specimens in the Department of Biology, Faculty of Science, Hirosaki University, Hirosaki.

Chromosone preparation

Metaphase chromosomes were obtained from the femoral bone marrow cells of the chloroform-anesthetized specimens. The procedure for chromosome preparation was almost the same as that previously described [26, 27]. For G- and C-band staining, the ASG [28] and BSG [29] methods were adopted. Analytical data on the conventionally-stained and G- and C-banded karyotypes are summarized in Table 1.

Electrophoresis

Five different tissues (kidney, liver, intestine, pancreas and skeletal muscle) which had been stored at −80°C were used for the electrophoretic study. The procedures for tissue preparation and polyacrylamide gel electrophoresis were almost the same as those described by Matsuoka [30, 31]. The following 14 different enzymes and general proteins (non-enzymatic proteins) were assayed, using supernatants of tissue homogenates: α-glycerophosphate dehydrogenase (α-GPDH), glucose-6-phosphate dehydrogenase (G6PD), hexose-6-phosphate dehydrogenase (H6PD), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), octanol dehydrogenase (ODH), 6-phosphogluconate dehydrogenase (6-PGD), xanthine dehydrogenase (XDH), superoxide dismutase (SOD), hexokinase (HK), aspartate aminotransferase (AAT), esterase (EST), amylase (AMY), leucine amino peptidase (LAP), and general protein (GP). G6PD, H6PD, LDH, ODH, 6-PGD, HK and AAT were assayed with the extract of the kidney; α-GPDH, MDH, XDH and SOD with that of the liver; AMY and LAP with that of the intestine and pancreas, and EST and GP with that of the skeletal muscle. As three enzymes (EST, LAP and AMY) and GP showed many polymorphic bands, these were assayed using a vertical slab gel apparatus (gel plate measured 1×160×140 mm with 8~20 sample slots). For other enzymes a disc gel apparatus (3×11×75 mm plastic column) was used. The stain mixtures for LDH and 6-PGD were prepared according to Shaw and Prasad [32] and that for α-GPDH according to Ayala *et al.* [33]. Stain recipes for other enzymes were those described previously [31, 34, 35]. GP was stained with Coomassie Brilliant Blue R-250 by the method of Matsuoka *et al.* [36].

RESULTS

Karyological analysis

All of the seven taxa of microtines examined had the diploid number of 56 (Table 1). A conventional karyotype of Caa is presented in Figure 1a as a standard karyotype representing these microtines. The karyotype consisted of 26 pairs of acrocentrics of gradually decreasing size, one pair of the smal-

TABLE 1. Analytic and cytologic data on the seven taxa of the Japanese red-backed voles examined in the present study

Species	No. of cells karyotyped			NFA	2n	Sex chromosome	
	Conv.	ASG	BSG			X	Y
Clethrionomys rutilus mikado	8	5	5	56	56	A	M*
C. rex	23	16	8	56	56	A	A
C. rufocanus sikotanensis	13	14	6	56	56	A	A
C. r. bedfordiae	48	57	24	56	56	A	A
C. andersoni andersoni	65	41	30	56	56	A	M
C. a. niigatae	18	15	14	56	56	A	M
Eothenomys smithi	15	12	11	56	56	A	St

* Shimba *et al.* [41], Tsuchiya and Yoshida [44] and Tsuchiya [37]. A, acrocentric; M, metacentric; St, subtelocentric; Conv., conventional staining; ASG, G-banding; BSG, C-banding; FNA, fundamental number of autosomes; 2n, diploid number of chromosomes.

lest metacentrics and the XY chromosomes. Small but easily detectable short arms were found only in the pair No. 3 which can be regarded as acrocentric judging from its arm ratio, 9.87 ± 0.71, though Tsuchiya [37] thought this chromosome to be subtelocentric. Thus, the NFA of Caa should be 56. Similar pattern of the small short arms was observed in all of the remaining taxa. Their autosomal constitution was almost identical with that of Caa, and hence their NFA was 56 in all the taxa examined (Table 1). The interspecific difference in the length and morphology of chromosomes was found only in the Y chromosomes. The X chromosomes were all acrocentrics of the same length.

A G-banded karyotype of Caa is presented in Figure 1b. Chromosomes are arranged according to the numbering system proposed by Gamperl [38] who has described the G- and C-banding patterns of the grey red-backed vole, *C. rufocanus*, and the bank vole, *C. glareolus*. As clarified in the previous report [25], the G-banding pattern of Caa is almost identical with that of these continental species. So, making this karyotype of G-bands standard, detailed pair matching analysis of G-banding pattern was made among the seven taxa (Fig. 2). As clearly demonstrated in the composite karyotype, almost perfect G-band homology was obtained in all the chromosome complements including the X chromosomes among the seven taxa, reflecting their phylogenetic kinship. The C-bands of Caa were all centromeric except for the Y chromosome which was entirely heterochromatic along its arms (Fig. 1b, insertion). There was no significant difference in the G- and C-banding patterns among the seven taxa, except for their Y chromosomes. The Y chromosomes showed distinct interspecific variations in their G- and C-banding patterns, but no detectable variation at the intraspecific or subspecies level (Fig. 3). On the basis of the size and morphology of the Y chromosomes the microtine taxa under present study could be classified into three groups as follows: *andersoni*-type carrying a metacentric Y (Caa and Can), *rufocanus*-type carrying an acrocentric one (Crb, Crs and Cre) and *smithi*-type carrying a subtelocentric one (Es). The rates of the Ys to the Xs (Y/X $\pm$ SD) were 0.28 ± 0.02 in Cre, 0.28 ± 0.03 in Crs, 0.30 ± 0.03 in Crb, 0.28 ± 0.03 in Caa, 0.27 ± 0.05 in Can and 0.41 ± 0.05 in Es. Thus, the Y chromosomes correspond to 27~30% of the X chromosomes in length except for Es, in which the Y was about 41% of its X chromosome length.

