CROSSING STUDIES AMONG SIX STRAINS OF *ANOPHELES SINENSIS*[1]

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ABSTRACT. Crossing experiments among 6 strains of *Anopheles sinensis* from Koniya, Kanoya, Karuizawa, Yomogita, Yakumo and Engaru were done by induced copulation; the number of eggs per female, the hatchability, and the fertility of the F_1 hybrids were investigated. The hybrid males between the Engaru strain and either the Kanoya or the Yakumo strain showed complete sterility. The hybrid females showed low fertility. Restoration of fertility of the hybrid females and males from those crosses were found in BC_2. No mature spermatozoa were observed in the testes of the hybrid males from the cross of the Engaru strain with the Kanoya strain. Therefore, the Engaru strain of *An. sinensis* is considered genetically to be a full species. The relationship between the Engaru strain of *An. sinensis* was discussed.

INTRODUCTION

Anopheles (An.) sinensis Wiedemann is the most widespread and common species of anopheline mosquito in Japan. It is also found in northeastern India, Malaysia, Thailand, Vietnam, Indonesia, China and Korea. It is the medically important vector of a filarial parasite, *Brugia malayi* Buckley, in China and Korea (Feng 1931, Kanda et al. 1975) and is presumed to be a vector of human malaria in southeast Asia. Morphological and epidemiological studies of the *An. hyrcanus* (Pallas) species group, to which *An. sinensis* belongs, have been done by Reid (1953, 1968) who gave an interpretation of the interspecific relationships within the species group. Otsuru and Ohmori (1960) reported the detailed morphology of the *An. sinensis* group in Japan. Harrison (1972) also proposed an interpretation of the relationships within the *An. hyrcanus* complex. Xu and Feng (1975) studied the *An. hyrcanus* group in China.

Some crossing experiments have been done with anopheline mosquitoes which are not easily identified morphologically, such as within the *An. gambiae* Giles complex (Davidson et al. 1967), the *An. maculipennis* Meigen complex (Kitzmiller et al. 1967) and the *An. punctulatus* Doenitz complex (Bryan 1973b). Additional hybridization experiments were carried out between other closely related species, such as *An. crucians* Wiedemann x *An. bradleyi* King (Kreutzer and Kitzmiller 1971), *An. punctipennis* Say x *An. perplexens* Ludlow (Kreutzer and Kitzmiller 1972) and *An. subpictus* Grassi x *An. litoralis* King (Darsie and Cagampang-Ramos 1973).

Recently Oguma and Kanda (1976) have succeeded in establishing laboratory strains of *An. sinensis* by using an induced copulation method similar to that described by Baker et al. (1962). This made possible genetic and more detailed morphological studies with respect to speciation in the *An. hyrcanus* species group. Kanda and Oguma (1977a, b) carried out crossing experiments between *An. sinensis* and *An. sineroides* Yamada, *An. sinensis* and *An. lesteri* Baisas and Hu. Furthermore, a morphological study of *An. sinensis* populations collected at 14 localities in Japan revealed that the Engaru and Tomakomai populations in Hokkaido had humeral pale spots (H p), a character which could not be found in the other *An. sinensis* populations (Kanda and Oguma 1976). Moreover, the mean frequency of the clasper movement in the male was 14.6 and 14.3 per copulation in the

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Engaru and Tomakomai strains respectively, while the other strains showed about 8.1 (Kanda and Oguma 1976). The salivary gland chromosomes and chromosomal polymorphism have been studied in 11 Japanese *An. sinensis* populations including Engaru. A new inversion, In2RB, was almost fixed in the Engaru population but not found in the other populations. The hybrid chromosomes obtained from the crosses between the Engaru and the other strains showed incomplete synapses along the whole lengths of all chromosome arms (Oguma 1976).

The results described above strongly suggest that there may be considerable genetic differences between the Engaru and the other *An. sinensis* populations. The purpose of the present study was to investigate the degree of the genetic divergence by crossing experiments.

