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STUDIES ON INTERSPECIFIC HYBRIDIZATION IN THE RODENTS, I. ARTIFICIAL INSEMINATION BETWEEN THE NORWAY RAT (♀) AND BLACK RAT (♂) AND THE RESULTING KARYOTYPES IN THE HYBRID BLASTOCYSTS[1)]

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To ascertain the relation between karyotype evolution and species differentiation is an important problem in the field of cytogenetics. Based on the karyological studies of 13 *Rattus* species, Yosida (1973), one of the present authors, has demonstrated that all of these species give clear evidence of being differentiated by karyotype evolution. This depends upon the pericentric inversion and the Robertsonian fusion from one species to another. Species differentiation of this group by karyotype evolution is also demonstrated by use of G-band staining (Yosida and Sagai 1973). The karyotype differences, however, are not always correlated directly to the species differentiation. For instance the black rats (*Rattus rattus*) are divided into three geographical variants according to the difference of chromosome numbers and karyotypes, viz., the Asian ($2n=42$), Ceylonese ($2n=40$) and Oceanian ($2n=38$) types. On the other hand, the Norway rat (*Rattus norvegicus*) and the black rat (Asian type) are characterized by having the same chromosome number ($2n=42$) (Oguma 1935; Makino 1943; Matthey 1953). Similarity of karyotypes in both species has also been demonstrated by conventional as well as G-band stainings. The karyotype of the Norway rat corresponded to one of the polymorphic karyotypes in the Asian type black rat which is conspicuous by having the acrocentric and subtelocentric polymorphism of the pairs Nos. 1, 9 and 13 (Yosida 1973; Yosida and Sagai 1973).

Species differences can be estimated by the result of hybridization experiments. As already reported, hybrids involving the three geographical variants of the black rat can easily be obtained in the laboratory by natural matings (Yosida *et al.* 1969, 1971b; Yosida 1976a, 1977b), but no hybrids between the black rat and the Norway rat could neither be obtained by the laboratory matings (Morgan 1909; Babcock 1949; Castle 1949), nor by artificial insemination (Hiraiwa and Yoshida 1955a, b). The degree of the differentiation between the two species seems to be evidenced by observing the degree of the development of fertilized eggs compulsively situated by means of the artificial insemination. Following this, studies on interspecies hybridization in rodents are being

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carried out with special regard to the cytogenetical evidences of the hybrid embryos. The present paper deals with an investigation on the hybrids between the Norway rat and the black rat by artificial insemination. As the karyotype in the hybrid blastocysts could be successfully analysed, the result of observations is added with a discussion on the differentiation of these two species.

MATERIALS AND METHODS

Animals: Several inbred strains (ACI, Fischer, Long-Evans, NIG, Wistar-King-A and Wistar-King-S) of the Norway rat were used. For the control, male and female rats were used, but only females were used in the interspecific hybridization. Male Asian type black rats (*Rattus rattus flavipectus*), which were collected in Hong Kong and bred in our laboratory for several years, were used in the present experiment.

Artificial insemination: Semen from the cauda epididymides of the black rat was suspended in a modified Krebs-Ringer bicarbonate solution. The oestrus cycles of the female rats were examined by the vaginal smears. When the rat was at the pro-oestrus stage of the cycle, the cervix uteri was stimulated by faradic current. The rat then was anesthesized with ether and 0.2 ml of the semen suspention ($28 \sim 126 \times 10^6$/ml) were injected into the uterine horns through each cervical canal by the hypodermic needle. When the rat was stimulated successfuly by the current, the vaginal smear invariably showed symptom of the pregnancy (called the pseudo-pregnancy). Among them some rats showed a real pregnancy by artificial insemination. If the insemination was unsuccessful, the rat came to show the oestrum which could easily be seen by continuous observation of the vaginal smear. As the control, the semen from the Norway rats was inseminated artificially into the female rat by the same technique as above.