Electrophoretic analysis

From the electrophoretic patterns of 14 different enzymes and general protein observed in and among the seven taxa of microtines, we assumed 26 genetic loci (Table 2). The major features of

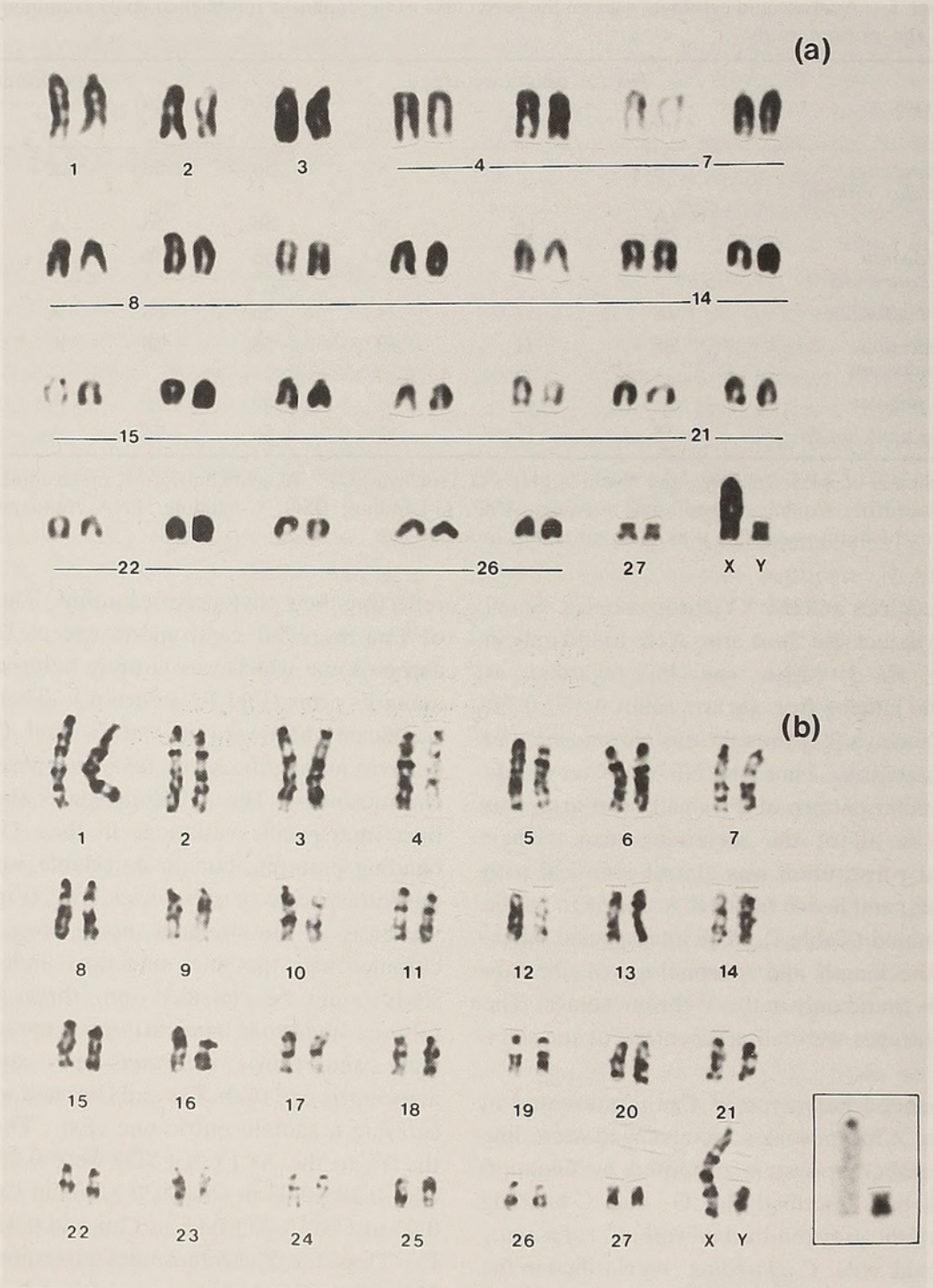

FIG. 1. Conventional (a) and G-banded (b) karyotypes of a male Japanese red-backed vole, *Clethrionomys a. andersoni*.

variation in these proteins are summarized as follows.

Eight enzymes: α-GPDH, G6PD, H6PD, ODH, 6-PGD, XDH, HK and AAT exhibited a single band of activity and they were all monomorphic. The bands of individual enzymes showed the same electrophoretic mobility in the seven taxa.

