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Evolutionary Relationships Between Laboratory Mice and Subspecies of *Mus musculus* Based on the Restriction Fragment Length Variants of the Chymotrypsin Gene at the *Prt-2* Locus

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Restriction endonuclease fragment length variants in mice were compared by Southern blot analysis using the cDNA probe pcXP33 for the chymotrypsin gene. The variants were detected in the restriction patterns generated by fragments from digestions with Bgl*II*, Eco*RI*, Hind*III*, Pst*I*, Sac*I, *and* Xba*I*. The set of protein phenotypes and the restriction patterns of chymotrypsin gene were examined in many laboratory strains and wild subspecies. Most laboratory strains (26 strains) are grouped into a set defined as Set 1, but only a few laboratory strains (AU/SsJ and five BALB/c sublines) are classified as belonging to Set 2. Of wild subspecies, only BRV-MPL (*M. brevirostris*) can be placed in Set 1, while DOM-BLG and SK/Cam (*M. domesticus*) belong in Set 2. The assignment of an appropriate set defined by the characteristics of the chymotrypsin gene has also been investigated in* M. musculus, *two Chinese subspecies,* M. yamashinai, M. molossinus, *and* M.

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castaneus, and the evolutionary relationship between laboratory mice and various subspecies of Mus *has been examined.*

KEY WORDS: mice; subspecies; chymotrypsin; restriction fragment length variants.

INTRODUCTION

Tryspin and chymotrypsin are digestive proteolytic enzymes of the pancreas. In the mouse, three genetic loci (*Prt-1, Prt-3,* and *Prt-6*) have been reported for trypsin and one locus (*Prt-2*) has been reported for chymotrypsin (Watanabe and Tomita, 1974; Watanabe *et al.*, 1976a, 1987). On the basis of analyses of the constitution of the genes at the *Prt-1* and *Prt-3* loci in various subspecies of *Mus musculus,* it was suggested that the ancestors of laboratory strains were probably wild mice from western European, related to *M. m. brevirostris* rather than to *M. m. domesticus* (Watanabe *et al.*, 1987). However, *M. brevirostris* may be only a regional group of *domesticus,* since Ferris *et al.* (1983) and Sage *et al.* (1986) have demonstrated that neither data from proteins nor data from mtDNA provide any genetic support for distinction between *M. brevirostris* and *M. domesticus.* The *Prt-2* locus is located on chromosome 8 (Watanabe *et al.*, 1976b), and three alleles, $Prt\text{-}2^a$, $Prt\text{-}2^b$, and $Prt\text{-}2^c$, have been described at the protein level (Watanabe *et al.*, 1987). $Prt\text{-}2^a$ has been found in all laboratory strains and also in most subspecies of *Mus musculus.* In contrast, $Prt\text{-}2^b$ has been identified only in Japanese wild mice, *M. m. molossinus,* and in Korean wild mice, *M. m.*

Table I. List of Laboratory Strains and the Countries Where They Are Established

Strain	Source
Laboratory inbred	
A/J, AKR/FuA, AU/SsJ, BALB/cAn, BALB/cByA, BALB/cCdA, BALB/cHeA, BALB/cRglA, CBA/ByA, CE/JA, C3H/He, C57BL/6J, C57BL/6ByA, C58/JA, DBA/2, and 129/SvJ	United States
DD/HeAf, DS, and TSI/A	West Germany
CFO, MAS/A, and SJL/JA	Switzerland
020/A	The Netherlands
RIII/SeA	France
WLL/BrAf	Norway
NZB	New Zealand
KK, N, NC, and SS	Japan
Laboratory closed colony	
ICR	United States
DDY	West Germany

Table II. List and Sources of the Subspecies of Wild Mice Examined[a]