Chromosome observation: Blastocysts were obtained from the uterus 5 days after insemination, and cultured for about 2 h in Eagle's minimum essential medium (MEM) with 15% calf serum. The blastocysts were treated with 1% sodium citrate for 20 min, and fixed with Carnoy 3:1 on slide glasses, dried and then stained with Giemsa. Five to 10 blastocysts obtained by dissection of the pregnant females were used for observation of chromosomes.

RESULTS

Artificial insemination with the semen of the back rat: Among 20 Norway rats which were inseminated artificially with the semen of the black rat, 19 showed the pseudo-pregnancy. Among them, only one half (9 rats) showed real pregnancy. Eight rats showing pseudo-pregnancy were selected at random and dissected to confirm the state of the impregnation and the number of embryos if the impregnation was successful (Table 1). Five of the 8 rats were confirmed impregnation, but the other 3 rats showed only the pseudo-pregnancy. One to 4 embryos (2.2 on average) were counted in the impregnated rats (Fig. 1). The number of embryos was significantly less than

Table 1. Results of interspecific crossing between the Norway rat, *Rattus norvegicus* (♀) and the black rat, *Rattus rattus* (♂) by artificial insemination

Exp. No.	Strain of rats used (♀)	No. of sperms inseminated* (per ml)	Bleeding period (days)	First pro-estrum or estrum after insemination	Date of dissection and No. of embryos	Pseudo-pregnancy	Pregnancy
1	Fischer	38×10^6		13th		+	−
2	〃	〃		11th		+	−
3	〃	〃		9th		+	−
4	〃	〃			11th-0	+	−
5	〃	78×10^6			8th-2	+	+
6	〃	〃	13~14th		14th-4	+	+
7	〃	〃	13~15th	20th		+	+
8	〃	〃			11th-0	+	−
9	〃	102×10^6		14th		+	−
10	〃	〃		19th		+	?
11	〃	〃		7th		−	−
12	〃	118×10^6			11th-0	+	−
13	〃	〃	14th		14th-2	+	+
14	〃	〃		13th		+	−
15	〃	126×10^6			9th-1	+	+
16	〃	〃	12th	13th		+	+
17	〃	〃			8th-?	+	+
18	Long-Evans	102×10^6	13~18th	17th		+	+
19	〃	〃	13th	14th		+	+
20	Wistar King-S	118×10^6				+	?

* 0.2 ml were injected into each rat.

the control experiment. No offspring was born in the case of the artificial insemination with the semen of the black rat. In 6 out of 9 rats in which the pregnancy was confirmed, bleeding appeared in their vaginal smears during 12th to 14th days after insemination. The bleeding continued for several days. This is a good indication whether or not the female was impregnated by artificial insemination.

Control experiment: As a control, the semen obtained from the male Norway rats was injected into the females of the same species. Among 25 rats treated 23 were impregnated successfully. A slight bleeding was often observed in the vaginal smear during 12th to 14th day after insemination if the fertilization had successfully taken place. The number of embryos impregnated and litter numbers obtained from 10 rats are shown in Table 2. The number of embryos confirmed by operation after insemination was 3 to 11 (6.3 on average) (Fig. 2). Offspring was born by 3 females with 4, 5 and 7 in their litter size which are almost normal.

Chromosomes: The Norway rat and the black rat together have 42 chromosomes and their karyotypes are similar as already noted by one of the present author (Yosida 1973). The Hong Kong black rat (*R. rattus flavipectus*) is characterized by having acrocentric pairs No. 1 to 13, metacentric pairs No. 14 to 20 and acrocentric X and Y (Yosida

