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Serum Transferrin Phenotypes of *Rana japonica* Distributed in Western Japan

By

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(With 2 Text-figures)

INTRODUCTION

The evidence that serum transferrins are polymorphic in amphibians has been extensively presented by FOX, DESSAUER and MAUMUS (1961), GUTTMAN (1967, 1969, 1972), BROWN and GUTTMAN (1970) and GUTTMAN and WILSON (1973) in *Bufo*, by CHALUMEAU-LE FOULEGOC, FINE and GALLIEN (1966, 1972), FINE, CHALUMEAU-LE FOULEGOC and AMOUCH (1967) and CHALUMEAU-LE FOULEGOC (1969) in *Pleurodeles*, by DESSAUER and NEVO (1969) in *Acris* and by PLATZ (1972) in *Rana*. It has been generally known that transferrins in amphibians are probably determined by series of allelomorphic genes, as those of mammals.

The purpose of the present study was to examine the polymorphism of serum transferrins of the Japanese brown frog, *Rana japonica* and to clarify the geographic distribution of its transferrin phenotypes in western Japan.

MATERIALS AND METHODS

Rana japonica GÜNTHER were collected from the following 12 localities in western Japan (Table 1): Tsuruga (1), Hashima (2), Santo-cho (3) in Hyogo Prefecture, Mihara (4), Fuchu-cho (5) and Hiroshima (6) in Hiroshima Prefecture, Yamaguchi (7), Sakaiminato (8), Mihonoseki-cho (9) in Shimane Prefecture, Kochi (10), Ooita (11) and Kagoshima (12). The dates of collection and the developmental stages of animals collected are presented in Table 1. The description of developmental stages follows those of *Rana pipiens* established by SHUMWAY (1940) and TAYLOR and KOLLROS (1946) for convenience sake. In the case of embryos, at least 20 egg masses were collected from each location, and 20 to 30 normally developing individuals were removed from each egg mass and reared in the laboratory. These embryos were raised until metamorphosis at room temperature in enameled pans which were 31 cm × 22 cm × 5.5 cm in size and contained Cl-free tap water. In the case of tadpoles, many normal individuals were collected from different places in each locality, and 25 of them were raised in enameled pans at room temperature. Tadpoles were fed on

TABLE 1
Collection sites, dates and developmental stages of *Rana japonica* specimens collected

Locality	Date	Stage at the time of collection
Tsuruga (1)	March, 1978	Embryos (st. 11～16)
Hashima (2)	March, 1978	Embryos (st. 14～18)
Santo-cho (3)	October, 1975	Adult frogs
Mihara (4)	October, 1976	Adult frogs
Fuchu-cho (5)	October, 1973	Adult frogs
	October, 1975	Adult frogs
Hiroshima (6)	November, 1974	Adult frogs
Yamaguchi (7)	November, 1975	Adult frogs
Sakaiminato (8)	March, 1976	Embryos (st. 16～19)
	June, 1977	Young frogs
Mihonoseki-cho (9)	March, 1976	Embryos (st. 16～19)
	June, 1977	Young frogs
Kochi (10)	March, 1976	Tadpoles (st. V～XII)
Ooita (11)	October, 1976	Adult frogs
Kagoshima (12)	March, 1976	Tadpoles (st. XX～XXI)

The number in parentheses corresponds to that on the map in Fig. 2.

boiled spinach, while frogs were fed on crickets.

From male and female frogs which were used in examining transferrin phenotypes, offspring were produced by means of artificial fertilization. The fertilized eggs were reared for approximately 3 months after metamorphosis in the laboratory.

Blood samples were obtained from the heart of frogs under ether anesthesia by using a slender glass tube containing a very small amount of heparin. Serum was separated by centrifuging at 3,000 r.p.m. for 10 minutes at room temperature. After centrifugation, the tubes were broken just above the erythrocyte-plasma interface and the supernatants were stored at −20°C for 1～3 days as crude serum samples.

Transferrin samples were prepared immediately before electrophoresis by acrinol pretreatment according to MORIWAKI, SADAIE and HIRASAWA (1974). The method of polyacrylamide gel electrophoresis developed by WRIGHT and MALLMAN (1966) was employed without any appreciable modification. Upon completion of electrophoresis, the gel was stained with amido black, destained electrophoretically and preserved in 7% solution of acetic acid (DAVIS, 1964). Transferrin was distinguished from albumin by difference in relative mobility in each gel.