Subspecies and stock designation	Site of original collection
Mus m. domesticus	
DOM-PGN	Pigeon, Canada
DOM-BLG	Pomorie, Bulgaria
SK/Cam	Skokholm, United Kingdom
Mus m. brevirostris	
BRV-MPL	Montpellier, France
Mus m. musculus	
MUS-BLG	Vrania, Bulgaria
MUS-NJL	Northern Jutland, Denmark
Chinese subspecies (unidentified)	
Sub-BJN	Beijing, China
Sub-SHH	Shanghai, China
Mus m. yamashinai	
Sub-ACI	Suwon, Korea
Sub-LAS	Suwon, Korea
Sub-KJR	Kojuri, Korea
Mus m. molossinus	
MOL-NEM	Nemuro, Japan
MOL-ANJ	Anjo, Japan
MOL-MOM	Nagoya, Japan
MOL-WAK	Wakayama, Japan
MOL-TSU	Tsushima, Japan
Mus m. castaneus	
CAS/J	Thailand
CAS-QZN	Quezon City, Philippines
CAS-TCH	Taichung, Taiwan

[a]Taxonomic assignment is according to the reports by Yonekawa *et al.* (1982) and Suzuki *et al.* (1986).

yamashinai, while *Prt-2*^{c} was detected in another species, *Mus caroli,* from Thailand.

In the present paper, we describe our analysis of the restriction fragment length variants in mice using the recombinant plasmid DNA (pcXP33) that carries rat chymotrypsin cDNA inserts as a probe (Bell *et al.,* 1984) and we discuss the evolutionary relationships between strains of laboratory mice and various subspecies of *Mus musculus.*

MATERIALS AND METHODS

Animals and Chemicals. The mice used are listed in Tables I and II. Restriction endonucleases, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, and *Xba*I, were

purchased from Toyobo Biochemicals. Proteinase K was obtained from Boerhinger–Mannheim Co., and nylon membrane was purchased from Pall Ultrafine Filtration Co. Two kinds of agarose, GP-36 and EPI-56, which were used for the electrophoretic separation of proteins and DNA respectively, were purchased from Nakarai Chemical Ltd. [α-^{32}P]dCTP was obtained from ICN Radiochemicals, and the nick-translation kit was from Amersham. Other reagents were commercial preparations of the highest purity available.

Gel Electrophoresis for Examination of Bands with Proteolytic Activity. Agarose gel electrophoresis of extracts from the pancreas and duodenum and the detection of proteolytic activity in bands have been described previously (Watanabe and Tomita, 1974; Watanabe *et al.*, 1976a).

Preparation of DNA. DNA was isolated from liver, kidney, or lung. Tissue was homogenized in 10 vol of buffer that contained 20 mM Tris–HCl at *p*H 8.0, 10 mM KCl, and 0.25 M sucrose. The homogenate was centrifuged at 300*g* for 15 min at 4°C, and the pellet was dissolved in 4 vol of 50 mM Tris–HCl (*p*H 8.0), 10 mM EDTA, and 1.25% sodium dodecyl sulfate (SDS). The solution was then incubated with proteinase K at a final concentration of 0.2 mg/ml at 45°C overnight. Nucleic acid was twice extracted from the mixture by the addition of an equal volume of phenol–chloroform–isoamyl alcohol (50:50:1) and was precipitated with 2 vol of ethanol at −20°C overnight. The pellet was dissolved in a buffer that contained 10 mM Tris–HCl (*p*H 8.0) and 1 mM EDTA.

Digestion with Restriction Endonucleases. Ten micrograms of DNA was digested in a total volume of 30 μl of reaction buffer with 20 U of restriction enzyme at 37°C for 2 hr.

Analysis by DNA Blotting. Electrophoresis on 0.9% agarose gels was carried out on slab gels (0.5 × 12 × 14 cm) in 0.09 M Tris, 0.09 M boric acid, and 2.5 mM EDTA at *p*H 8.5 for 4 hr at 75 V. After electrophoresis each gel was stained with 0.1 μg/ml ethidium bromide. Transfer of DNA fragments to a nylon membrane was performed as described by Southern (1975). The chymotrypsin gene from the rat has been cloned and sequenced by Bell *et al.* (1984). The plasmid DNA (pcXP33) was nick-translated in the presence of [α-^{32}P]dCTP (Rigby *et al.*, 1977) to give a specific activity of 1–3 × 10^8 cpm/μg. Hybridization was performed essentially as described by Wahl *et al.* (1979) except that the hybridization mixture contained 50% formamide and did not contain dextran sulfate. Prehybridization was carried out for 4–5 hr at 42°C and hybridization was allowed to take place for 20 hr at 42°C. Hybridizing fragments were visualized by autoradiography with Kodak XAR-5 film.