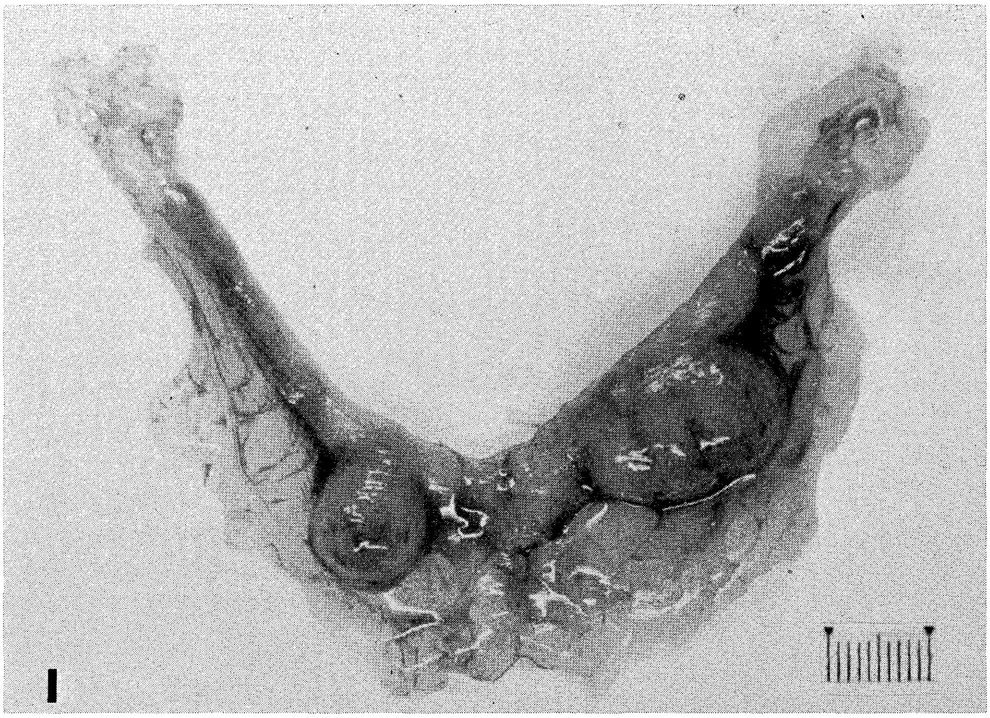

Fig. 1. Uterine swelling of the Norway rat with hybrid embryos 14 days after artificial insemination, which developed by injection of semen of the black rat. Four swellings, one in the left and 3 in the right, were observed (Exp. No. 6 in Table 1).

Table 2. Control experiment of artificial insemination in which the semen of the Norway rats was injected into the females of the same species

Exp. No.	Strain of rat		Date of dissection after insemination (days)	No. of embryos confirmed	Pregnancy	No. of rats born
	Female	Male*				
1	Long-Evans	ACI	—		+	4
2	〃	〃	9th	6	+	
3	NIG	〃	8th	9	+	
4	ACI	〃	9th	3	+	
5	Fischer	Fischer	10th	7	+	
6	ACI	ACI	11th	3	+	
7	Long-Evans	〃	14th	11	+	
8	F_1 (ALB×LE)	Wistar-King-S	7th	5	+	
9	NIG	Long-Evans	—		+	5
10	Wistar-King-A	Wistar-King-A	—		+	7

* 0.2 ml of 10^7/ml semen suspension was injected into each rat.