MATERIALS AND METHODS

The mosquitoes used in this study were collected at Koniya, Kanoya, Karuizawa, Yomogita, Yakumo and Engaru (Figure 1). Although the details of the laboratory techniques have been described in a previous paper (Oguma and Kanda 1976), the rearing methods are briefly described below. Blood-fed females of *An. sinensis* were obtained by using an aspirator during the night or day in pigpens, barns and cowbarns. Two days after collection females were etherized and more than half of each of the wings was cut off by a knife. Then those gravid females were transferred to containers (6 cm in diameter, 3 cm in depth) with wet filter paper. The eggs laid on the filter paper were transferred to a white pan (40 × 25 × 6 cm) with deionized water. Pulverized food for larvae, consisting of wheat germ, dry yeast and mouse food (1 : 1 : 1 in weight) was sprinkled on the water surface. It takes about 10 days before emergence at 25±1°C, 80% R. H. The adult nutriment was furnished by cotton pads saturated with about 3% sugar solution. Human blood was supplied to 3-day-old female mosquitoes and each blood-fed female was artificially inseminated with a 3-day-old male.

In each cross a female was mated with a male using induced copulation. Crosses of the possible combinations were attempted among 6 strains, but some of the crosses could not be done because these strains could not be synchronously established and some of the strains could be maintained only for a few generations. Nineteen of the 36 possible crosses were successful. The Engaru strain could not be used for backcrosses, because of much difficulty in the establishment of this strain even for the first generation.

We attempted to obtain more than 5 egg batches for each cross in this experiment. However, fewer batches were actually obtained in some crosses because some females did not oviposit due to insufficient development of their ovaries, or some females died before oviposition. The number of eggs laid and their hatchability were recorded. The testes preparations were made as follows: each 30 adult hybrid males and females taken approximately 3 days after emergence were dissected in normal saline and their testes removed and transferred to a small drop of saline on a slide, the slides were examined on a phase contrast microscope. The restoration of fertility of the Engaru × Kanoya and the Kanoya × Engaru hybrids was tested.

RESULTS

The results of intra- and interstrain crosses are given in Table 1 and the results of backcrosses in Table 2. As shown in Tables 1 and 2, 1 to 10 egg batches were obtained in each successful cross. The number of eggs per female varied between 100 and 200, therefore females appeared to have deposited eggs normally in all crosses. The mean hatchability varied from 15.5 to 83.4% in intra- and interstrain crosses as shown in Table 1. No difference was observed in the sex ratio. The fertility of the females and the males of F_1 hybrids among the 5 strains,

