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Experimental Crossing between *Bursaphelenchus lignicolus* and *Bursaphelenchus mucronatus*

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Introduction

So far, two kinds of *Bursaphelenchus* have been reported[6,7,8] in Japan, related to an epidemic wilting of pine trees.

The pine wood nematode, *Bursaphelenchus lignicolus* Mamiya et Kiyohara (marked as B.l. in the following) is severely pathogenic to domestic pines. On the other hand, *B. mucronatus* Mamiya et Enda (B.m. in the following), so-called the false pine wood nematode in Japanese nomenclature is non- or weak- pathogenic. At the early stage in the survey of these nematodes, B.l. was assumed to have been found only in the coastal regions of Central and Western Japan, but B.m. was strictly limited to Northern Japan. However, it has gradually become clear that B.m. is also distributed in Central and Western Japan, although scantly on unhealthy trees. In an extreme instance[10], the nematode was noted to be coexisting with B.l. in a single tree. Otherwise, B.l. spread gradually into the northern districts and inland areas of the infested districts. For example, B.l. jumped into a district of Northern Japan in 1975 through the work of the vector, the Japanese pine sawyer, *Monochamus alternatus* Hope[14]. Under these circumstances, it is quite possible that B.l. and B.m. come into contact and cross naturally. However, little has been known on the results of the crossing, and its effect on the epidemiology caused by B.l. Therefore, I tried to cross these nematodes experimentally and evaluate the meaning of the results.

Materials and Methods

Nematode populations

Artificially cultured populations of the nematodes, isolated at various seasons from various districts in Japan were used in the experiments. Both of the species are capable of being cultured with fungal mycelia on agar media. A strain of *Botrytis cinerea* Pers. which is not pathogenic to any plant host was used as the bait fungus for the test populations. The respective test population is defined below:

B.l. No. 1: Isolated by me from an adult pine sayer which emerged in June, 1973, from a log of Japanese black pine (*Pinus thunbergii* Parlatore), collected at Uranouchi, Susaki City, Kochi Prefecture, in Oct., 1972.

B.l. No. 2: Isolated by me, in June, 1975, from a log of Japanese red pine (*P. densiflora* Siebold et Zuccarini), collected at Kubokawa Town, Kochi Pref.

B.l. No. 3: Isolated by me, in Sept., 1975, from a log of Japanese black pine, collected at Namikata Town, Ehime Pref.

B.l. No. 4: Isolated by me in Sept., 1976, from a log of Japanese black pine, collected at Misaki Town, Ehime Pref.

B.l. No. 5: Isolated by me, in Dec., 1976, from a log of Japanese black pine, collected at Hariki, Kochi City, Kochi Pref.

B.l. No. 6: Isolated by me, in Jul., 1977, from an adult pine sawyer, delivered to me by Mr. Takeuchi of Kochi Prefectural Forest Exp. Sta., Tosayamada Town, Kochi Pref., immediately after the emergence of the beetle at the station.

B.m. No. 1: Isolated by me, in Oct., 1971, from a log of Japanese five-needled pine (*P. parviflora* S. et Z.), collected at Saijo City, Ehime Pref.

B.m. No. 2: Delivered to me as a tube culture, 1975, by Mr. Kiyohara, of Kyushu Branch, Forest and Forest Products Research Institute (marked as FFPRI), who isolated it from a log of Japanese red pine, collected at Kusu Town, Ooita Pref., in Sept., 1971.

B.m. No. 3: Delivered to me as a tube culture in 1975, by Mr. Mineo, of Kansai Br., FFPRI, who isolated it from an adult pine sawyer, which emerged from a log of Japanese black pine which had been collected in June, 1974, at Kaike, Yonago City, Tottori Pref.

B.m. No. 4: Delivered to me in 1976, as a tube culture, by Dr. Mamiya, of Division of Forest Protection, FFPRI, who isolated it from a log of Japanese black pine, collected at Yachiyo City, Chiba Pref., in April, 1971.

B.m. No. 5: Delivered to me as a tube culture in 1976, by Mr. Shoji, of Tohoku Br., FFPRI, who isolated it from a log of Japanese red pine, collected at Rifu Town, Miyagi Pref., in Oct., 1976.

Localities where the test populations were collected are shown in Fig. 1.

Crossing methods

The bait fungus was cultured on potato-dextrose-agar (PDA), at 25°C for a week in a Petri dish. The agar medium on which the fungus grew was cut into small cubes, about 2×2 mm. To every fungus cube in a sterilized glass dish, settled in a sterilized Petri dish was added with a few drops of sterilized water. After cultured at 25°C for a week in a test tube, every test population was set going to Baermann's funnel and prepared as a suspension in distilled water. A larva of the 3rd stage or the younger of the nematodes was picked at random from the suspension and released into water around the fungus cube.