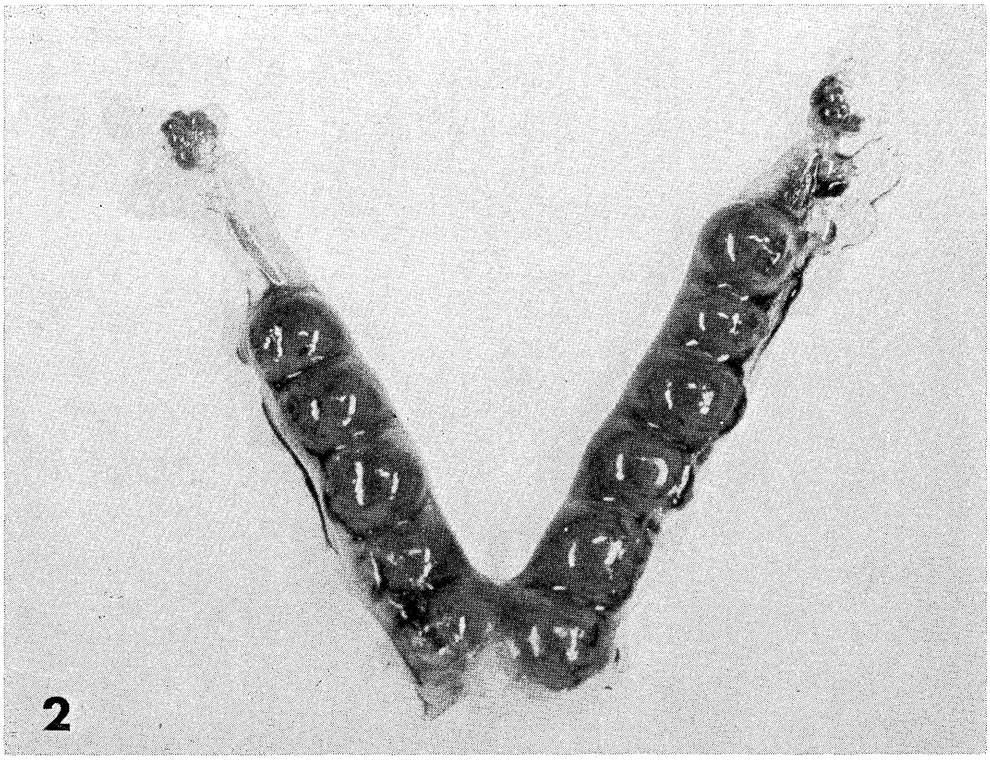

Fig. 2. Uterine swellings with Norway rat embryos 14 days after artificial insemination-developed by injection of semen of the Norway rat (control). Eleven embryos are counted (Exp. No. 7 in Table 2).

et al. 1971b). On the other hand, although the Norway rat and the black rat have similar karyotypes, pairs Nos. 1, 9 and 13 (pairs Nos. 1, 13 and 12 according to the nomenclature of Committee for Standard Karyotype of the Rat; 1973) always show subtelocentrics (Yosida 1973). Hybrids between these two species, thus, are expected to be determined by observation of their karyotypes whether it consists of each haploid set from the Norway rat and the black rat. Five blastocysts examined invariably showed 42 chromosomes, among which the pairs Nos. 1, 9 and 13 are remarkable by acrocentric and subtelocentric heteromorphism which are good proofs of the hybrid between the Norway rat and the black rat (Fig. 3).

Chromosome complement in the blastocysts from the control experiment was usually characterized by having a regular karyotype of the Norway rat which is detectable by presence of the subtelocentric pairs Nos. 1, 9 and 13 (Fig. 4).

DISCUSSION

The basic karyotype of the black rat is characterized by 42 chromosomes consisting of 13 acrocentric pairs (No. 1 to 13), 7 metacentric pairs (No. 14 to 20), and acrocentric X any Y. Among them pairs 1, 9 and 13 are polymorphic in respect to acrocentric