LDH showed five-banded pattern in all taxa. As this enzyme is known to be a tetrameric protein, the five bands could be interpreted as five different

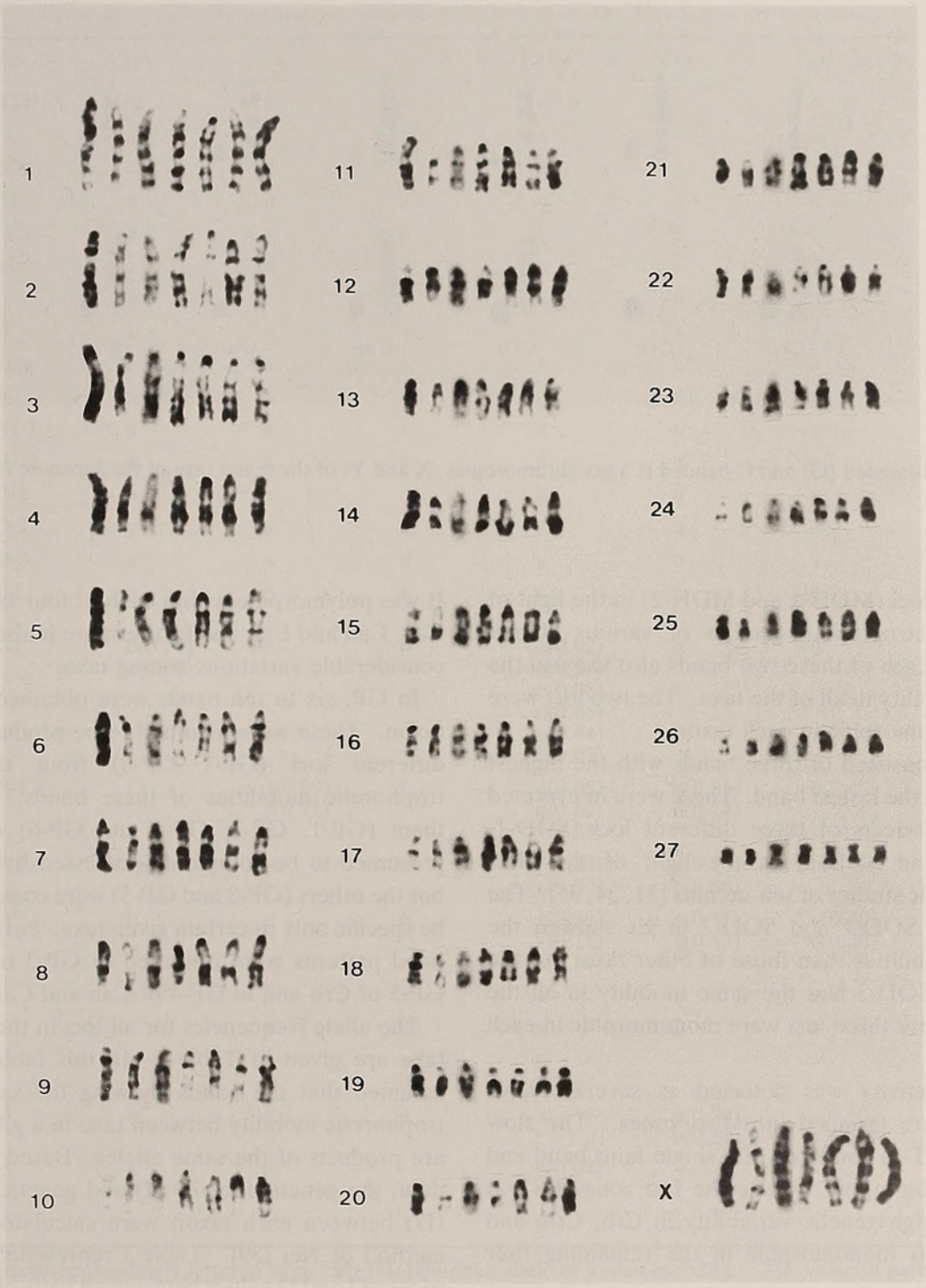

FIG. 2. Pair-matching of G-banded haploid chromosomes from each of seven taxa of the Japanese red-backed voles. From left to right; *Clethrionomys rutilus mikado, C. rex, C. rufocanus sikotanensis, C. r. bedfordiae, C. andersoni andersoni, C. a. niigatae* and *Eothenomys smithi*.

tetramers consisting of two polypeptides produced by two codominant alleles at two different loci (LDH-1 and LDH-2). Each of these bands showed the same mobility in all taxa and the two loci were monomorphic in each taxon.

MDH showed two active bands, of which the faster band was markedly high in activity. These two bands were interpreted as the products of two

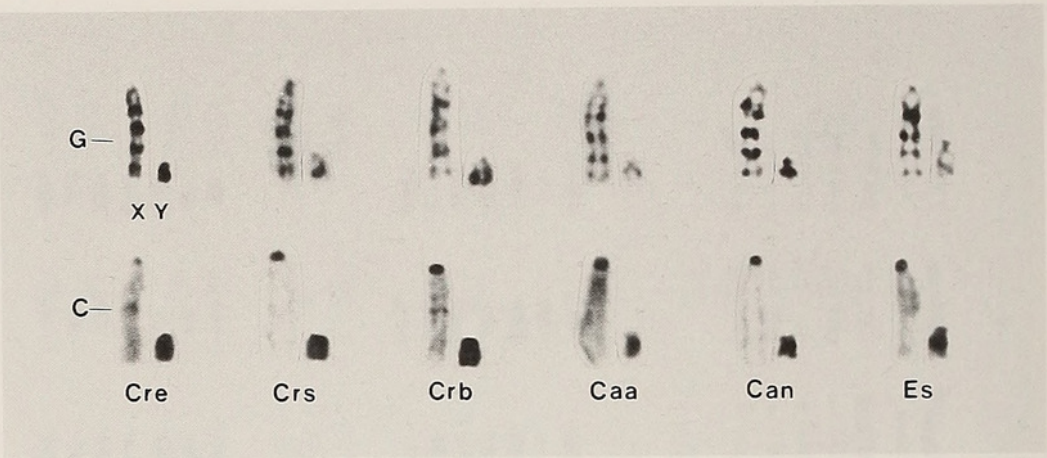

FIG. 3. G-banded (G) and C-banded (C) sex chromosomes, X and Y, of the seven taxa of the Japanese red-backed voles.

different loci (MDH-1 and MDH-2) in the light of other electrophoretic studies of various animal groups. Each of these two bands also showed the same mobility in all of the taxa. The two loci were also monomorphic in each taxon.