The Petri dish holding the larva, was kept at 25°C in relatively saturated humidity, for a week in dark incubator. After incubation, test individuals were classified into male, female and immature. The latter individuals were all discarded for the crossing. Five vigorous virgin males of a population of B.l. and five similar females of B.m. were released and incubated at 25°C, on a fresh mat of the bait fungus cultured on PDA in Petri dish. Reciprocal crossing (i.e. the mixture of five virgin males of B.m. and five females of B.l.) was also prepared. After a week, all nematodes, flowing down from every crossing dish, were counted, examined and classified into two sexes and larva. Recovery and inspection of nematodes were carried out every day, for a week beginning on the next day after the commencement of the inspection. A similar survey was carried out on the dish kept for 2 weeks after cross starting. For intra-species crossing, 5 virgin males and females in a species, prepared by a method similar to the above mentioned were mixed. For back-crossing, females recognized as true hybrids of the 2 species, were released in a Petri dish and mixed with virgin males of B.l. or B.m. As no decisive male hybrid was obtained, back-crossing between a hybrid male and each of the parent females was not carried out.

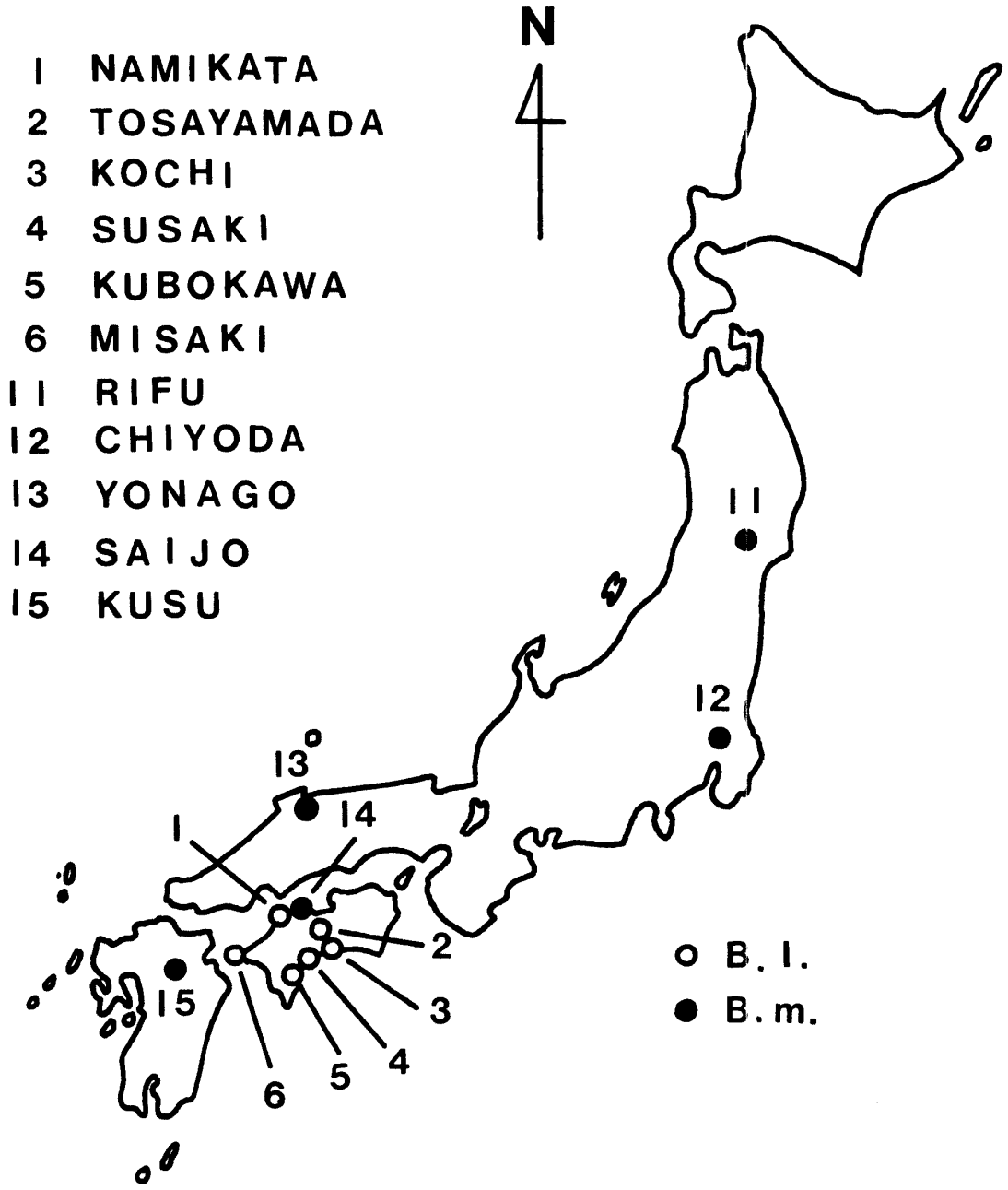

Fig. 1. Distribution of the localities from where test nematode populations were isolated. (B.l.: *Bursaphelenchus-lignicolus* B.m.: *B. mucronatus*)

Results

Intra-species crossing

Results of intra-population crossing of B.l. and B.m. as intra-species crossing are shown in Fig. 2. By 5 male×5 female, test populations of B.l. were multiplied into about 1,500–23,000 in a week, and about 25,000–582,000 in two weeks, on average. In B.m., similar crossing was multiplied into about 500–1,900, and about 2,600–36,000 in one and two weeks, respectively. The multiplication rate was different for different populations, and B.l. was multiplied faster than B.m. However, the results of intra-population crossing in B.m. are more noteworthy, when compared with those of inter-species crossing between B.l. and B.m., as shown later.