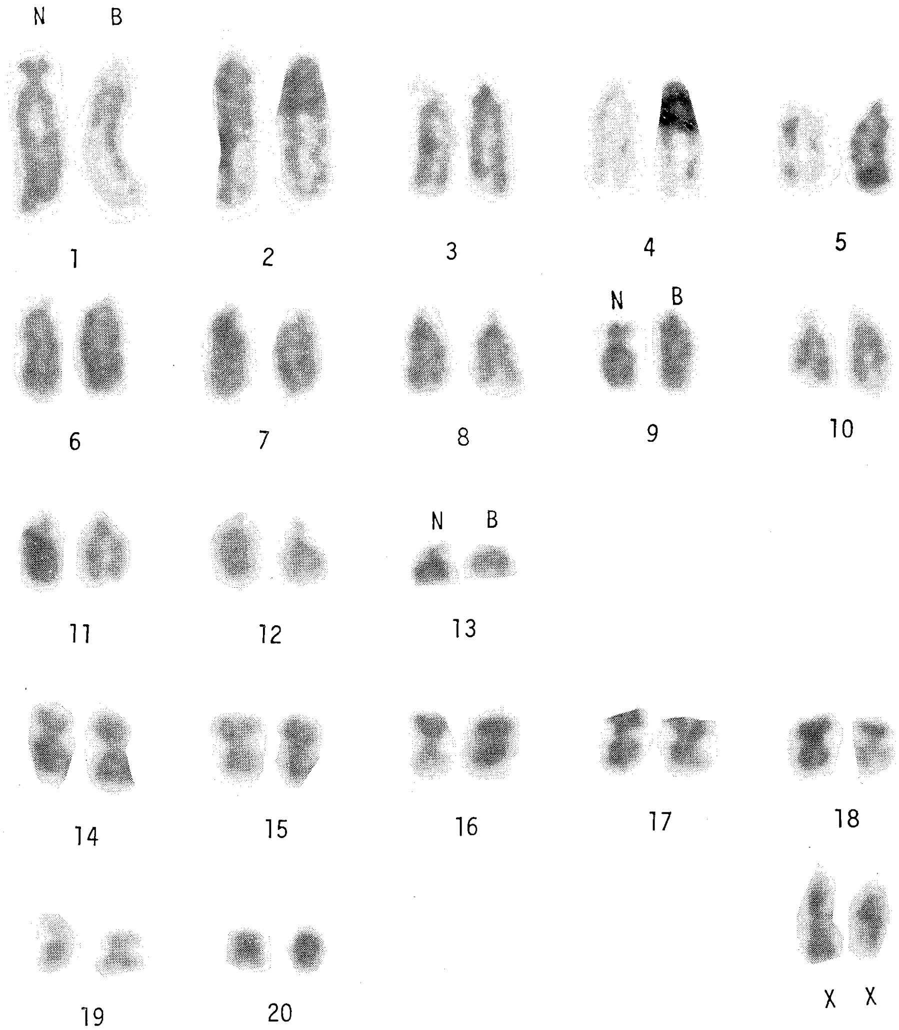

Fig. 3. Karyotype of the hybrid blastocyst developed by artificial insemination between the Norway rat (♀) and the black rat (♂) ($2n=42$). Among 20 autosome pairs, Nos. 1, 9 and 13 are heteromorphic in respect to acroccentric and subtelocentric, which were derived from the black rat (B) and the Norway rat (N), respectively.

and subtelocentric chromosomes (Yosida *et al.* 1965, 1971a; Gropp *et al.* 1970a; Yosida 1976b, 1977a). Besides the chromosome polymorphism, this species is conspicuous by having three geographical variants due to the translocation of some acrocentric chromosomes; viz., Asian ($2n=42$), Ceylonese ($2n=40$) and Oceanian types ($2n=38$) (Yosida *et al.* 1969, 1971b, 1974a). Although the chromosome number and karyotypes of the black rats are markedly different by geographical variants, the hybrids between them involving all variants can easily be obtained in the laboratory by natural matings (Yosida *et al.* 1969, 1971b; Yosida 1976a, 1977b).