SOD consisted of three bands with the highest activity in the fastest band. These were interpreted as the products of three different loci (SOD-1, SOD-2 and SOD-3) in the light of the electrophoretic studies of sea-urchins [31, 34, 35]. The bands of SOD-1 and SOD-2 in Es showed the faster mobilities than those of other taxa, but the band of SOD-3 had the same mobility in all the taxa. These three loci were monomorphic in each taxon.

EST activity was detected as several bands which were grouped into two zones. The slow zone (EST-1) consisted of a single faint band and was monomorphic. While the fast zone (EST-2) showed high genetic variability in Crb, Crm and Es, it was monomorphic in the remaining four taxa.

LAP showed only a single active band in Crm, but in the remaining six taxa the enzyme consistently appeared as two active bands. These band patterns were interpreted to be controlled by two different loci (LAP-1 and LAP-2). LAP-1 was polymorphic in four taxa (Crb, Cre, Caa and Es) and LAP-2 in three taxa (Crb, Caa and Es).

AMY activity was detected as one to two bands. It was polymorphic within each of four taxa (Crb, Caa, Can and Es), and furthermore it also showed considerable variations among taxa.

In GP, six to ten bands were obtained in each taxon. These were assumed to be products of six different loci (GP-1～GP-6) from the electrophoretic mobilities of these bands. Four of them (GP-1, GP-2, GP-4 and GP-6) could be presumed to be commonly possessed by all taxa, but the others (GP-3 and GP-5) were considered to be specific only to certain given taxa. Polymorphic band patterns were observed in GP-3 of Crs, in GP-5 of Crb and in GP-4 of Caa and Can.

The allele frequencies for all loci in these seven taxa are given in Table 2. In this table, it was assumed that the bands showing the same electrophoretic mobility between taxa in a given locus are products of the same alleles. Based on these data, the genetic identity (I) and genetic distance (D) between each taxon were calculated by the method of Nei [39]. Table 3 represents the matrices of I and D values between all pairs of the taxa examined. As evident from this table, the I value between Caa and Can, which allopatrically inhabit the hilly countries of northern Honshu (Tohoku district) and the high mountaines (more than 2,000 m above the sea level) of central Honshu (Kanto and Chubu districts), respectively, is the highest ($I = 0.951$). The I values between Crb,Crs, Cre and Crm, all of which are distributed in Hokkaido and

TABLE 2. Allele frequencies at various genetic loci in the seven taxa of microtines

Locus	Crm	Cre	Crs	Crb	Caa	Can	Es
α-GPDH	a	a	a	a	a	a	a
G6PD	a	a	a	a	a	a	a
H6PD	a	a	a	a	a	a	a
ODH	a	a	a	a	a	a	a
6-PGD	a	a	a	a	a	a	a
XDH	a	a	a	a	a	a	a
HK	a	a	a	a	a	a	a
AAT	a	a	a	a	a	a	a
LDH-1	a	a	a	a	a	a	a
LDH-2	a	a	a	a	a	a	a
MDH-1	a	a	a	a	a	a	a
MDH-2	a	a	a	a	a	a	a
SOD-1	a	a	a	a	a	a	b
SOD-2	a	a	a	a	a	a	b
SOD-3	a	a	a	a	a	a	a
EST-1	a	b	b	c	c	c	c(0.71) d(0.29)
EST-2	b(0.50) e(0.50)	e	b	a(0.02) b(0.38) c(0.08) d(0.06) e(0.44) f(0.02)	e	e	c(0.29) e(0.71)
LAP-1	f	d(0.29) e(0.71)	f	a(0.05) d(0.62) f(0.33)	b(0.86) d(0.14)	b	c(0.14) d(0.43) f(0.43)
LAP-2	—	b	b	b(0.43) c(0.57)	a(0.14) b(0.86)	b	d(0.57) e(0.43)
AMY	b	e	h	f(0.39) g(0.11) h(0.50)	a(0.47) c(0.53)	a(0.50) c(0.50)	b(0.21) d(0.29) e(0.50)
GP-1	a	a	a	a	a	a	a
GP-2	a	a	a	a	a	a	a
GP-3	b	b	a(0.50) b(0.50)	b	—	—	—
GP-4	a	a	a	a	a(0.79) b(0.21)	a(0.50) b(0.50)	a
GP-5	a	d	—	b(0.04) c(0.96)	—	—	d
GP-6	a	a	a	a	a	b	a

Alleles are correspondingly lettered from "a", this being the allele of lowest mobility. The value in parenthesis represents the frequency of each allele in taxon. The scientific name of each taxon is shown as abbreviation described in Materials and Methods.

its neighbouring islands, are relatively high with the range of 0.803～0.895. On the other hand, the I values between Es and the six taxa of the genus *Clethrionomys* are in the range of 0.713～0.792 and lower than the values mentioned above. Figure 4 shows the biochemical dendrogram for the seven taxa constructed from the genetic distance matrix of Table 3 using the unweighted pair-group arithmetic average (UPGMA) clustering method of Sneath and Sokal [40]. The biochemical dendrogram indicates the following: (1) The seven taxa studied here are divided into three clusters.