OBSERVATION

I. Identification of transferrin phenotypes

Transferrin phenotypes were first identified in electrophoretic pattern of serum transferrin samples prepared from *Rana japonica* collected from Hiroshima. In

these electrophoretic patterns, the most intensely stained component was albumin (Fig. 1). The advancing edge of albumin invariably moved about 68 to 71 mm from the origin. The second intensely stained component was located about midway between the origin and the base end of the gel. There was a thin band shortly before this band, while one or two thin bands appeared immediately behind it. Besides, a few bands were usually found between this group of bands and the albumin.

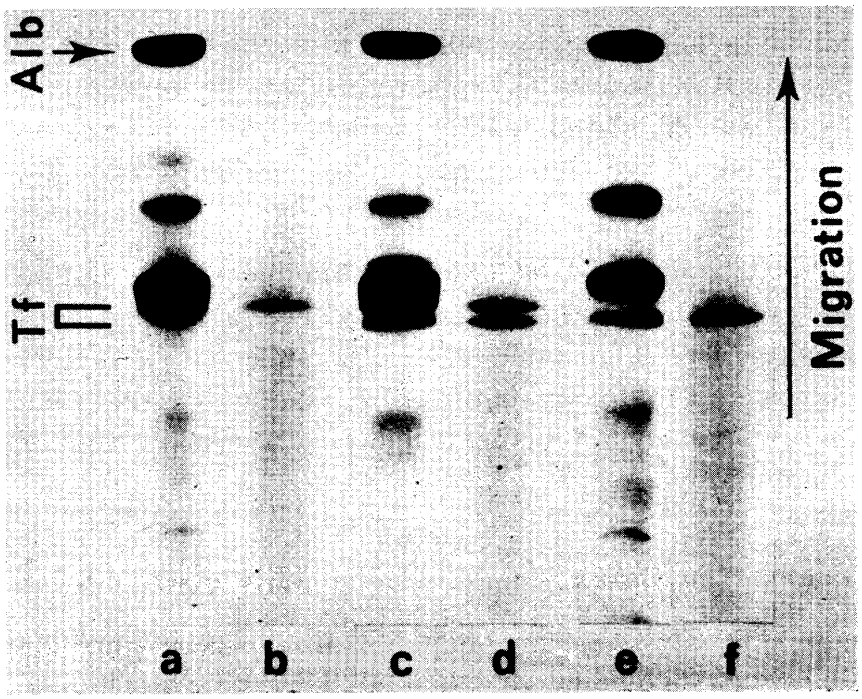

Fig. 1. Electrophoretic patterns of transferrin.
a and b. TfAA c and d. TfAB e and f. TfBB a, c and e. Crude sera b, d and f. Sera pretreated with acrinol and ethanol Tf, Transferrin Alb, Albumin

Transferrins of these serum components were identified by pretreatment with acrinol. This procedure demonstrated one or two bands situated immediately behind the second intensely stained band to be transferrin components.

The results of electrophoretic experiments showed that the serum transferrins of *Rana japonica* were divided into three phenotypes. The first phenotype showed a fast moving band which was 0.57～0.59 in relative mobility. This phenotype was designated TfAA. The second phenotype showed a slowly migrating band, 0.52～0.55 in relative mobility, and was designated TfBB. The third phenotype showed two bands which were apparently a combination of the above two bands. Thus, this third phenotype was designated TfAB.

A total of 101 frogs were electrophoretically examined, and it was found that 54, 35 and 12 were TfAA, TfAB and TfBB in phenotype, respectively. The frequencies of TfAA, TfAB and TfBB expected from HARDY-WEINBERG formulation were 0.504, 0.412 and 0.084, respectively. These frequencies agreed on

TABLE 2
Distribution of transferrin phenotypes in males and females of the Hiroshima population

Phenotype	No. of frogs		
	Total	Female	Male
TfAA	54	30	24
TfAB	35	18	17
TfBB	12	6	6
Total	101	54	47

the whole with those found in frogs collected from the field. The frequencies of Tf^A and Tf^B were 0.71 and 0.29, respectively. As well-known in various kinds of vertebrates, it was evident that the transferrin alleles were autosomal, since there were no differences in phenotype frequency between males and females (Table 2).