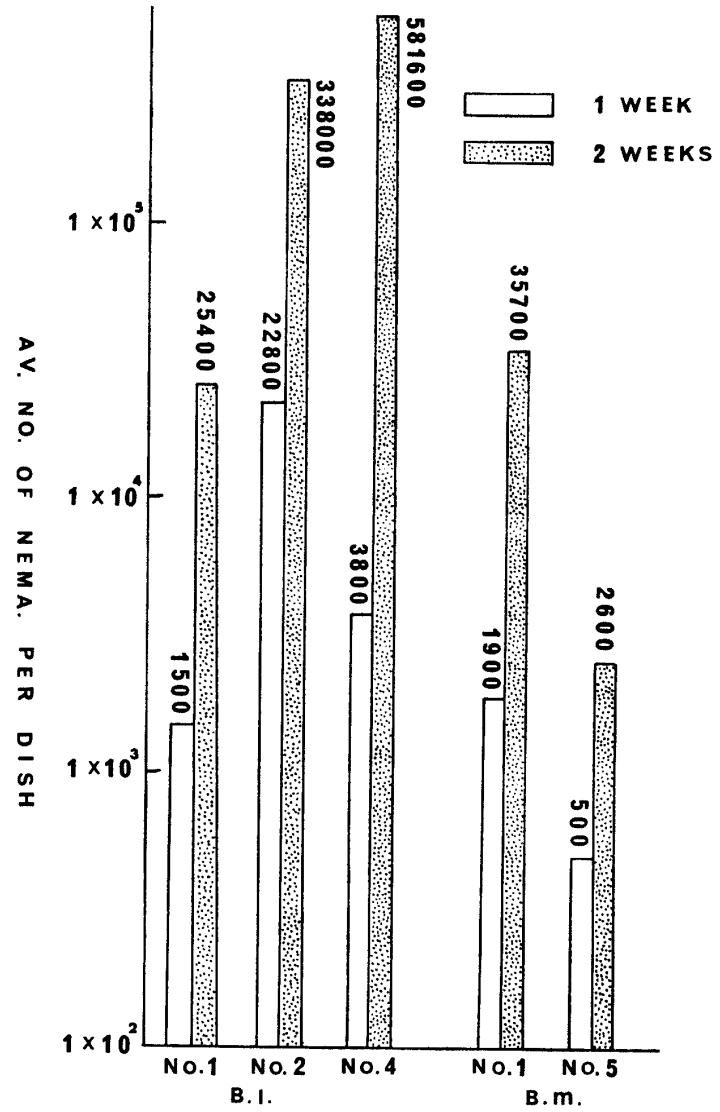

Fig. 2. Numbers of *Bursaphelenchus lignicolus* (B.l.) and *B. mucronatus* (B.m.) inspected one week and 2 weeks after cross starting (Nos. 1–5 show the population number of the test species).

Inter-species crossing

Results of B.l.×B.m. are shown in Figs. 3–4. In Fig. 3, average numbers of larva, male and female by B.l. male×B.m. female and the reciprocal crossing examined at one week and two weeks after the starting of the cross are shown. In Fig. 4, the numbers of female classified by the form of tail end are shown. The numbers of male and female in the Figs., include the numbers of the parents, used in the crossing, respectively. Theoretically, larva is to be classified into male or female. However, the number of larva was specially listed, because the existence of larva provides a useful proof of successful hybridization by itself. It was postulated also that almost all the individuals active at the inspection dates were re-collected by the isolation method. When one or both of the following results were obtained, hybridization between the two species was recognized to be successful.

1) Larva was found.

2) Total number of male or that of female exceeded 5 in a test dish, respectively.

Significance of difference between the results was examined by *t*-test (5% level). The main