TABLE 3. Genetic identities (above diagonal) and genetic distances (below diagonal) between the seven taxa of microtines

Taxa	Crm	Cre	Crs	Crb	Caa	Can	Es
Crm	—	0.803	0.824	0.844	0.769	0.713	0.736
Cre	0.219	—	0.842	0.855	0.823	0.771	0.789
Crs	0.194	0.172	—	0.895	0.816	0.763	0.713
Crb	0.170	0.157	0.111	—	0.891	0.829	0.783
Caa	0.263	0.195	0.203	0.115	—	0.951	0.792
Can	0.338	0.260	0.270	0.188	0.050	—	0.729
ES	0.307	0.237	0.338	0.245	0.233	0.316	—

The scientific name of each taxon is shown as abbreviation described in Materials and Methods.

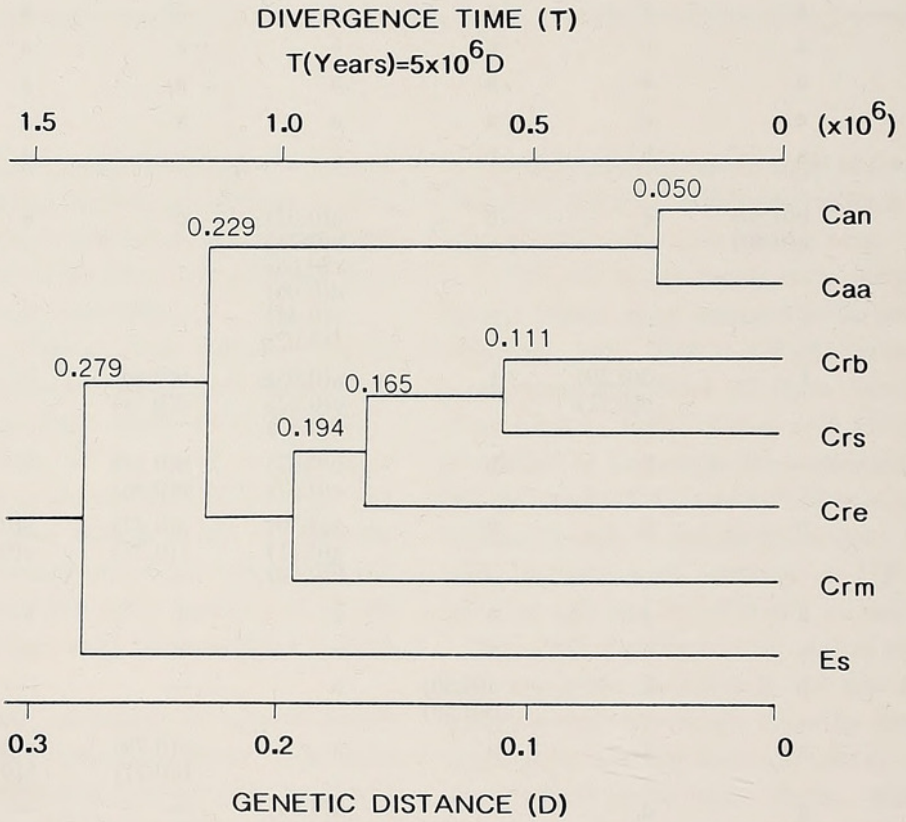

FIG. 4. A biochemical dendrogram showing the phylogenetic relationships among the seven taxa of the Japanese microtine voles.

The first cluster consisted of two *andersoni* subspecies (Caa and Can), the second cluster four taxa (Crb, Crs, Cre and Crm), and the third cluster only Es. (2) Of the seven taxa, Caa and Can of the first cluster is the most closely related to each other (D =0.050). (3) In the second cluster, Crb and Crs are the most closely related to each other (D= 0.111), and Cre is more closely related to Crb and Crs than Crm. However, the differences of the D values are small in all pair-groups of these four taxa. (4) Es is slightly differentiated from the first and second clusters, and the mean D value between Es and two large clusters consisting of six taxa of the genus *Clethrionomys* is 0.279.

The proportion of polymorphic loci (P) in each taxon was as follows: 4.0% for Crm and Crs, 3.8%

for Cre, 19.2% for Crb, 16.7% for Caa, 8.3% for Can and 20.0% for Es. The mean value of P was 10.9%. The expected mean heterozygosity per locus (H) in each taxon was as follows: 2.0% for Crm and Crs, 1.6% for Cre, 8.9% for Crb, 5.5% for Caa, 4.2% for Can and 10.2% for Es. The mean value of H was 4.9%.

DISCUSSION

The karyotypes of the seven taxa of microtines here studied were essentially identical, except for the Y chromosome of Can, to those of the previous observation [37, 41–44]. The Y chromosomes of the *andersoni* complex were metacentric in both Caa abd Can, as far as the present specimens are concerned. Hsu and Benirschke [42] regarded the Y chromosome of Can as the smallest metacentric element, but Tsuchiya [37] as an acrocentric one. Our finding is, therefore, consistent with the former view. These fects may be suggestive of the polymorphic nature of the Y chromosome of this taxon, just indicating an additional case of the parallelism of the Y chromosome variabillity reported in three *Clethrionomys* species; *C. glareolus, C. rutilus* and *C. rufocanus* [45]. However, it is still uncertain whether the Y chromosome variants have been established as the interpopulation polymorphism or not. Further examination of the specimens from various localities is necessary for elucidation of this subject. The Y chromosome of Cre showed a similar but somewhat indistinct variation: it was regarded as a subtelocentric element by Tsuchiya [37], and as an acrocentric one in the present specimens. Further, he is of opinion that the X chromosomes of Cre, Caa and Es are subtelocentric. But they were all acrocentric in the present study (Fig. 3). These minor discrepancies may be not essential but attributable to the technical matters or how to set up the criteria for chromosome morphology.