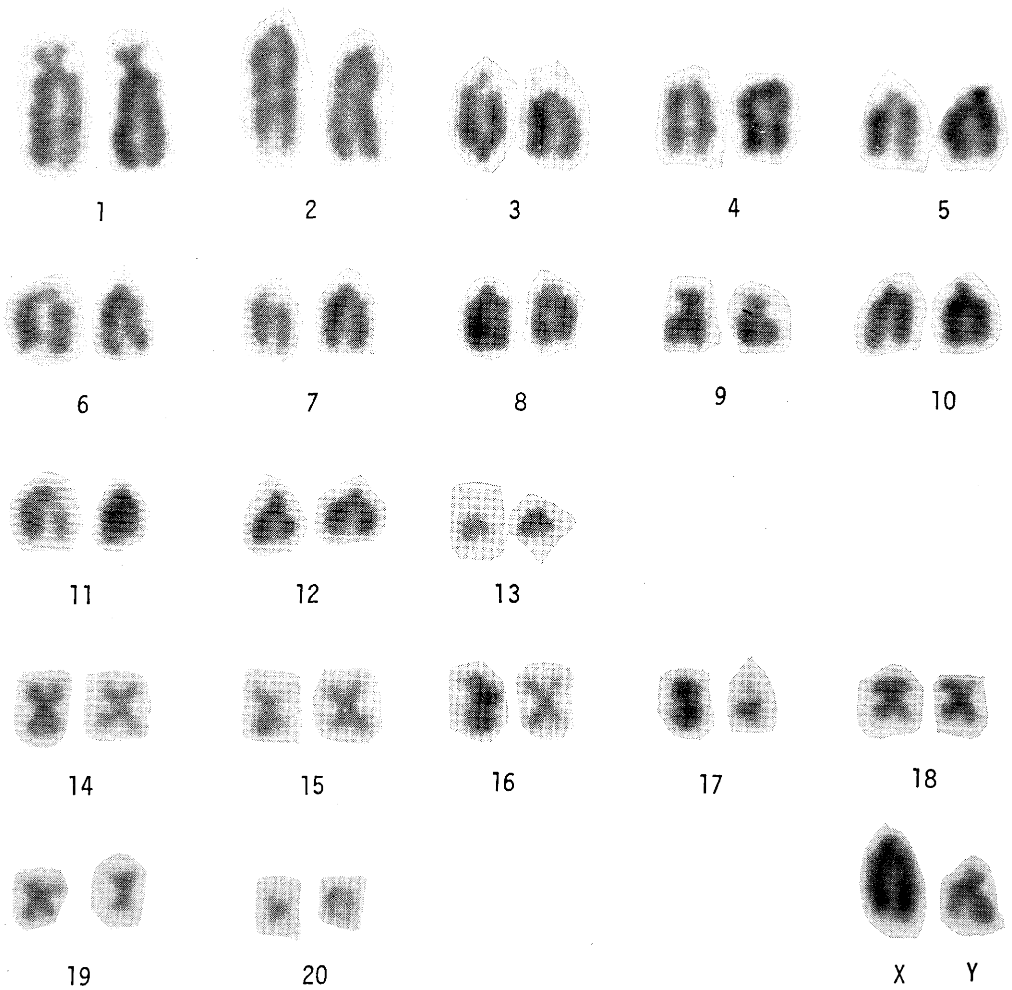

Fig. 4. Karyotype of the Norway rat in the blastocyst developed by artificial insemination with semen of the Norway rat. Pair 1, 9 and 13 chromosomes are subtelocentric homomorphic.

Based on the observation of karyotypes in 13 *Rattus* species one of the present author (Yosida 1973; Yosida and Sagai 1973) pointed out that the chromosome numbers of these species are counted as $2n=32$, 34, 38, 40, and 42. Each species had its unique karyotype. The karyotype evolution in these species was evidenced as a rusult of pericentric inversion and also by the Robertsonian fusion, as seen in the polymorphic and geographic variants in the black rats. The number of chromosomes in the Norway rat which is a close relative of the black rat had 42 chromosomes as shown by many investigators (Oguma 1935; Makino 1943; Matthey 1953 and others) since finding by Painter (1926). Similar karyotypes in both species have been demonstrated by use of conventional and banding stainings (Yosida 1973; Yosida and Sagai 1973). The electrophoretic pattern of the serum transferrin in the black rat is also highly polymorphic in the black rat (Moriwaki *et al.* 1969, 1975; Yosida *et al.* 1971c, 1974b). There are two types of transferrin; fast-moving (R-type) and slow-moving (C-type). The Norway rat has a similar pattern to the fast moving type of the black rat. Differences in amino acid composition of serum transferrin among various species of *Rattus* have been examined by Moriwaki *et al.* (1971). According to them the difference between *Rattus norvegicus* and *Rattus rattus* (Asian type with TfR or TfN) is much larger than that between the Oceanian type and the Asian type black rats (*R. rattus*). Although the Norway rat and the black rat are similar in karyotype and serum transferrin composition, the hybrids between them could not be obtained by laboratory matings (Morgan 1909; Babcock 1949; Castle 1949). Artificial insemination is commonly used to the large domestic animals. In mice and rats this technique has been used in experimental works. Hiraiwa and Yoshida (1955a, b) have attempted to get the interspecific hybrids between the Norway rat and the black rat by artificial insemination, but could not get any hybrids between them. Hybrid embryos, however, can develop to the maximum age of the 14th to 15th days after insemination, but always the embryos died as shown in the present work.