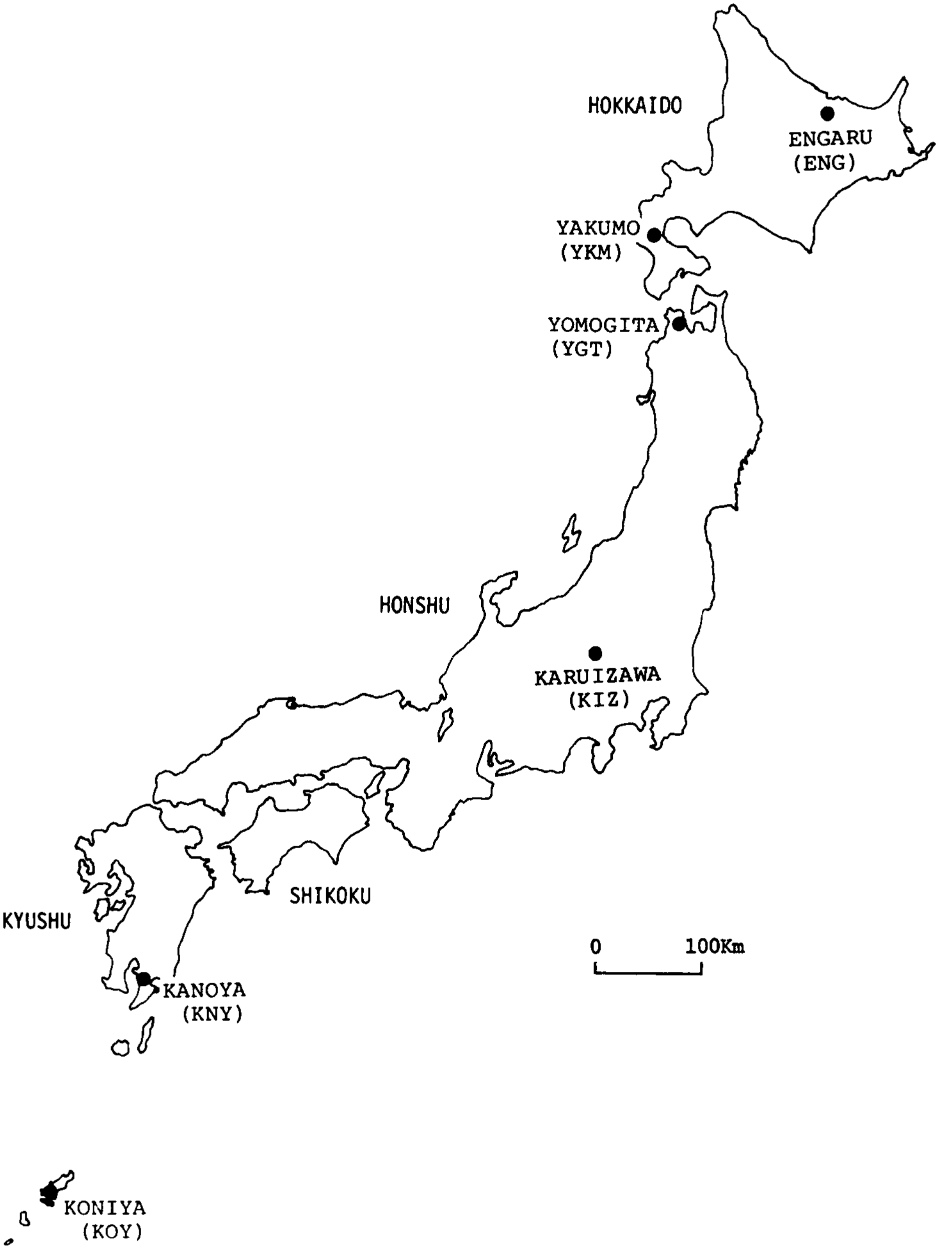

Fig. 1. Collecting sites of anopheline mosquitoes mentioned in the text.

Table 1. Egg laying capacity and hatchability among 6 strains of *Anopheles sinensis*

Female x Male	No. of egg batches	Total no. of eggs	Hatchability (%)
ENG x ENG	10	1609	83.4
YKM x YKM	4	821	60.2
YGT x YGT	2	354	42.1
KIZ x KIZ	3	675	53.9
KNY x KNY	4	940	31.4
KOY x KOY	5	1331	57.3
Intra strain crosses			
ENG x YKM	5	1177	59.0
YKM x ENG	5	1678	61.5
ENG x KNY	5	873	47.9
KNY x ENG	5	1417	42.2
YKM x KNY	6	1512	26.8
KNY x YKM	6	1963	61.4
YGT x YKM	3	380	58.2
KIZ x KNY	2	348	15.5
KNY x KIZ	4	779	67.0
KIZ x KOY	4	1278	57.0
KOY x KIZ	7	2247	76.5
KNY x KOY	7	1634	50.1
KOY x KNY	5	1900	42.3

excluding the Engaru strain, was normal as compared with the controls. The fertility of females of the F_1 hybrids involving the Engaru strain decreased sharply and the hybrid males were sterile (Table 2).

The testes of the F_1 hybrids were examined. The normal testes of the Engaru and Kanoya strain are shown in Fig. 2, 3, 4 and 5. The testes of the F_1 hybrid from the Engaru × Kanoya and Kanoya × Engaru crosses can be seen in Fig. 6 and 7, the size of the reproductive system appeared to be almost normal as compared to those of normal strains. The difference between the two kinds of hybrids in the process of spermatogenesis was observed. Fig. 8 shows the testis of the F_1 hybrid from the Kanoya and Engaru cross, very few spermatids are found. On the other hand, the testes of the F_1 hybrids from the Engaru and Kanoya cross have spermatocytes (round cells) only. The ovaries of the hybrids were apparently normal.

The restoration of fertility of the F_1 females and males from the crosses between the Engaru and Kanoya and between the Kanoya and Engaru strains were tested (Table 3). The results revealed that the fertility of the females was completely restored in BC_2 with a hatchability of about 50% and that of the males as well as evidenced by high fertilization rates.