As shown by the composite karyotype of Figure 2, the seven taxa studied are almost identical to each other in their G- and C-banding pattern as well as in their conventional karyotypes, though their Y chromosomes showed three types of interspecific variations; *andersoni*-type, *rufocanus*-type and *smithi*-type (Table 1 and Fig. 3). These findings plainly signify that all of these microtines are the descendants from a common ancestral form, and with the only exception of Y chromosome, no karyotypic differentiation has proceeded in the course of phenotypic differentiation. As clearly demonstrated from the pair-matching analysis of G-bands between Caa and *C. rufocanus* from near Gallivare, Sweden [38], the red-backed voles of Japan could be closely related to the grey red-backed vole, *C. rufocanus* which is widely but sporadically distributed from Scandinavia to Siberia, Sakhalin and Hokkaido. In view of these facts, it may be most likely that the seven taxa of microtines studied have derived from a *rufocanus*-like ancestor of the Eurasian continent.

The biochemical dendrogram (Fig. 4) demonstrated that the seven taxa of microtine voles are genetically divided into three groups. This relationship is well consistent with their distribution in Japan: Caa and Can are distributed in northern and central Honshu, Crs, Crb, Cre and Crm are endemic to Hokkaido and its neighbouring islands, and Es inhabits Kyushu, Shikoku and southwestern Honshu. Further, the biochemical dendrogram consisting of three clusters is also consistent with the morphological variations of their Y chromosomes: metacentric in the first cluster, acrocentric in the second one and subtelocentric in the third one. The present electrophoretic results seem to be compatible, in a broad sense, with the taxonomic system of Imaizumi [15] based on the phenotypic characters. He classified, in the light of the rooting pattern of molars, these microtine taxa into three different genera: the genus *Aschizomys* including Caa and Can, the genus *Clethrionomys* including Crs, Crb, Cre and Crm, and the genus *Eothenomys* including Es. However, this classification is out of harmony, in a strict sense, with our taxonomic system in some respects. He regarded *andersoni* and *niigatae* as two distinct species of the genus *Aschizomys*. As already mentioned, the D value between the two taxa is markedly low ($D=0.050$) and comparable to the D values observed between conspecific geographic populations in many other animals [46–48]. Judging from their highly close similarity at molecular and chromosomal level, *andersoni* and *niigatae* should be considered as two subspecies of the same

species. Further, the mean D value (D=0.229) between the *andersoni* complex (Caa and Can) and the four taxa of red-backed voles (Crs, Crb, Cre and Crm) is much lower than the D values reported between different genera of many other animals and rather at the subspecies or closely related species level [46–48]. Since they are also very similar to each other in phenotypic and karyological aspects, these microtine taxa should be considered as the congeneric members of *Clethrionomys,* as proposed by Corbet [14].

In the cluster consisting of the *rufocanus* complex (Crb and Crs), Cre and Crm, Crb has the closest affinity with Crs and the D value between them is the lowest (D=0.111), which is comparable to the D values observed between subspecies of many other animals [46–48]. Taking their distribution areas and high genetic similarity into consideration, it seems very reasonable to regard the insular (Rishiri Island) taxon Crs and the mainland (Hokkaido) taxon Crb as conspecific, though these two have been regarded as distinct species by Imaizumi [15]. It is clear from the electrophoretic results that Crm had already differentiated prior to the evolutionary divergence of the *rufocanus* complex and Cre. These biochemical findings may substantiate the phenotypic evidence that Crm significantly differs in the molar pattern as well as in the body size from *rufocanus* complex and Cre. However, the high I values found between these four taxa strongly suggest that *rufocanus, rex* and *rutilus* are distinct but closely related species each other.

With respect to the taxonomic allocation of the *andersoni* complex (Caa and Can), Aimi [9] proposed that *andersoni* (including *niigatae*) should be referred to the genus *Eothenomys* on the basis of the craniometry and the occlusal pattern of molars. If *andersoni* and *smithi* are congeneric with each other, then *rufocanus, rex* and *rutilus* also must necessarily be included in the genus *Eothenomys,* judging from the biochemical dendrogram shown in Figure 4. But,the view that the *rufocanus*-group must be included in the genus *Eothenomys* may be hard to accept even from usual taxonomic criteria because of the clear difference in rooting of the cheek-teeth. On the whole, our classification system as to the generic allocation of the *andersoni* complex is consistent not with that of Aimi [9], but with that of Corbet [14] who classified the *andersoni* complex as a member of the genus *Clethrionomys.* According to Corbet [14], the *Eothenomys* species closely resemble *Clethrionomys,* only being distinguished mainly by the absence of rooting of the cheek-teeth even in old age. Further, the *Eothenomys* species are distributed mainly in China, and quite rarely in the Korean Penninsula and Japan, and the *Clethrionomys* species are widely distributed to the northern regions of the Eurasian Continent. Thus, it would be most probable that the genus *Eothenomys* might have diverged from the red-backed voles, *Clethrionomys,* by modifying the rooting pattern of molars somewhere in the Eurasian Continent and the ancestral population of Es might have migrated into Japan through a southern route, or the Korean Penninsula. The genetic differentiation of the genus *Eothenomys* from the lineage of the genus *Clethrionomys* may well be reflected in our biochemical dendrogram.