In the case of interspecific hybridization among related species in mammals, some hybrids died at the embryonic stage as seen in the mating between the rabbit and hare (Chang 1965), the goat and sheep (Hancock *et al.* 1968; Alexander *et al.* 1967) and also the ferret and the mink (Chang 1968). On the other hand, some crosses between the horse and the donkey (Benirschke *et al.* 1962), the brown bear and the polar bear, the alpaca and the vicuna (Benirschke 1967) tended to give viable hybrids. In the case of rodents, the hybrids between the Romanian hamster (*Mesocricettus newtoni*) and the Syrian hamster (*M. auratus*) (Raicu and Bratosin 1968) and the house mouse (*Mus musculus*) and the tabacco mouse (*M. poschiavinus*) (Gropp *et al.* 1970b; Tettenborn and Gropp 1970) were successfully produced. Three species of southern Australian *Rattus* (*R. fuscipes*, *R. greyii* and *R. assimilis*) were also easily mated with each other and viable hybrids were obtained in natural matings (Horner and Taylor 1965).

Based on the karyotype analysis in the hybrid embryos developed by natural matings or artificial insemination, it can be said that a complete chromosome constitution consisting of each haploid set from both parents is nessessary to produce viable hybrids. In the case of the hybrids between the Norway rat and the black rat, the karyotype of the hybrid embryo seems to be set up completely, but the hybrids died at the embryonic stage. The same situation found in the ferret and the mink (Chang *et al.* 1969). The question is why are some interspecific hybrids successful and others are not. There are two explanations for the embryonic death in the interspecific hybridization; one of them is the immunological reaction in the female parent of the hybrid embryo which developed by mating between two different species, and the other is due to the developmental disorder of the hybrid itself by genic unbalance. In the case of black rat and Norway rat, the karyotype in both species is very similar, but a large difference between them has been found in the C-banding pattern in the chromosomes (Yosida and Sagai 1975; Yosida 1975). Large and clear C-bands were found near the centromeric portion of all chromosome pairs in the black rats, although a polymorphic change was found in the band. On the other hand, the band in the Norway rat was very small and unclear. It is known that the C-band region on the chromosome is composed of constitutive heterochromatin (Padue and Gall 1970; Jones 1970; Arrighi and Hsu 1971). Heterochromatin seemed to offer direct evidence of the degree of nuclear differentiation (Brown and Nur 1964; Mittwoch 1967), and of the regulation of gene expression in higher organisms (Frenster 1969). Based on these considerations, one of causes in the embryonic death in the hybrids seems to be the difference of the constitutive heterochromatin between the different species.

SUMMARY

Semen of the black rat was inseminated artificially into the female Norway rat by routine technique. Among 20 rats, 9 showed pregnancy. Five impregnanted rats were dissected on 8 to 14 days after insemination and one to 4 embryos were found. No offspring were born in the case of the artificial insemination. In the control in which the semen of the Norway rat was inseminated artificially into the female Norway rat, pregnancies were obtained at high frequency and offspring were successfully born as in natural matings. Chromosomes of the hybrid blastocysts showed 42 chromosomes consisting of two haploid sets, one from the Norway rat and the other from the black rat.

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