DISCUSSION

Sexual isolation and hybrid sterility are important events in the study of species formation among closely related species. These aspects of speciation may generally be examined in the laboratory. However, in the case of *An. sinensis* and its related species the details of isolation cannot be analyzed in the laboratory because these strains have to be maintained by induced copulation; only hybrid sterility was analyzed in this experiment. According to Mayr's (1963) classification of isolating mechanisms, hybrid sterility is the "inability of most hybrids to produce the normal number of viable gametes." The results of the fertility test of F_1 hybrids from the crosses Engaru × Kanoya and Engaru × Yakumo are examples of that definition. That is to say, the males of F_1 hybrids are sterile, the females show very low fertility, a basis for strong reproductive isolation. Crossing experiments were made among 6 localities from the Japanese archipelago. There were 19 crosses among 36 possible crosses, and it could be assumed that the strains without the Engaru strain belonged to the same species group. It is of great interest to compare the relationship between *An. sinensis s. l.* and the Engaru type of *An. sinensis* with the results obtained in other closely related species of anopheline mosquitoes. Harrison (1972) reported that the *An. hyrcanus* species group consists of 14 taxa in southeast Asia. Unfortunately, genetic studies of the *An. hyrcanus* species group have now only started, and at this time the phylogenetic position of the Engaru type of *An. sinensis* with reference to the other taxa is unknown.

Morphological differences between *An.*

Table 2. Egg laying capacity and hatchability in various backcrosses and F_1 x F_1 crosses

Female x Male	No. of egg batches	Total no. of eggs	Hatchability (%)
Backcrosses or F_1 x F_1 crosses			
(ENG x YKM)F_1 x YKM	3	131	5.3
YKM x (ENG x YKM)F_1	7	2141	0
(YKM x ENG)F_1 x YKM	5	1137	15.6
YKM x (YKM x ENG)F_1	6	1722	0
(ENG x KNY)F_1 x KNY	5	1017	5.5
KNY x (ENG x KNY)F_1	8	874	0
(KNY x ENG)F_1 x KNY	5	931	2.7
KNY x (KNY x ENG)F_1	6	1679	0
(YKM x KNY)F_1 x (YKM x KNY)F_1	3	650	51.2
(KNY x YKM)F_1 x YKM	5	1241	52.2
YKM x (KNY x YKM)F_1	5	1126	68.7
(YGT x YKM)F_1 x YKM	4	700	37.4
YKM x (YGT) x YKM)F_1	5	1482	44.7
(KIZ x KNY)F_1 x KNY	6	1649	30.6
KIZ x (KIZ x KNY)F_1	1	276	41.3
(KNY x KIZ)F_1 x KNY	6	2359	40.7
KIZ x (KNY x KIZ)F_1	6	1567	53.2
(KIZ x KOY)F_1 x (KIZ x KOY)F_1	5	1836	58.9
(KNY x KOY)F_1 x (KNY x KOY)F_1	8	1534	74.3
(KOY x KNY)F_1 x (KOY x KNY)F_1	7	1762	59.9
(KOY x KIZ)F_1 x (KOY x KIZ)F_1	7	2273	83.2

gambiae A and B, *An. crucians* and *An. bradleyi* were very slight. In the *An. punctulatus* complex, it was impossible to distinguish *An. farauti* Laveran No. 1 from No. 2 by the proboscis which is ordinarily the most useful character for identification of the species of the complex (Bryan 1973a). Morphological differences between *An. sinensis s. l.* and the Engaru type of *An. sinensis* could not be found, although 15% of the Engaru population of *An. sinensis* happened to have the humeral pale spots (H p) in both wings (Kanda and Oguma 1976).

The testes of the hybrid between *An. farauti* No. 1 and No. 2 (Bryan 1973b), *An. crucians* and *An. bradleyi* (Kreutzer and Kitzmiller 1971) were small and underdeveloped, and no normal sperm were found. The testes of some hybrids in the *An. gambiae* complex were normal in size but the testes contents were a mixture of spermatozoa and mature-looking spermatozoa (Davidson et al. 1967). On the

Table 3. The restoration of the fertility from the crosses between the Engaru and Kanoya

Crosses	No. of egg batches	To.al no. of eggs	Hatchability (%)
[(KNY♀ x ENG♂)♀ x KNY♂]♀ x KNY♂	5	996	56.5
KNY♀ x [(KNY♀ x ENG♂)♀ x KNY♂]♂	6	1167	27.3
[(ENG♀ x KNY♂)♀ x KNY♂]♀ x KNY♂	5	1015	58.4
KNY♀ x [(ENG♀ x KNY♂)♀ x KNY♂]♂	6	1275	30.0