In general, biochemical dendrogram shows not only the phylogenetic relationships, but also the sequence of evolutionary divergence. According to Nei [49], genetic distance (D) corresponds well with the divergence time (T) from the common ancestor, and T of two taxa can be estimated by $T = 5 \times 10^6$ D (year). Applying this equation to our biochemical dendrogram, each divergence time may be calculated as follows: 1.4 million years (MY) for *Eothenomys* and *Clethrionomys,* 1.1~1.2 MY for the two large groups of *Clethrionomys,* 0.5~1.0 MY for the four taxa of *Clethrionomys* endemic to Hokkaido, and 0.25 MY for the two *andersoni* subspecies.

For further clarification of the phylogenetic relationships among the Japanese microtine voles, it would be needed to synthetically examine the chromosomes, genetic distances and the phenotypic characters of all the taxa including *C. montanus, C. imaizumii* and *E. kageus* which could not be dealt with in this study.

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REFERENCES

1 Thomas, O. (1905) On some new Japanese mammals presented to the British Museum by Mr. R. Gordon-Smith. Ann. Mag. Nat. Hist., Ser. 7., **15**: 487–495.

2 Thomas, O. (1905) Abstract. Proc. Zool. Soc. London, **23**: 18–19.

3 Hinton, M. A. C. (1926) Monograph of the voles and lemmings (Microtinae), living and extinct. Vol. 1, pp. 246–248, 257–262, 437–438. British Museum (N. H.), London.

4 Kuroda, N. (1931) A new locality and scientific name of red-backed vole, *Eovotomys niigatae*. Zool. Mag., **43**: 661–666 (in Japanese).

5 Imaizumi, Y. (1960) Coloured illustrations of the mammals of Japan. pp. 128–139. Hoikusha, Tokyo. (in Japanese).

6 Jameson, E. W. Jr. (1961) Relationships of the red-backed voles of Japan. Pacific Sci., **15**: 594–604.

7 Miyao, T., Morozumi, T., Hanamura, H., Akahane, H. and Sakai, A. (1964) Small mammals on Mt. Yatsugatake in Honshu. III. Smith's red-backed vole (*Eothenomys smithi*) in the subalpine forest zone on Mt. Yatsugatake. Zool. Mag., **73**: 189–195 (in Japanese with English summary).

8 Tanaka, R. (1971) A research into variation in molar and external features among a population of the Smith's red-backed vole for elucidation of its systematic rank. Japn. J. Zool., **16**: 163–176.

9 Aimi, M. (1980) A revised classification of the Japanese red-backed voles. Mem. Fac. Sci. Kyoto Univ., Ser. Biol., **8**: 35–84.

10 Ota, K. (1956) The Muridae of the islands adjacent to Hokkaido. Mem. Fac. Agr. Hokkaido Univ., **2**: 123–136 (in Japanese with English summary).

11 Abe, H. (1968) Growth and development in two forms of *Clethrionomys*. I. External characters, body weight, sexual maturity and behaviour. Bull. Hokkaido Forest Exp. Station, No. **6**: 69–89 (in Japanese with English summary).

12 Abe, H. (1973) Growth and development in two forms of *Clethrionomys*. II. Tooth characters with special reference to phylogenetic relationships. J. Fac. Agr. Hokkaido Univ., **57**: 229–254.

13 Abe, H. (1973) Growth and development in two forms of *Clethrionomys*. III. Cranial characters, with special reference to phylogenetic relationships. J. Fac. Agr. Hokkaido Univ., **57**: 255–274.

14 Corbet, G. B. (1978) The mammals of the Palaearctic region: A taxonomic review. Cornell Univ. Press, London·Ithaca, pp. 97–100.

15 Imaizumi, Y. (1979) Classification of the Japanese myomorphs. Nat. Anim., **9**: 2–6 (in Japanese).

16 Imaizumi, Y. (1972) Land mammals of the Hidaka Mountains, Hokkaido, Japan, with special reference to the origin of an endemic species of the genus *Clethrionomys*. Mem. Natl. Sci. Mus. (Tokyo), **5**: 131–149 (in Japanese with English summary).

17 Tokuda, M. (1941) A revised monograph of the Japanese and Manchou-Korean Muridae. Biogeographica, Transact. Biogeogr. Soc. Japan, **4**: 1–156.

18 Imaizumi, Y. (1971) A new vole of the *Clethrionomys rufocanus* group from Rishiri Island, Japan. J. Mammal. Soc. Japan, **5**: 99–103.

19 Greenbaum, I. F. (1981) Genetic interactions between hybridizing cytotypes of the tent-making bat (*Uroderma bilobatum*). Evolution, **35**: 306–321.

20 Bohlin, R. G. and Zimmerman, E. G. (1982) Genic differentiation of two chromosomal races of the *Geomys bursarius* complex. J. Mammal., **63**: 218–228.

21 Hafner, J. C., Hafner, D. J., Patton, J. L. and Smith, M. F. (1983) Contact zones and the genetics of differentiation in the pocket gopher *Thomomys bottae* (Rodentia: Geomyidae). Syst. Zool., **32**: 1–20.