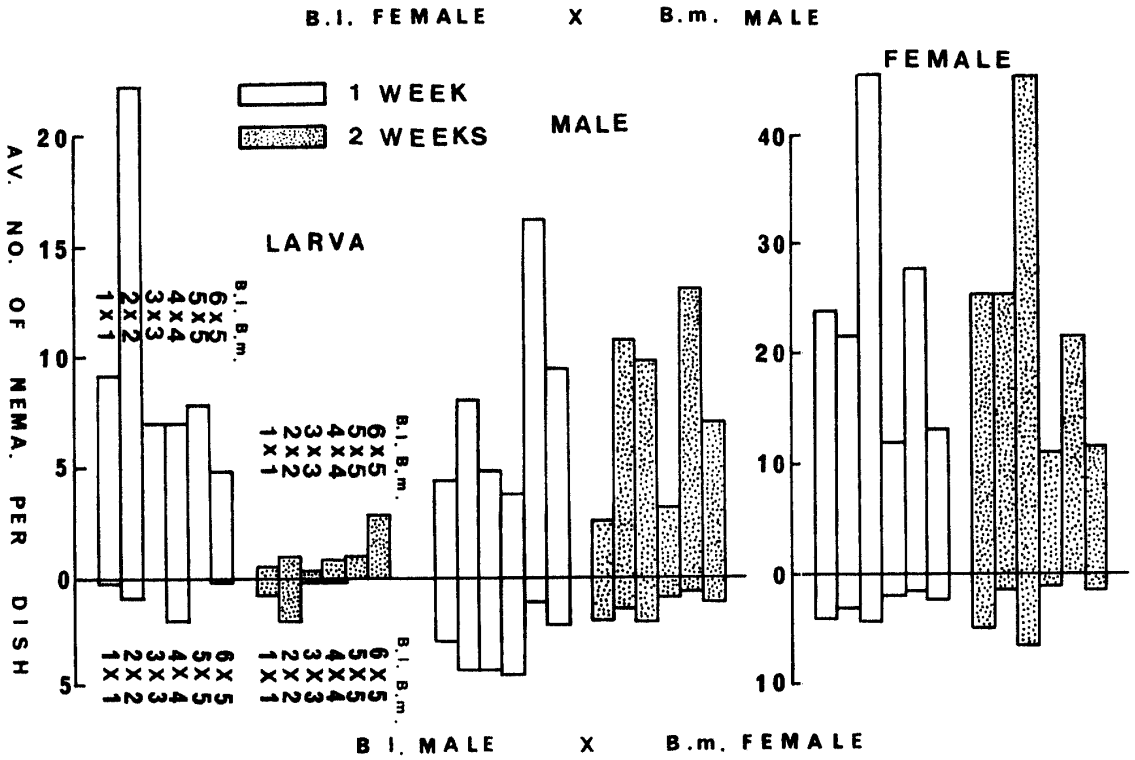

Fig. 3. Crossing results between *Bursaphelenchus lignicolus* (B.l.) and *B. mucronatus* (B.m.) one and 2 weeks after cross starting (Nos. 1–6 show the population numbers of the test species).

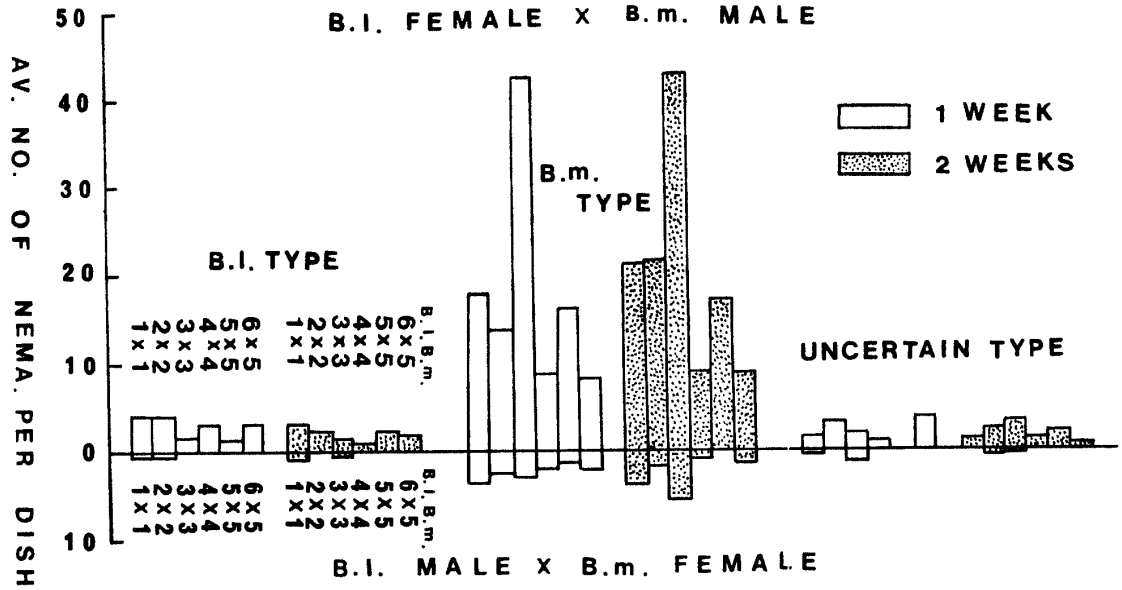

Fig. 4. Number of females, classified by the form of tail end, in B.l. Female × B.m. male and the reciprocal crossing (Nos. 1–6 show the population number of the test species).

results shown in Figs. 3–4 and other ones which were not presented here are as follows:

1) Successful hybridization was observed in 18 dishes among 54 ones, crossing B.l. male and B.m. female (33.3%). On the other hand, hybridization was recognized in 60 dishes among the

total of 65 dishes under the reciprocal crossing (92.3%).

2) The number of total nematodes by B.l. female×B.m. male, detected at every inspection date was significantly larger than that by the reciprocal crossing.

3) Time between the first and the second inspection dates made no statistical difference in the total nematodes, total males and total females in either crossing combination.

4) The number of larva detected at one week after the starting of cross in B.l. female×B.m. male was larger than that detected at the next week. The numbers of larva detected at the 2 inspection dates in the reciprocal crossing were not different from each other.

5) The numbers of male, detected at the both inspection dates in B.l. female×B.m. male were larger than those detected at the 2 dates in the reciprocal crossing, respectively.

6) The numbers of female in B.l. female×B.m. male detected at the both dates were larger than those of the reciprocal crossing for the same dates, respectively.