II. Inheritance of transferrin phenotypes

Offspring were produced from 10 matings of seven combinations among males and females having the three transferrin phenotypes (Table 3). There were no remarkable differences in viability of offspring between different kinds of matings. In these matings, 75.4 to 98.4 percent of normally cleaved eggs attained completion of metamorphosis.

TABLE 3
Viability of the offspring produced from 10 matings of seven combinations among three transferrin phenotypes

Phenotype of parents		No. of eggs	No. of normally cleaved eggs	No. of hatched tadpoles (%)	No. of frogs (%)
Female	Male				
TfAA	TfAB	72	66	65 (98.5)	62 (93.9)
TfAB	TfAA	69	67	65 (97.0)	62 (92.5)
TfBB	TfAA	60	55	52 (94.6)	46 (83.6)
TfBB	TfBB	64	63	63 (100.0)	62 (98.4)
		75	70	66 (94.3)	55 (78.6)
TfBB	TfAB	75	73	73 (100.0)	67 (91.8)
TfAB	TfBB	85	78	74 (94.9)	65 (83.3)
		68	63	62 (98.4)	58 (92.1)
TfAB	TfAB	75	65	62 (95.4)	49 (75.4)
		63	57	53 (93.0)	47 (82.5)

Parentheses show the percentage of normally cleaved eggs.

The transferrin phenotypes were examined in the frogs about three months after completion of metamorphosis. The results are presented in Table 4. In two matings between TfBB and TfBB, a total of 105 offspring were all of TfBB. In a mating, TfBB × TfAA, 11 offspring were all of TfAB.

On the other hand, genotypic segregation occurred in the offspring resulting from three kinds of matings, TfAA × TfAB, TfAB × TfBB and TfAB × TfAB. In two matings between TfAA and TfAB, a total of 101 offspring consisted of 48 TfAA and 53 TfAB individuals. In three matings between TfBB and TfAB, a total of 168 offspring consisted of 78 TfAB and 90 TfBB individuals. In two matings between TfAB and TfAB, a total of 50 offspring consisted of 13 TfAA, 22 TfAB and 15 TfBB individuals. In these three kinds of combinations, the ratios of different phenotypes found in the offspring were approximately 1 : 1 or 1 : 2 : 1 as expected.

The sex of 435 offspring in total whose transferrin phenotypes were examined is presented in Table 5. Of these frogs, 213 were females, 219 were males and the remaining three were transforming from female to male. No remarkable

TABLE 4

Distribution of transferrin phenotypes in the offspring produced from 10 matings of seven combinations

Phenotype of parents		No. of matings	No. of offspring			
Female	Male		Total	TfAA	TfAB	TfBB
TfAA	TfAB	1	60	29	31	0
TfAB	TfAA	1	41	19	22	0
		2	101	48	53	0
TfBB	TfAA	1	11	0	11	0
		1	11	0	11	0
TfBB	TfBB	1	53	0	0	53
TfBB	TfBB	1	52	0	0	52
		2	105	0	0	105
TfBB	TfAB	1	64	0	32	32
TfAB	TfBB	1	52	0	24	28
TfAB	TfBB	1	52	0	22	30
		3	168	0	78	90
TfAB	TfAB	1	26	6	12	8
TfAB	TfAB	1	24	7	10	7
		2	50	13	22	15

TABLE 5

Distribution of transferrin phenotypes in male and female offspring produced from 10 matings of seven combinations

Phenotype	No. of frogs			
	Total	Female	Hermaphrodite	Male
TfAA	61	33	2	26
TfAB	164	77	0	87
TfBB	210	103	1	106
	435	213	3	219

differences were found between males and females in the ratios of transferrin phenotypes. Thus, the alleles for transferrin phenotypes were evidently autosomal.

III. Geographic distribution of transferrin phenotypes

The distribution of the three transferrin phenotypes (TfAA, TfAB and TfBB) in 12 localities is presented in Table 6. The frequency of transferrin alleles in each population is shown in Fig. 2.

In the Tsuruga population, transferrin phenotypes were examined by using 60 young frogs which had been raised from embryos collected in the field. Of these frogs, 33 (55.0%) were of TfAA, 6 (10.0%) of TfBB and 21 (35.0%) of TfAB. Gene Tf^A was 0.73 in frequency.

Transferrin phenotypes in the Hashima population were also examined by using 60 young frogs raised from embryos collected in the field. The results showed that 24 (40.0%) of them were of TfAA, 10 (16.7%) of TfBB and 26 (43.3%) of TfAB. Gene Tf^A was 0.62 in frequency.