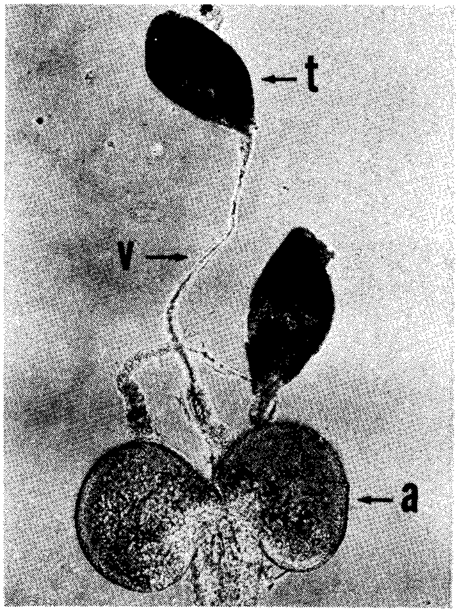

Fig. 2. The normal male reproductive system of the Engaru strain. t = testis; v = vas deferens; a = accessory gland.

Fig. 3. Normal sperm from the ruptured vas deferens of the Engaru strain. s = sperm.

other hand, the testes of the hybrid between the Engaru and Kanoya strain were almost normal in size, no sperm could be found. There seemed to be no correlation between the size of testes of the hybrids and the degree of spermatogenesis.

The female hybrids between *An. gambiae* A and B showed fertility as high as in the controls, but the male hybrids were sterile (Davidson et al. 1967). The fertility of female hybrids between *An. crucians* and *An. bradleyi* was 41.8% (the control was 60.9%, calculated from the data by Kreutzer and Kitzmiller 1971), but the male hybrids were sterile. The hybrid adults were almost all sterile in the crosses between *An. farauti* No. 1 and No. 2 (Bryan 1973b). The hatchability in the F_1 hybrids between the Engaru strain and *An. sinensis s. 1.* strain was normal, and the larvae developed to adults. The hybrid males were completely sterile, while the hybrid females showed considerably decreased fertility. It was only 7.3% (the control was 54.7%).

In the salivary gland chromosome of the *An gambiae* A × B hybrids, 2R had asynaptic regions but the other autosomal arms were synaptic. The X-chromosomes were completely asynaptic in one case, but partially synaptic in the other cases (Davidson 1964, Davidson et al. 1967). There was considerable lack of synapsis, but there were large synaptic areas in each of the autosomal arms in the *An. crucians* × *An. bradleyi* hybrids (Kreutzer and Kitzmiller 1967). All F_1 hybrids of the *An. farauti* No. 1 × No. 2 had asynaptic areas on all autosomal arms, although the X-chromosome contained partially synaptic regions in some cases (Bryan and Coluzzi 1971). The salivary gland chromosomes of the hybrids from *A. sinensis s. 1.* × Engaru strain showed a high degree of asynapsis along the whole length. From the data of the salivary gland chromosomes and the fertility of the hybrids, it can be assumed that the depression in fertility of the females seems to be correlated with the degree of asynapsis of the

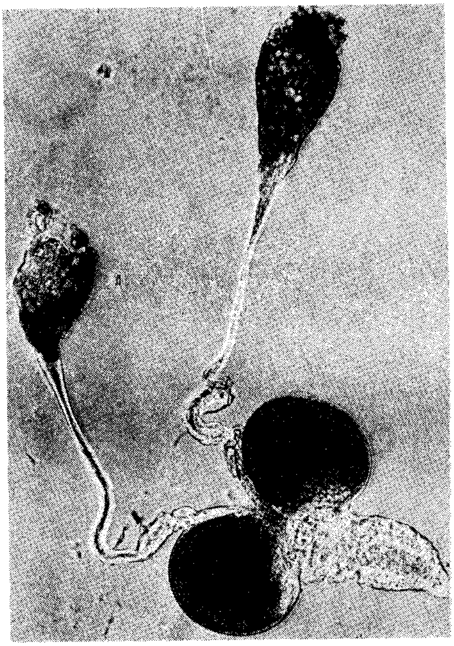

Fig. 4. The normal male reproductive system of the Kanoya strain.