7) The numbers of female detected at the 2 dates in B.l. female×B.m. male were larger than those of males, detected at the 2 dates, in the 2 cross combinations, respectively.

8) The number of female with B.l. type tail end in B.l. female×B.m. male, detected at each inspection date was larger than those of similar female in the reciprocal crossing, detected at the 2 dates.

9) The numbers of female with B.m. type tail end in B.l. male×B.m. female, detected at the 2 dates were larger than those of female with B.l. type tail end in similar cross combination, detected at the 2 dates, respectively.

10) The numbers of female with B.m. type tail end in B.l. female×B.m. male, detected at the 2 dates were larger than those of female with B.l. type tail end in the same cross combination, detected at the 2 dates, respectively.

11) The numbers of female with B.m. type tail end in B.l. female×B.m. male, detected at each inspection date was larger than those of female with B.m. type tail end as well as than those with B.l. type tail end in the reciprocal crossing, detected at the 2 dates.

12) It is suggested that the form of B.m. type tail end in F_1 female is a genetically dominant phenotype in B.l. female×B.m. male, because the females occupied most of the total females detected at the both inspection dates in the crossing. The females with B.m. typed tail end reached 64.8–93.4 (av. 75.2)% of total females (including parent females) and 77.6–90.3 (av. 82.9)%, respectively in the one or two weeks after the starting of cross. It was also observed that the number of females with B.l. typed end never exceeded 5, per a dish in 63 dishes among the total of 65 in B.l. female×B.m. male.

Back-crossing

Back-crossing was carried out only between hybrid female, obtained by B.l. female×B.m. male and the male of either B.l. or B.m., because no sure hybrid males were obtained and the hybrid females obtained by B.l. male×B.m. female were not abundant. Main results of the back-crossing were as follows:

1) Multiplication of active individuals was not observed in any of the total 15 test dishes.

2) Two larvae were found in a test dish, although as dead.

3) Copulation between a hybrid female and a male of B.l. was observed (Photo Z in Plate 2).

In addition to these results, conception of hybrid females holding B.m. typed tail end, were occasionally observed in B.l. female×B.m. male (Table 1).

Judging from the above mentioned results, it was concluded that the possibility of back-crossing

Table 1. Rate of pregnant hybrid females in total hybrid females, counted at 2 weeks after the cross starting in B.l. female × B.m. male

Crossing combination	Total of* hybrid female	Total of pregnant hybrid female	Rate of pregnant hybrid female in total hybrid female (%)
B.l. No. 4 Female × B.m. No. 4 Male	71–82	1	1.2–1.4
B.l. No. 5 Female × B.m. No. 5 Male	138–156	2	1.3–1.4
B.l. No. 6 Female × B.m. No. 5 Male	45–49	2	4.1–4.4

* Total of females except the parent females, or sum total of females exceeded 5 in a test crossing dish

between the hybrid female obtained by B.l. female × B.m. male and either male of B.l. or B.m. was very rare, though not nil.

Deformation of nematode body

In the course of microscopic observation of the individuals obtained by the crossings, deformed individuals were occasionally found. The followings were the types of deformation:

1) Partial swelling of the skin
2) Crooking of the tail
3) Absence or underdevelopment of sex organ (chiefly in male)
4) Disease caused by fungi or bacteria

Item 1 and 2 might be related to Item 4. The rates of deformation in the larva, male and female, are shown in Table 2. From these results, it was concluded that the larva, male and female were all subject to deformation, with no detection of difference in the deformation rate.

Form of hybrid

Larva

No difference was observed between the larval form, produced by B.l. female × B.m. male and those by the reciprocal crossing. In the growing process of larva which turned into the female, the tail end form was first dull conical until reaching about 500 μm in total length, then transforming itself into with a distinct mucrone (Photos B–D and F–H in Plate 1).

Male

Hybrid males produced by the 2 crossings were not morphologically different from each other. They were not to be distinguished from each parent male too.

Female

Hybrid females produced by B.l. female × B.m. male were not morphologically separated from adult female of B.m. Occasionally, the female with a mucrone which was noted at the tail end and shorter than that of a typical B.m. female but longer than that of B.l. female (intermediate), and those with a type obviously different from the female of the respective species (abnormal type) were observed. Examples of the abnormal type are shown in Photos I–L of Plate 1.

Hybrid females obtained by B.l. male × B.m. female were not morphologically different from those obtained by the reciprocal crossing.

Table 2. Rate of deformed individuals in total nematodes, obtained by B.l. female × B.m. male

Insp. date after cross start. (week)	Crossing combination	Total number of nema. obtained	Rate of deformed nematodes (%)*		
			Larve	Male	Female
1	B.l. No. 2 female × B.m. No. 2 male	155	13.6	4.2	0
	B.l. No. 3 female × B.m. No. 3 male	171	4.8	14.2	2.9
	B.l. No. 4 female × B.m. No. 4 male	135	4.8	4.5	1.4
	B.l. No. 5 female × B.m. No. 5 male	413	3.2	1.6	1.4
	B.l. No. 6 female × B.m. No. 5 male	136	8.3	4.3	4.6
2	B.l. No. 2 female × B.m. No. 2 male	112	33.3	15.6	9.1
	B.l. No. 3 female × B.m. No. 3 male	221	0	7.7	1.1
	B.l. No. 4 female × B.m. No. 4 male	119	0	4.0	5.7
	B.l. No. 5 female × B.m. No. 5 male	285	0	2.9	2.3
	B.l. No. 6 female × B.m. No. 5 male	109	7.1	0	6.9
		Average	7.3	4.2	2.9