TABLE 6

Frequencies of transferrin phenotypes in 12 populations

Locality	No. of frogs	Phenotype (Expected number)			Alleles (%)	
		TfAA	TfAB	TfBB	Tf^A	Tf^B
Tsuruga	60	33 (32.0)	21 (23.7)	6 (4.4)	73	27
Hashima	60	24 (23.1)	26 (28.3)	10 (8.7)	62	38
Santo-cho	47	36 (36.4)	11 (9.9)	0 (0.7)	88	12
Mihara	48	26 (27.0)	20 (18.0)	2 (3.0)	75	25
Fuchu-cho	111	50 (48.4)	46 (49.8)	15 (12.8)	66	34
Hiroshima	47	18 (17.5)	21 (22.4)	8 (7.1)	61	39
Yamaguchi	56	54 (53.8)	2 (2.2)	0	98	2
Sakaiminato	73	31 (32.8)	36 (32.3)	6 (7.9)	67	33
	50	25 (25.2)	21 (20.6)	4 (4.2)	71	29
Mihonoseki-cho	58	22 (23.8)	30 (26.7)	6 (7.5)	64	36
	45	19 (20.2)	22 (19.9)	4 (4.9)	67	33
Kochi	57	32 (30.4)	19 (22.5)	6 (4.2)	73	27
Ooita	47	21 (21.7)	22 (20.5)	4 (4.8)	68	32
Kagoshima	35	21 (20.2)	11 (12.8)	3 (2.0)	76	24

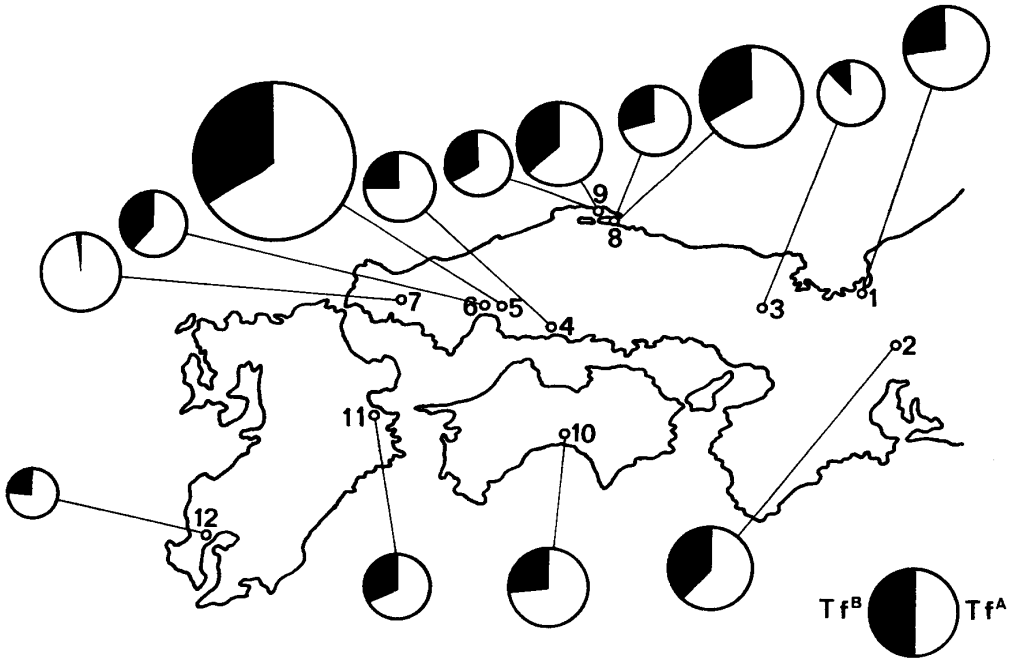

Fig. 2. Geographic distribution of transferrin alleles among *Rana japonica* populations.

In the Santo-cho population, the transferrin phenotypes of 47 frogs collected from the field were analyzed. It was observed that 36 (76.6%) were of TfAA and the remaining 11 (23.4%) of TfAB. Gene Tf^A was 0.88 in frequency.