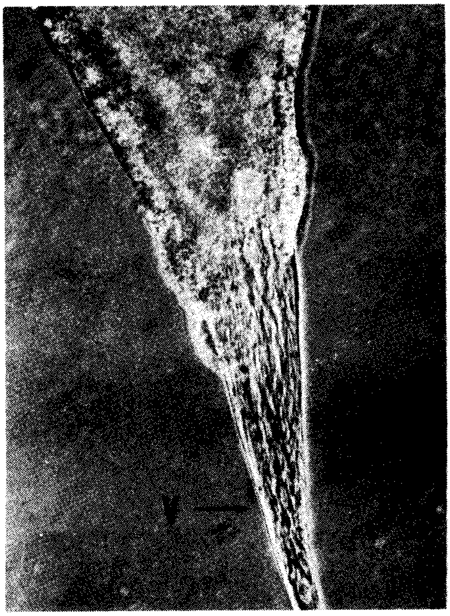

Fig. 5. High-power view of the normal testis of the Kanoya strain and beginning of the vas deferens showing the mature spermatozoa.

salivary gland chromosomes in the hybrids. Namely, the synapsis is almost complete except for the X-chromosomes in the *An. gambiae* A-B hybrids, less complete in the *An. crucians-An. bradleyi* hybrids, a high degree of asynapsis in Engaru-*An. sinensis s. l.* hybrids and almost asynaptic in *An. farauti* No. 1-No. 2 hybrids.

Briefly comparing the above 3 cases with each other, the results of hybridization experiments and salivary gland chromosomal analysis indicate that the relationship between the *An. sinensis s. l.* and the Engaru strain of *An. sinensis* is not as close as compared to *An. gambiae* A and B, and *An. crucians* and *An. bladleyi* but is closer than *An. farauti* No. 1 and No. 2.

Although more studies of the systematic relationships among the members of the *An. hyrcanus* species group are needed, the results of these investigations suggest that the Engaru population of *An. sinensis* may be considered genetically to represent a different species from *An. sinensis s. l.*

The distribution of the Engaru type of *An. sinensis* (Oguma and Kanda 1977) appears to be similar to a relationship in *Drosophila* which has been reported by Moriwaki et al. (1967); *D. bifasciata* Pomini inhabits extensive areas in Europe and Asia including Japan, while *D. imaii* Moriwaki and Okada which is a sibling species of *D. bifasciata,* can be collected only in Hokkaido. Only minor morphological differences can be observed between these 2 species of *Drosophila* and strong sexual isolation is operating between them; the F_1 females are fertile but the males are completely sterile. The salivary gland chromosomes of hybrids of the two species show incomplete synapsis or are partially synaptic. Yamaguchi (1973) suggested that *D. imaii* was closer to *D. bifasciata* of Italy and Switzerland than that of Japan, as suggested from the kind of inversion systems. Similarly it

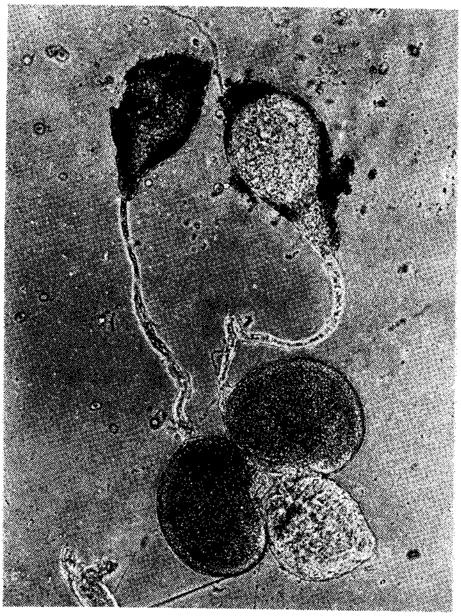

Fig. 6. The male reproductive system of the hybrid from the cross between the Engaru strain female and the Kanoya strain male.

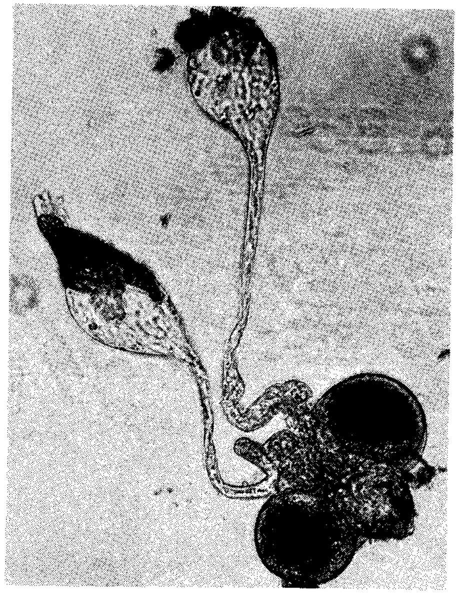

Fig. 7. The male reproductive system of the hybrid from the cross between the Kanoya strain female and the Engaru strain male.

