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Studies on Morphological and Physio-Ecological Variations of the Reniform Nematode, *Rotylenchulus reniformis* Linford and Oliveira, 1940 with an Emphasis on Differential Geographical Distribution of Amphimictic and Parthenogenetic Populations in Japan

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Abstract: The geographical distribution and polymorphism in morphological and biological characters of the reniform nematode, *Rotylenchulus reniformis*, in Japan were studied. The northern limit of habitat of this nematode was found on the 14 °C isothermal line of annual average-air temperature. Three morphologically different groups were ascertained which corresponded to three biological types based on male frequency. Incidence of males was consistent within populations and was not affected by environmental factors. Sexual attraction of males by females indicated reproductive isolation between the male-numerous type (MNT) and male-rare type (MRT) or male-absent type (MAT). Reproduction was amphimictic in the MNT and parthenogenetic in the MRT and MAT. Larval development in both MRT and MAT, but not that of MNT populations, was inhibited at 34 °C. Differences in host preference were also observed among populations.

There are genetic and non-genetic variations in morphological and other characters of animal species (Mayr et al., 1953). Thorne and Allen (1959) considered variations as commonly observed phenomena in nematode individuals and populations, and discussed variations occurring in both individuals and populations as the result of natural selection within ancestral species during evolution. They also discussed the taxonomic significance of physiological and morphological variations involved in genetic changes. De Coninck (1962) emphasized contributions of the experimental analysis of nematode variations to the progress of nematode taxonomy.

Since the plant-parasitic nematodes were broadly realized to be very important as pathogens infecting crops and causing yield reduction in Europe in the last half of the 19th century, much research focussed on the study of physiological intra-specific variation, especially differences in host preference between nematode populations or strains, and much information and knowledge have been accumulated on various nematode species (Sturhan, 1971a). Identification of intraspecific variation based on differential host preferences has not only had practical significance to control nematodes or reduce crop injuries by means of field management through use of non-host plants or resistant varieties but also contributed to discovery of morphological new species and development of more comprehensive methods of nematode classification and taxonomy (Chitwood, 1949; Christie and Albin, 1944; Franklin, 1951; Goffart, 1930; Liebscher, 1892; Schmidt, 1930). In contrast, research on other physiological and ecological characters within species and populations has been limited to a few nematode species (Baines et al., 1969; Daulton and Nusbaum, 1961, 1962; Foot, 1978; Franco, 1979; Webster and Hooper, 1968).

Morphological features of a nematode include its distinctive shape and size, and(or) characters within some range of variation, which are determined genetically but also affected by environmental factors (Thorne and Allen, 1959). Major environmental factors prone to influence morphological variations in many nematode species include host plants (foods), nutrient condition, temperatures, and population densities (Evans and Fisher, 1970a, 1970b; Goodey, 1952; Gysels and Bracke, 1964).

Rotylenchulus reniformis, dealt with in the present paper, is a nematode that was first described by Linford and Oliveira as a new genus and species parasitizing cowpea (*Vigna sinensis* Endl.) roots in the areas of pineapple cultivation of Hawaii (Linford and Oliveira, 1940). This species is polyphagous and lives on several hundred crop plants other than cowpea, such as pineapple, potato, tomato, cucumber, soybeans, sweet potato, cotton, castor oil plant, sugar cane, coffee, and so forth (Ayala, 1962; Ayala and Ramirez, 1964; Birchfield, 1962; Birchfield and Martin, 1968; Bird et al., 1973; Castillo et al., 1977; Heald, 1978; Jones et al., 1959; Linford and Yap, 1940; Nath et al., 1969; Rebois and Johnson, 1973; Rebois et al., 1978; Rodríguez, 1974; Sigh, 1975; Sikora and Schlosser, 1973; Sivakumar and Seshadri, 1971b; Sturhan, 1971b; Swarup et al., 1967; Valdez, 1968).