RESULTS

The restriction endonuclease fragment length variants in mice were clearly detectable by Southern blot analysis using the chymotrypsin cDNA probe pcXP33, which contains 370 base pairs of the open reading frame corresponding to amino acids 33 to 156. The variants were detected in the restriction patterns generated by digestions with *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, and *Xba*I, whereas in the case of digests with *Bam*HI, *Eco*RV, and *Kpn*I no difference in restriction patterns was observed in any mice examined.

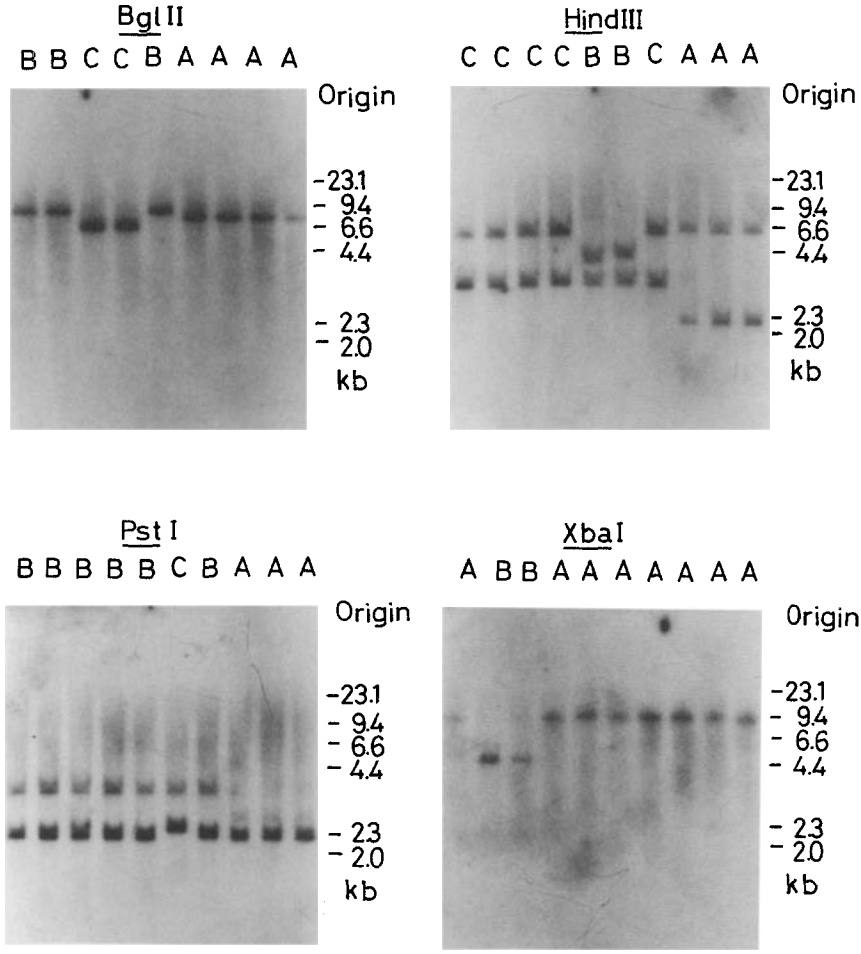

Fig. 1. The restriction endonuclease fragment length variants of the chymotrypsin gene from mice examined by Southern blot analysis using a rat chymotrypsin cDNA pcXP33 probe.

Table III. Restriction Fragment Length Variants of the Chymotrypsin Gene from the Mouse

Restriction enzyme	Fragment length (kb)		
	A	B	C
*Bgl*II	7.0	8.0	6.0
*Eco*RI	7.6 7.3	8.3 7.3	
*Hin*dIII	5.8 2.3	4.2 3.3	5.8 3.3
*Pst*I	3.0 2.4	3.6 2.4	3.6 2.0
*Sac*I	11.0	4.2	
*Xba*I	9.3	4.3	