Transferrin phenotypes of frogs distributed in Hiroshima Prefecture were analyzed in three populations, Mihara, Fuchu-cho and Hiroshima. In the Mihara population, 26 (54.2%) of 48 frogs were of TfAA, 2 (4.2%) of TfBB and the remaining 20 (41.7%) of TfAB. Gene Tf^A was 0.75 in frequency. In the Fuchu-cho population, 50 (45.1%) of 111 frogs were of TfAA, 15 (13.5%) of TfBB and 46 (41.4%) of TfAB. Gene Tf^A was 0.66 in frequency. In the Hiroshima population, 18 (38.3%) of 47 frogs were of TfAA, 8 (17.0%) of TfBB and 21 (44.7%) of TfAB. Gene Tf^A was 0.61 in frequency.

In the Yamaguchi population, transferrin phenotypes were examined by using 56 frogs collected from the field. It was observed that 54 (96.4%) of them were of TfAA and the remaining two (3.6%) of TfAB. Gene Tf^A was 0.98 in frequency.

Transferrin phenotypes in the Sakaiminato population were first examined by using 73 young frogs raised from embryos collected in the field. The results showed that 31 (42.5%) of them were of TfAA, 6 (8.2%) of TfBB and the remaining 36 (49.3%) of TfAB. Gene Tf^A was 0.67 in frequency. In the following year, transferrins were analyzed in 50 young frogs collected from the same locality. It was observed that 25 (50.0%) of them were of TfAA, 4 (8.0%) of TfBB and 21 (42.0%) of TfAB. Gene Tf^A was 0.71 in frequency. Transferrin phenotypes were also examined in the Mihonoseki-cho population which was situated 8 km from the Sakaiminato population in Tottori Prefecture. The results showed that of 58 young frogs raised from embryos collected in the field, 22 (37.9%)

were of TfAA, 6 (10.3%) of TfBB and 30 (51.7%) of TfAB. Gene Tf^A was 0.64 in frequency. In the following year, transferrin analyses were made in 45 young frogs collected from the same locality. It was observed that 19 (42.2%) were of TfAA, 4 (8.9%) of TfBB and 22 (48.9%) of TfAB. Gene Tf^A was 0.67 in frequency.

Transferrin phenotypes in the Kochi population were examined by using 57 young frogs raised from tadpoles collected in the field. It was observed that 32 (56.1%) of them were of TfAA, 6 (10.5%) of TfBB and 19 (33.3%) of TfAB. Gene Tf^A was 0.73 in frequency.

In the Ooita population, transferrin phenotypes were examined by using 47 frogs collected from the field and it was found that 21 (44.7%) were of TfAA, 4 (8.5%) of TfBB and 22 (46.8%) of TfAB. Gene Tf^A was 0.68 in frequency.

In the Kagoshima population, transferrin phenotypes were examined by using 35 young frogs raised from metamorphosing tadpoles collected in the field. Of these frogs, 21 (60.0%) were of TfAA, 3 (8.6%) of TfBB and 11 (31.4%) of TfAB. Gene Tf^A was 0.76 in frequency.

The sex of a total of 794 frogs whose transferrin phenotypes were determined is presented in Table 7. These frogs consisted of 419 males and 375 females. Of 417 frogs with TfAA phenotypes, 190 were females and 227 males. Of 306 frogs with TfAB phenotypes, 150 were females and 156 males, while of 71 frogs with TfBB phenotypes, 35 were females and 36 males. It was evident that there was nearly an equal number of males and females in each of the three transferrin phenotypes.

TABLE 7

Distribution of transferrin phenotypes in males and females of each of the 12 populations

Locality	TfAA		TfAB		TfBB		Total	
	Female	Male	Female	Male	Female	Male	Female	Male
Tsuruga	19	14	11	10	2	4	32	28
Hashima	13	16	8	16	5	2	26	34
Santo-cho	18	18	6	5	0	0	24	23
Mihara	13	13	9	11	0	2	22	26
Fuchu-cho	23	27	23	23	8	7	54	57
Hiroshima	10	8	11	10	5	3	26	21
Yamaguchi	7	47	2	0	0	0	9	47
Sakaiminato	17	14	18	18	4	2	39	34
	15	10	12	9	0	4	27	23
Mihonoseki-cho	10	12	13	17	4	2	27	31
	11	8	10	12	2	2	23	22
Kochi	15	17	11	8	2	4	28	29
Ooita	11	10	12	10	1	3	24	23
Kagoshima	8	13	4	7	2	1	14	21
	190	227	150	156	35	36	375	419