* Deformed larvae, males and females were divided by total detected larvae, males and females, respectively

Discussion

Total number of nematode, detected at the two inspection dates even in the crossings where hybridization occurred, was very small and was not different from each other (Figs. 3–4). This result indicates that successive multiplication did not occur vigorously after the first hybridization between the parental species. Results of the back-crossing also support this indication. As mentioned in Introduction, the contact of B.l. with B.m. in nature is possible. However, it has been assumed that the contact and following hybridization may not cause any serious effect upon the wilting epidemiology caused by B.l., because the result of hybridization is not fecund and the effect of heterosis or the appearance of any pathologically stronger hybrid than others, for examples, may scarcely be expected. Kishi[4] reported that the detection of B.m. approached nil after the invasion of B.l., as if the former were cleared by the latter in the areas where B.m. had been frequently observed before the occurrence of the epidemic. This observation indicates that B.m. gives little effect upon the activity of B.l. Deformed nematodes were occasionally found in larva and hybrid female

(Table 2), and some of them were similar to those reported by Sturhan[16] and Erikkson[2]. It looks also that the deformation ratios shown in Table 2 are quite larger than those of ordinary populations of the test 2 species. And this is the reason why the deformation is assumed to be related with the hybridization.

Concerning the hybridization between different species or races in nematodes, the following works have been published in past decades, so far as I could range over literatures. Nigon and Dougherty[11] attempted to hybridize *Rhbditis briggsae* and *R. elegans,* both of which were hermaphiditic and similar in morphology. However, these attempts uniformly failed and the workers concluded that the failure offered the final proof to the independence of the two species. Mulvey[9] mentioned that the female of *Heterodera trifolii,* a parthenogenetic nematode may be impregnated by the male of *H. schachitii,* a bisexual nematode, but no males were found to be among many offsprings of several impregnated females of *H. trifolii.* Smart and Darling[15] reported that two potato races of *Ditylenchus destructor* were not to be interbred with a mushroom race of the species, which had been known as *D. myceliophagus* and is not different morphologically from the potato races. Potter and Fox[13] mentioned successful hybridization between *Heterodera schachtii* and *H. glycines.* Ladygina[5] failed to obtain a hybrid between *Ditylenchus destructor* and *D. dipsaci,* according to the report of Erikkson[2]. The following results of interbreeding between the races of *D. dipsaci* by some workers are noteworthy in relation with my own results. Sturhan[16] published a report on successful interbreeding between several races and strains of the species. Erikkson[2] reported that the red clover and lucerne races of the species are capable of interbreeding and producing a fertile progeny. Webster[18] mentioned that several races of the species hybridized successfully while other races did not. He also reported that the hybrids were multiplied, on average, less than the parental races but their host ranges were not related to those of the parental races. Erikkson[3] again attempted to hybridize 10 races of *D. dipsachi* in 44 different combinations and clarified that fertile hybrid populations were obtained in most of the combinations and he indicated that at least some of the hybrid populations combine the host range characteristics of the parental races. However, he also mentioned that there was a consistent failure in some cases where one combination was successful but the reciprocal one with the same 2 races failed. Similar results had been obtained in the experiments of Webster[18].

Turning to the crossing of other nematodes, again, Parrott[12] reported that the populations of *Heterodera rostochiensis* is capable of being divided into two partially incompatible crossing groups represented by pathotype A and E, and she suggested that *H. rostochiensis* should be regarded as two species. She also mentioned that A type males were comparatively successful in crossing with E type females. Webley[17] showed experimentally that the larval length, the spear length and the distance between the crescentic valves of the median bulb and the excretary pore are significantly shorter in the 2nd stage larvae of A pathotype than in B and E types of *H. rostochiensis.* According to Akhurst[1], interspecific crosses among 7 species in *Deladenus,* almost always produced hybrids and often in no less number than intra-specific crosses. However, *D. wilsoni* from hymenopterous parasitoids did not produce a larger number of hybrids with any other species. Although only 15% of the eggs deposited by the F_1 hybrids of *D. rudyi* (UJ7) male × *D. imperialis* female produced larvae, no difficulty was experienced in maintaining a culture of these hybrids and monitoring the fecundity of succeeding generations. The results of interbreeding in some nematodes are so complicated as above mentioned.

If B.l. and B.m. did not cross at all, in my experiments, the 2 species should be separated as distinctly different species, because the both females keep morphologically slight difference in the

form of tail end, from each other. If the F_1 and its offsprings were multiplied vigorously, the 2 species could be regarded as a single species, because the difference in the form of tail end is negligible and has not been confirmed as a criterion to the classification in *Bursaphelenchus*. My cross experiments showed an intermediate result between two postulations above mentioned. The poor results of B.l. male × B.m. female and the occurrence of aberrant individuals in the offsprings of B.l. female × B.m. male make me consider some crossing results by the workers above mentioned. Therefore, from my experimental results, I got to the conclusion that some room for suspicion is still left on the separation of B.l. and B.m. as different species.