Examples of restriction patterns and a summary of the different types of pattern are presented in Fig. 1 and Table III, respectively. Three patterns (A, B, and C) were observed in the case of fragments produced by *Bgl*II, *Hin*dIII, and *Pst*I, while two patterns (A and B) were obtained when *Eco*RI, *Sac*I, and *Xba*I were used. Although there are two types of protein variants showing different electrophoretic mobilities, the strain distributions in the phenotype of the protein were not consistent with those of any types of the restriction pattern (Table IV), indicating that the variable site in the protein variants is different from the polymorphic sites detected in the DNA variants. Genomic DNA encoding rat chymotrypsin has been isolated and sequenced (Bell *et al.*,

Table IV. The Set of Protein Phenotypes and Types of Restriction Fragment Length Variants of the Chymotrypsin Gene from Subspecies of Wild Mice

Strain	Set	Protein phenotype	Restriction type					
			*Bgl*II	*Eco*RI	*Hin*dIII	*Pst*I	*Sac*I	*Xba*I
BRV-MPL and DOM-PGN	1	A	A	A	A	A	A	A
DOM-BLG and SK/Cam	2	A	C	B	C	B	B	B
MUS-BLG, MUS-NJL, sub-BJN, and sub-SHH	3	A	B	B	B	B	B	A
Sub-ACI, sub-LAS, sub-KJR, MOL-NEM, MOL-ANJ, MOL-MOM, and MOL-WAK	4	B	B	B	B	B	B	A
MOL-TSU, CAS/J, CAS-QZN, and CAS-TCH	5	A	B	B	B	C	B	A

1984), but that from the mouse has not. At the present time, as we did not construct the restriction maps of mouse chymotrypsin genomic DNA, it remains to be determined whether the polymorphic sites are in coding or noncoding regions.

Since all of several polymorphic sites are involved in one chymotrypsin genome, their variants behave as a set of genetic elements in the mouse populations. In order to analyze the genetic relationships between mice, the set of protein phenotypes (Watanabe *et al.*, 1987) and the types of restriction fragment variants of the chymotrypsin genomic gene were examined for many laboratory strains and wild subspecies of mice (Tables IV and V). Most of the laboratory strains can be grouped into Set 1, which includes the following characteristics: A in protein phenotype, A type in *Bgl*II, A in *Eco*RI, A in *Hin*dIII, A in *Pst*I, A in *Sac*I, and A in *Xba*I. In contrast, only the AU/SsJ strain and five BALB/c sublines (BALB/cAn, BALB/cByA, BALB/cCdA, BALB/cHeA, and BALB/cRglA) were classified as belonging to Set 2, being A, C, B, C, B, B, and B, respectively. Of European wild subspecies, BRV-MPL (*M. brevirostris*), as well as DOM-PGN (a strain of *M. domesticus* from Canada), could be placed unequivocally in Set 1 with most of the laboratory strains. DOM-BLG and SK/Cam, which are *M. domesticus* from Bulgaria and the United Kingdom, resepectively, fell into Set 2 with AU/SsJ and the five BALB/c sublines. Two *M. musculus* strains of the MUS-BLG and MUS-NJL subspecies were classified as belonging to Set 3, being A, B, B, B, B, B, and A. As the populations of Asian wild mice have sometimes been shown to possess unique genetic characters different from those of European wild subspecies and laboratory mice, we chose several strains representative of Asian wild subspecies and examined the restriction fragment length variants of their chymotrypsin genes. The Chinese subspecies clearly belonged to Set 3, as did *M. musculus*. The Korean wild subspecies (*M. yamashinai*) and four Japanese wild strains (*M. molossinus*) belonged to a fourth set, Set 4, having

Table V. The Set of Protein Phenotypes and Types of Restriction Fragment Length Polymorphism of the Chymotrypsin Gene in Laboratory Mouse Strains

Strain	Set	Protein phenotype	Restriction type					
			*Bgl*II	*Eco*RI	*Hin*dIII	*Pst*I	*Sac*I	*Xba*I
26 laboratory strains[a]	1	A	A	A	A	A	A	A
6 laboratory strains[b]	2	A	C	B	C	B	B	B

[a] A/J, AKR/FuA, CBA/ByA, CE/JA, C3H/He, C57BL/6J, C57BL/6ByA, C58/JA, DBA/2, 129/SvJ, DD/HeAf, DS, TSI/A, CFO, MAS/A, SJL/JA, 020/A, RIII/SeA, WLL/BrAf, NZB, KK, N, NC, SS, ICR, and DDY.