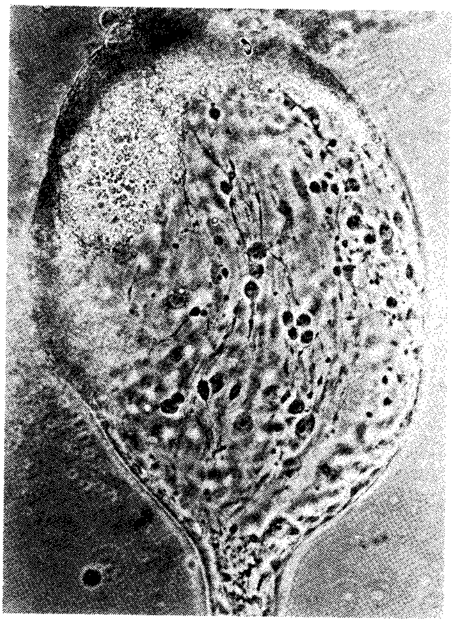

Fig. 8. High-power view of the testes of the hybrid from the cross between the Kanoya strain female and the Engaru strain male. Contents showing very few spermatids only.

seems that the Engaru type of *An. sinensis* might have invaded Hokkaido from the Asiatic continent rather than diverging sympatrically from *An. sinensis s. l.* Xu and Feng (1975) revised the members of the *An. hyrcanus* species group in China and reported 9 species including 1 new subspecies. If the Engaru type of *An. sinensis* exists in China as well as in Japan, it will be revealed by crossing experiments. It will be very interesting to know how *An. sinensis s. l.* and the Engaru type of *An. sinensis*, or other sibling species, are distributed on the Asiatic continent.

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INCREASED PREVALENCE OF *CULEX TARSALIS* IN OHIO AND ITS IMPLICATIONS [1]

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ABSTRACT: Fifteen new county distribution reports for *Cx. tarsalis* were recorded for Ohio in 1977. *Cx. tarsalis* has now been reported from 28 of Ohio's 88 counties. This species was collected in significantly higher numbers in 1977 than in the previous 7 years. Serologic evidence of hemagglutination inhibiting antibodies to WE in juvenile pigeons for 3 consecutive years and the increased prevalence of *Cx. tarsalis* indicate this species should be closely monitored for possible involvement with western encephalitis in Ohio.

INTRODUCTION

Culex tarsalis Coquillett was regarded as a rare mosquito in Ohio by Venard and Mead (1953). Parsons et al. (1972) reported this species as occurring in 10 Ohio counties based on extensive light trap collecting during the years 1965 to 1971. In 1977 an unusual increase in the abundance of *Cx. tarsalis* was noted and resulted in the establishment of 15 new county records, increasing the known distribution of this species to 28 of 88 Ohio counties. Detection of this increased population density and new distribution records is directly attributed to an ongoing statewide St. Louis encephalitis (SLE) surveillance program, directed by the Ohio Department of Health.

The surveillance program was instituted in 1976 as a result of an SLE epidemic which swept through the midsection of the United States in 1975, leaving Ohio with 416 known human cases, including 29 fatalities. A network of surveillance sites in 24 counties was established to monitor SLE virus activity in mosquitoes and birds. The goal of this surveillance program was to provide an early warning of significant SLE activity so that appropriate and effective mosquito control efforts could be mobilized.

METHODS

The Vector-borne Disease Unit (Ohio Department of Health, Communicable Disease Division) and cooperating local health departments made mosquito and bird blood sample collections on a biweekly basis at each surveillance site. Mosquito collection techniques described by Sudia and Chamberlain (1962, 1967) were adopted for the program. These techniques included use of CDC Miniature light traps supplemented with dry ice (Newhouse et al. 1966), shelter collections from daytime resting sites, evening collections in bird coops, and human biting collections. All collections were processed according to methods described by Sudia and Chamberlain (1967) and Sudia et al. (1970). Surveillance sites selected in 1976 were used again in 1977 with only minor changes.

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