The poor results of B.l. male × B.m. female, the abundance of female in the F_1 between B.l. female and B.m. male and the appearance of deformation in the hybrid may also the problems which should be researched.

Summary

Since *Bursaphelenchus lignicolus* (marked as B.l.), the pine wood nematode, and *B. mucronatus* (marked as B.m.), nonpathogenic wood nematode, are both easily cultured on agar medium with a fungal bait, they were crossed experimentally. The both species hybridized with each other producing F_1. More F_1 were produced in B.l. female × B.m. male than in the reciprocal crossing. However, the number of F_1, even in the successful crossing, was very limited and the total number, inspected the two weeks after the starting of the cross was not different from that a week after the starting. Any example of back-crossing between each parent male and the hybrid female produced by B.l. female × B.m. male was scarcely successful. However, conception and oviposition by the last mentioned female were occasionally observed. In F_1, obtained by B.l. female × B.m. male, the number of female was larger than that of male, on average, and the form of tail end of the hybrid female was usually similar to that of B.m. female.

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Explanation of photos

(Scale No mark: 10 μm, 1: 100 μm, 2: 50 μm, 3: 25 μm)

Plate 1

A–D : Larvae produced by B.l. female × B.m. male
- A : Active state
- B–D: Development of mucrone (from B to D)

E–H : Larvae, produced by B.l. male × B.m. female
- E : Active state
- F–H: Development of mucrone (from F to H)

I–L : Abnormal tail end form of F_1 female, produced by B.l. female × B.m. male
- I–J: Tail end with eccentrically developed mucrone
- K : Tail end with thicker mucrone than that of ordinary B.m. females
- L : Tail end with split (or forked) mucrone

Plate 2

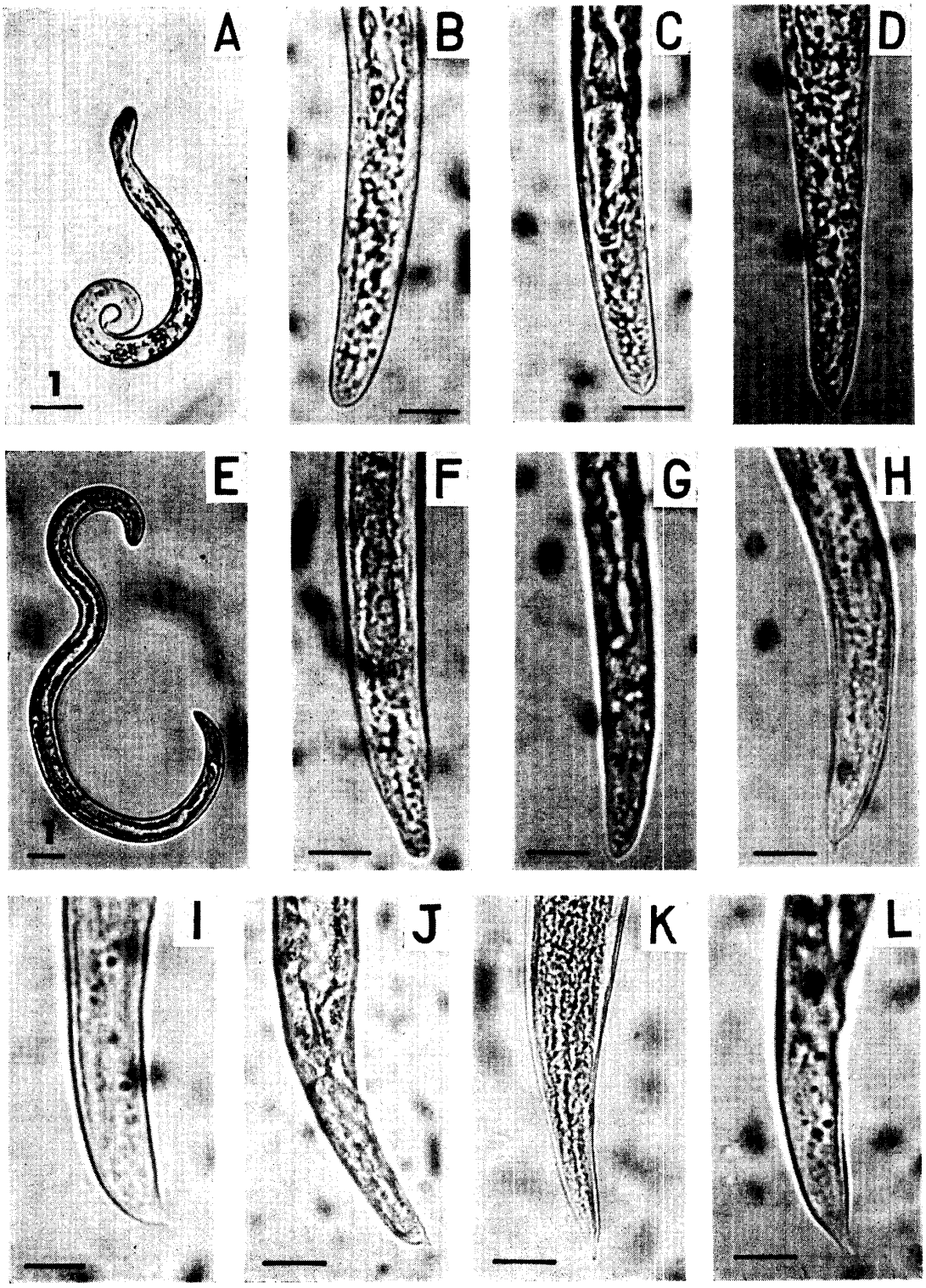
M–P : Aberrance of larva, produced by B.l. female × B.m. male
- M: Crooking of tail
- N : Swelling of skin at various parts of whole body
- O : Hollowing of tail
- P : Split of tail end