[b] AU/SsJ, BALB/cAn, BALB/cByA, BALB/cCdA, BALB/cHeA, and BALB/cRglA.

characteristics B, B, B, B, B, B, and A, and a *M. molossinus* strain, MOL-TSU, and three strains of *M. castaneus* from Thailand, the Philippines, and Taiwan belonged to Set 5, having characteristics A, B, B, B, C, B, and A.

We estimated the number of nucleotide substitutions in the different fragments of the five sets. One nucleotide substitution was expected between every pair of types in the three types that generated different *Bgl*II restriction fragments. One nucleotide substitution was also expected between the two types that generated different *Eco*RI fragments. In the *Hin*dIII digests, one nucleotide exchange was estimated between the A and the C types and also between the B and the C types, while two were estimated between the A and the B types. In the case of *Pst*I fragments, one nucleotide substitution was estimated between the A and the B types and also between the B and the C types, while two were estimated between the A and the B types. Between the two types identified by both the *Sac*I and the *Xba*I digests, one nucleotide exchange was expected. From these results, genetic divergence among the five sets was calculated by the procedure of Nei and Li (1979), and the dendrogram was drawn as shown in Fig. 2; in this case, the difference of protein phenotype was not taken into account in the degree of genetic distance, as it is not exactly clear that the different protein phenotypes had arisen from a nucleotide substitution in a site different from any of the restriction sites. The genetic relationship between the members of Set 1 and those of the other four sets is the most distant. Next, Set 2 is more distant from the other three sets. The relationship among Sets 3, 4, and 5 is very close; Sets 3 and 4 seem to be closest.

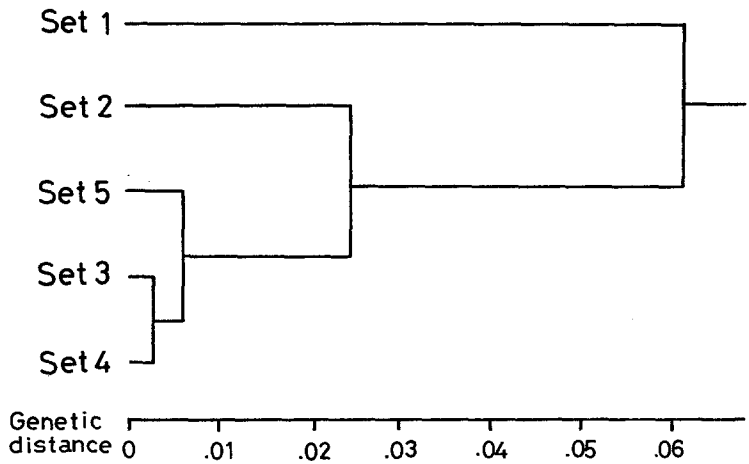

Fig. 2. Dendrogram showing the genetic relationship among the five sets of chymotrypsin genes.

DISCUSSION

Since DNA variants include the differences in sequence that correspond not only to amino acid substitutions but also to those that cause no exchanges of amino acids in exons and even in noncoding regions of introns, they are much more useful for finding genetic variations, as shown, for instance, in serum amyloid A proteins (Taylor and Rowe, 1984). DNA variation in mice was previously reported in mitochondrial DNA (mtDNA), and the reports on mtDNA variations have demonstrated that strains of laboratory mice were derived primarily from the European subspecies *Mus m. domesticus* (Yonekawa *et al.*, 1980, 1982, Ferris *et al.*, 1982, 1983). The *M. brevirostris* type of mtDNA has been found only in NZB sublines and CF1 mice (Yonekawa *et al.*, 1982; Wakana and Kato, personal communication), and other laboratory strains have all been shown to be of the *M. domesticus* type. Furthermore, there is evidence that wild mice from various locales show a high level of restriction-site variations of mtDNA associated with their rapid evolution, but no such variations are found among most laboratory strains (Ferris *et al.*, 1982, 1983). It has been proposed from these findings that only one female of *M. domesticus* contributed to the generation of most laboratory strains. However, as Suzuki *et al.* (1986) have pointed out, the mtDNA variations may not reflect the true evolutionary relationship between subspecies because mtDNA is only maternally inherited, and therefore, it is essential to examine nuclear DNA also. Suzuki *et al.* (1986) intensively investigated the restriction fragment length variants of the nontranscribed spacer region with ribosomal DNA (NTS) from various subspecies of *Mus musculus*. In their study, they found that most laboratory strains examined also possessed the *M. domesticus* type of NTS. The fact that some nuclear genes in laboratory mice are derived from *M. m. domesticus* is in accord with the results from the mitochondrial genomes.