DISCUSSION

FOX, DESSAUER and MAUMUS (1961) reported that each of two American toad species, *Bufo fowleri* and *Bufo valliceps,* possessed a single transferrin and that the transferrins of the two species migrated at strikingly different rates. No intraspecific variation in transferrin was observed by them. GUTTMAN (1967) observed that the transferrin components of two African toad species, *Bufo regularis* and *Bufo rangeri,* were polymorphic. All toads of both species examined by him possessed at least two electrophoretically distinct transferrins. The transferrin components of two Argentine toad species, *Bufo arenarum* and *Bufo spinulosus,* were examined by BROWN and GUTTMAN (1970). While *Bufo spinulosus* possessed only one transferrin which was the slowest in mobility, *Bufo arenarum* possessed four fast-migrating transferrin components which distinctly differed from one another in mobility. Each toad possessed one or two of these four transferrin components.

GUTTMAN (1969) reported that the transferrin components of six species of the *Bufo americanus* group were polymorphic. In 77 specimens examined, 13 molecular types of transferrin components appeared. Thereafter, the transferrin components of one *Bufo americanus* population from southwestern Ohio were examined by GUTTMAN and WILSON (1973). The results showed that 185 individuals collected from this population revealed 35 phenotypes determined by 13 transferrin alleles.

DESSAUER and NEVO (1969) examined the transferrin components of two cricket frog species, *Acris crepitans* and *Acris gryllus,* and confirmed that they were polymorphic. While *Acris gryllus* possessed two codominant alleles, *Acris crepitans* possessed five codominant alleles. According to DESSAUER and NEVO, three divergent population groups were definable within *Acris crepitans* on the basis of the distribution of specific transferrins. The transferrins of *Acris crepitans* were remarkably slower in mobility than those of *Acris gryllus.* PLATZ (1972) reported on the transferrin components of *Rana pipiens berlandieri* from Texas. Serum samples of individuals revealed either a slow migrating band, a slightly faster band, or both. The frequencies of the band patterns suggested that the slow and fast bands were produced by alleles at the same locus. There was a close fit between the observed frequencies and the theoretical values based on HARDY-WEINBERG equilibrium. The frequency of the slow transferrin band was about $0.6 \sim 0.9$ and appeared to be different between two forms of *Rana pipiens berlandieri.*

By the present study, it became evident that the transferrin components of *Rana japonica* collected from 12 localities in western Japan were polymorphic. In each of these populations, there were always two or three transferrin phenotypes which were determined by two codominant alleles, Tf^A and Tf^B. These genes were autosomal and gene Tf^A was far more predominant over Tf^B. The frequency of Tf^A was $0.61 \sim 0.76$ in 10 of the 12 populations, while it was 0.88

and 0.98 in the other two populations, the Santo-cho population and the Yamaguchi population. Although these figures of frequencies merely showed approximate values, as the number of individuals in each population was generally small, the Santo-cho and Yamaguchi populations seemed to be peculiar in paucity of gene Tf^B. In the other populations, males and females were nearly equal in number and the ratio of Tf^A to Tf^B frequencies seemed to be almost constant. In the Yamaguchi population, 47 individuals were males and nine were females, and all the males possessed phenotype TfAA. Of the females, seven were of TfAA and the other two of TfAB. The male preponderance in number was probably attributable to earlier hibernation of females than that of males in November when they were collected. It seems necessary to investigate the frequency distributions for the transferrin alleles in a larger area surrounding each of Santo-cho and Yamaguchi as well as in eastern Japan in order to clarify further the existence of local differences in *Rana japonica*.

SUMMARY

1. Serum transferrins were examined by combination of acrinol pretreatment and polyacrylamide gel electrophoresis in 12 populations of *Rana japonica* collected from western Japan.

2. Serum transferrins were polymorphic and revealed three phenotypes determined by two autosomal, codominant alleles, Tf^A and Tf^B. The band determined by gene Tf^A was faster in migration than that determined by gene Tf^B. Genotypic segregation occurred in the offspring resulting from three kinds of matings, TfAA × TfAB, TfAB × TfBB and TfAB × TfAB, as expected.

3. The frequency of gene Tf^A was 0.61 ~ 0.76 in 10 of the 12 populations examined, while it was 0.88 and 0.98 in the other two populations, the Santo-cho population and the Yamaguchi population.

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