Q–S : Tail end aberrance of male, detected in the offsprings of B.l. female × B.m. male
- Q : Absence of spicule
- R : Underdevelopment of spicule
- S : Abnormal form of tail end

T–V : Aberrance of tail end of F_1 female, produced by B.l. female × B.m. male
- T : Hollowing of tail
- U–V: Crooking of tail

W–Y: Nematode attacked by a fungus, detected in the offsprings of B.l. female × B.m. male and the parasite
- W: Attacked nematode (male)
- X : Fungal body in the nematode
- Y : Spores of the fungus

Z : Copulation between B.l. male and F_1 female, produced by B.l. female × B.m. male


A
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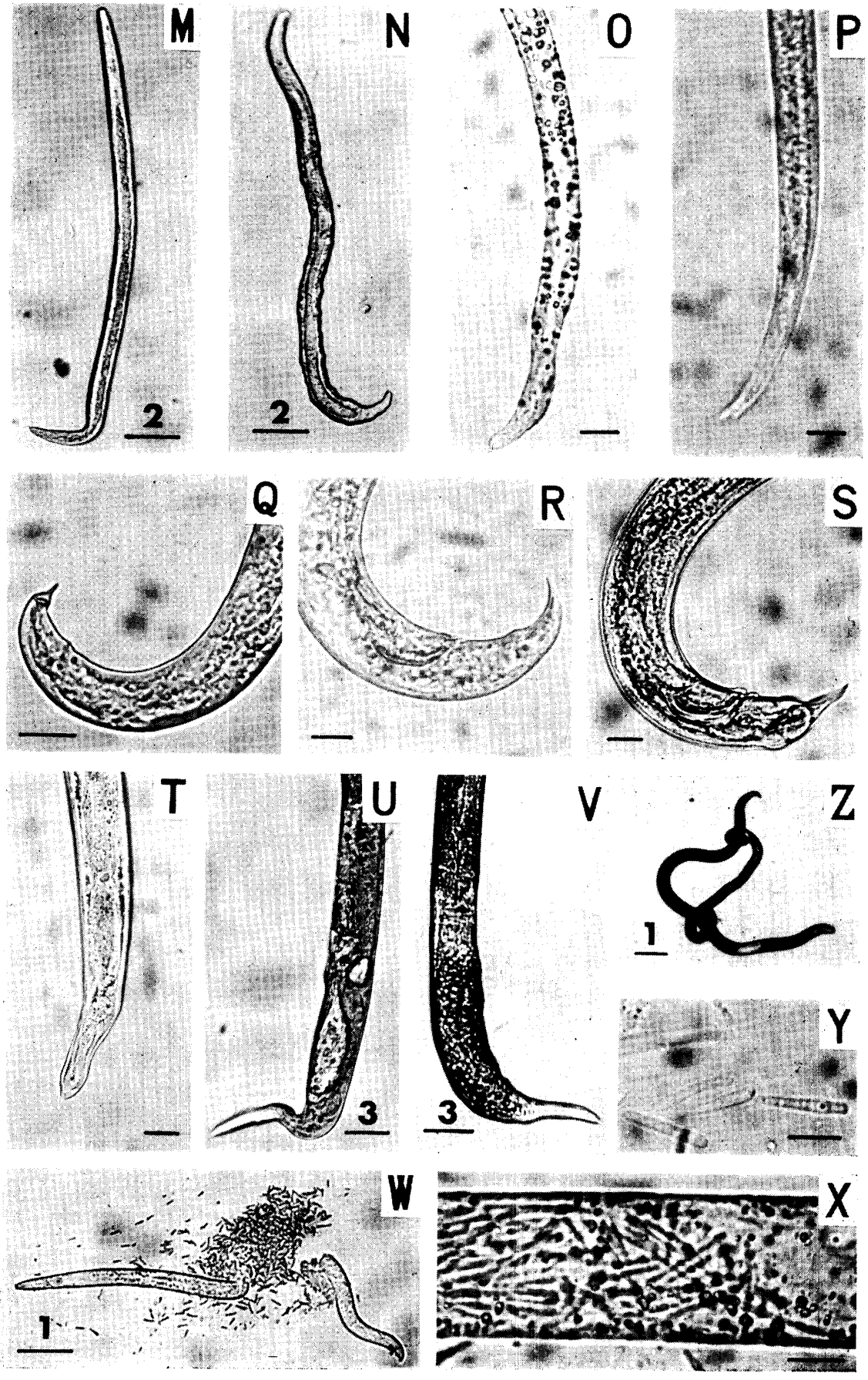
M
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