Since previous studies of variations in either proteins (Bonhomme *et al.*, 1984) or mtDNA polymorphism (Ferris *et al.*, 1983) failed to detect marked differences between *M. domesticus* and *M. brevirostris,* these subspecies are often combined as a single taxonomic unit, *M. m. domesticus.* However, according to the old morphological classification of Schwarz and Schwarz (1943), mtDNA polymorphism (Yonekawa *et al.*, 1982), and heterogeneity of ribosomal DNA (Suzuki *et al.*, 1986), *M. domesticus* and *M. brevirostris* do, in fact, have different genetic characteristics. The protein phenotype at the *Prt-1* and *Prt-3* loci in many strains of laboratory mice coincides not with the *M. domesticus* phenotype but with that of *M. brevirostris* (Watanabe *et al.*, 1987). Furthermore, in the present study, the set of protein phenotypes and types of restriction fragment length variants of the chymotrypsin gene at the *Prt-2* locus in most laboratory strains is consistent with that of *M. m. brevirostris,* since they are all grouped into Set 1. Only a *M. domesticus* strain

from Canada belonged in Set 1, even though Selander and Yang (1969) indicated that the wild mice in North America, which were introduced from Europe, might be *M. brevirostris*. Both strains of *M. domesticus* from Bulgaria and the United Kingdom are classified into Set 2 as are AU/SsJ and five BALB/c sublines. As shown in Tables IV and V, the nucleotide sequences of the chymotrypsin genes in Sets 1 and 2 are expected to be clearly different. So many differences in their sequences are not considered to have occurred in the course of breeding of laboratory strains but are assumed to have resulted from the variations in wild populations. Therefore, laboratory strains might be mixed as the result of a cross between *M. domesticus* mice and *M. brevirostris* mice.

AU/SsJ and five BALB/c sublines are grouped in Set 2. AU/SsJ is well known to possess Hbb^p (Morton, 1966), which has not been found in western European wild mice but has been detected in Asian wild mice (Japan, Korea and China), Polish and Bulgarian wild mice, and wild mice in Israel, Greece, and Italy (Ritte and Neufeld, 1982; Miyashita *et al.*, 1985). In the present study, it has also been demonstrated that BALB/c sublines have a chymotrypsin gene that differs from that of many other laboratory strains.

Asian wild mice such as the Chinese subspecies, *M. yamashinai, M. molossinus,* and *M. castaneus,* appear to be closely related genetically from the DNA restriction patterns of their chymotrypsin genes. *M. molossinus* (a Japanese wild mouse) is divided between Set 4 and Set 5. Since *M. yamashinai* and the Chinese subspecies are classified into Set 4 and *M. castaneus* is in Set 5, *M. molossinus* must be derived and mixed from *M. yamashinai* or the Chinese subspecies and *castaneus*. This result is consistent with the investigations of mtDNA polymorphism (Yonekawa *et al.*, personal communication) and the distribution of Hbb^p (Miyashita *et al.*, 1985) in *M. molossinus* captured in several places in Japan. The Chinese wild subspecies are also divided between Set 3 and Set 4. The set of some Chinese subspecies was the same as those of two strains of *M. musculus* from Bulgaria and Denmark, because they are classified as belonging to Set 3. The area associated with mice in Set 3 is very large, and the wide distribution is similar to the geographical distribution of heterogeneity of ribosomal DNA (Suzuki *et al.*, 1986). *M. musculus* seems to function to mediate the gene migration between European wild mice and Asian wild mice.

The difference in protein phenotypes (A and B) transcribed from the chymotrypsin gene did not coincide entirely with the restriction types of the DNA. For example, as seen in the correlation between Set 1 and Sets 2, 3, and 5, even if the protein phenotype is A, the restriction types are different from one another. Conversely, although the protein phenotypes are different from each other in that Set 3 is A and Set 4 is B, their restriction types were all the same.

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