22 Gothran, E. G. and Smith, M. H. (1983) Chromosomal and genic divergence in mammals. Syst. Zool., **32**: 360–368.

23 Yamakage, K.. Nakayashiki, N., Hasegawa, J. and Obara, Y. (1985) G-, C- and N-banding patterns on the chromosomes of the Japanese grass vole, *Microtus montebelli montebelli*, with special attention to the karyotypic comparison with the root vole, *M. oeconomus*. J. Mammal. Soc. Japan, **10**: 209–220 (in Japanese with English summary).

24 Obara, Y. and Yoshida, I. (1985) A case of X-autosome translocation in the Japanese red-backed vole, *Clethrionomys andersoni andersoni*. Chrom. Inf. Serv., **39**: 3–5.

25 Obara, Y. (1986) G-band homology between the Japanese red-backed vole, *Clethrionomys a. andersoni* and the grey red-backed vole, *C. rufocanus*. Chrom. Inf. Serv., **40**: 7–9.

26 Obara, Y. (1982) Comparative analysis of

karyotypes in the Japanese mustelids, *Mustela nivalis namiyei* and *M. erminea nippon*. J. Mammal. Soc. Japan, **9**: 59–69.

27 Saitoh, M. and Obara, Y. (1986) Chromosome banding patterns in five intraspecific taxa of the large Japanese field mouse, *Apodemus speciosus*. Zool. Sci., **3**: 785–792.

28 Sumner, A. T., Evans, H. J. and Buckland, R. A. (1971) New technique for distinguishing between human chromosomes. Nature New Biol., **232**: 31–32.

29 Sumner, A. T. (1972) A simple technique for demonstrating centromeric heterochromatin. Exptl. Cell Res., **75**: 304–306.

30 Matsuoka, N. (1981) Phylogenetic relationships among five species of starfish of the genus *Asterina*: An electrophoretic study. Comp. Biochem. Physiol., **70B**: 739–743.

31 Matsuoka, N. (1985) Biochemical Phylogeny of the sea-urchins of the family Toxopneustidae. Comp. Biochem. Physiol., **80B**: 767–771.

32 Shaw, C. R. and Prasad, R. (1970) Starch gel electrophoresis of enzymes: A compilation of recipes. Biochem. Genet., **4**: 297–320.

33 Ayala, F. J., Powell, J. R., Tracey, M. L., Mourão, C. A. and Pérez-Salas, S. (1972) Enzyme variability in the *Drosophila willistoni* group. IV. Genetic variation in natural populations of *Drosophila willistoni*. Genetics, **70**: 113–139.

34 Matsuoka, N. (1987) Biochemical study on the taxonomic situation of the sea-urchin, *Pseudocentrotus depressus*. Zool. Sci., **4**: 339–347.

35 Matsuoka, N. and Suzuki, H. (1987) Electrophoretic study on the taxonomic relationship of the two morphologically very similar sea-urchins, *Echinostrephus aciculatus* and *E. molaris*. Comp. Biochem. Physiol., **88B**: 637–641.

36 Matsuoka, N., Ohnishi, K., Takahashi, T., Oka, K. and Hori, S. H. (1983) Evidence for the homology of hexose 6-phosphate dehydrogenase and glucose 6-phosphate dehydrogenase: Comparison of the amino acid compositions. Comp. Biochem. Physiol., **76B**: 811–816.

37 Tsuchiya, K. (1981) On the chromosome variations in Japanese cricetid and murid rodents. Honyurui Kagaku, **42**: 51–58 (in Japanese).

38 Gamperl, R. (1982) Chromosomal evolution in the genus *Clethrionomys*. Genetica, **57**: 193–197.

39 Nei, M. (1972) Genetic distance between populations. Amer. Natur., **106**: 283–292.

40 Sneath, P. H. A. and Sokal, P. R. (1973) Numerical Taxonomy. Freeman, San Francisco.

41 Shimba, H., Ito, M., Obara, Y., Kohno, S. and Kobayashi, T. (1969) A preliminary survey of the chromosomes in field mice, *Apodemus* and *Clethrionomys*. J. Fac. Sci. Hokkaido Univ., Ser. 6, Zool., **17**: 257–262.

42 Hsu, T. C. and Benirschke, K. (1974) An Atlas of Mammalian Chromosomes. Vol. 8., Folio 370, 371. Springer-Verlag, New York/Heidelberg/Berlin.

43 Sonta, S., Hayata, I. and Kobayashi, T. (1971) A chromosome survey of the red-backed mouse, *Clethrionomys rufocanus bedfordiae*, in Hokkaido, with a note of a karyotypically abnormal specimen. Proc. Japan Acad., **47**: 679–682.

44 Tsuchiya, K. and Yoshida, T. H. (1971) Chromosome survey of small mammals in Japan. Ann. Rep. Natl. Inst. Genet., **21**: 54–55.

45 Vorontsov, N. N., Lyapunova, E. A., Borissov, Y. M. and Dovgal. V. E. (1980) Variability of sex chromosomes in mammals. Genetica, **52/53**: 361–372.

46 Avise, J. C. (1976) Genetic differentiation during speciation. In "Molecular Evolution" Ed. by F. J. Ayala, Sinauer Associates, Massachusetts, pp. 106–122.

47 Ferguson, A. (1980) Biochemical Systematics and Evolution. Blackie, Glasgow.

48 Ayala, F. J. (1982) Population and Evolutionary Genetics: A Primer. The Benjamin/Cummings Publishing Company, California.

49 Nei, M. (1975) Molecular Population Genetics and Evolution. North-Holland, Amsterdam.