In Japan, Yokoo and Tanaka described in 1954 a new species named as *Tetylenchus nicotiana* from a

tobacco field of Wakimoto district, Akune city, Kagoshima prefecture. This species was characterized as having only females extracted from soil and no males. In 1960, Saegusa and Matsumoto identified nematodes extracted from fields in Nagano, Chiba, Shizuoka, Nara, and Nagasaki prefectures, as *R. reniformis* Linford and Oliveira, and provided a description of morphological characteristics of males. At about the same time, Nakasono et al. (1960) reported high density of nematodes (second-stage juveniles and young females) of the genus *Rotylenchulus* from soil in a great burdock growing field in Tokorozawa city of Saitama prefecture with adult females parasitizing roots of the plant. However, no males were extracted from the same field, and they reported that the nematode was morphologically very similar to *T. nicotiana*, reported by Yokoo and Tanaka in 1954. In 1963, Ohshima et al. extracted abundant *Rotylenchulus* nematodes from upland fields in Nagasaki prefecture. It was very interesting that their nematode populations from various crop fields were grouped into two different types, one of which commonly had males and females and another that had only females without males. In 1962, Baker synonymized *T. nicotiana*, mentioned above, with *Rotylenchulus*. Nakasono and Ichinohe (1967) also recognized *T. nicotiana* as synonymous with *Rotylenchulus* and gave a redescription of *R. nicotiana*. In this paper they adopted the criterion of the male's presence or absence in *Rotylenchulus* populations for separating species.

In 1968, Dasgupta et al. revised the genus *Rotylenchulus* based on nematode specimens collected from around the world with additional descriptions of six new species (*R. anamictus, R. clavicaudatus, R. leptus, R macrodoratus, R. macrosomus, and R. variabilis*). In this paper, they synonymized not only *T. nicotiana* Yokoo & Tanaka, 1954 with *R. reniformis* but also the following species: *R. elisensis* Carvalho, *R. queirozi* Lordello & Cesnik, *R. leiperi* Das, *R. stakmani* Husain & Khan (Carvalho, 1957; Das, 1960; Husain and Khan, 1965; Lordello and Cesnik, 1958; Yokoo and Tanaka, 1954). Accordingly, nine species, including two known species [*R. borealis* (Loof and Oosternbrink, 1962) and *R. parvus* (Williams, 1960)] other than *R. reniformis*, were considered as distinct species of the genus. It was interesting that *R. reniformis* was characterized to be polymorphic and to include various populations that differed in the frequency of male occurrence.

Furthermore, those populations without males are known from areas of Southeast Asia, the southern United States, and more than 10 other areas of the world including Central and South America (Dasgupta et al., 1968). Morphological variations of nematodes in those populations are conspicuous for a wide range of measurements. Some populations show values very close to the value for the type specimen and others do not. There are also some intermediate nematodes between the type and the larger ones. It was concluded that this situation possibly showed only variations of the nematode as a polymorphic species but did not show any distinctiveness among mixed species, and(or) the species might include some sibling species. Additionally, since differences in the frequency of male emergence among populations appeared to result from environmental changes, the presence or absence of males was considered to have no value for separating species.

In sum, the morphological polymorphism of *R. reniformis* discussed here suggested to the author that this species might consist of diverse populations, which were different in physiological and ecological characteristics. When the author came across this nematode in the 1960s, knowledge and information on the biology (physiological and ecological aspects) of *R. reniformis* were sparse. Under these circumstances, the author considered that usefulness of the morphological study, as applied until the present time, would have limited value for the progress of taxonomy on this nematode species. Instead, it would be more fruitful to conduct comparative and analytical studies on the biology, especially on the physiological and ecological aspects, such as geographical distribution, variations of sex ratio, male's role in reproduction, possibility of crossing between populations (reproductive isolation), and various other properties.

In view of this, the present study dealt with *R. reniformis* Linford & Oliveira, 1940, which was known to occur at high population densities in vegetable fields, especially in sweet potato fields in Japan, and also, often associated with soil-borne diseases of great burdock. Its objective was to elucidate the biological significance of polymorphism in this nematode by means of field surveys and experimental analysis of populations, which had been collected from several representative localities in Japan and in the United States (Hawaii and Texas).

The study, starting with a survey of the geographical distribution of the nematode in Japan, first showed the northern limit of habitat to be on the 14 ˚C isothermal line running near Tokorozawa district in the Kanto area with a wide distribution in southern areas, such as the southern shore line of Honshu, Shikoku, and many localities in Kyushu island. Secondly, three morphologically different groups of nematode populations (small, moderate, and large bodies) corresponded to three biological types (male-numerous, male-rare, and male-absent types) in terms of differences in male occurrence rates. Thirdly, male occurrence was suggested to be determined genetically but

not environmentally and reproduction was evidently amphimictic in the male-numerous type but parthenogenetic in both the male-rare and male-absent types. Reproductive isolation between the male-numerous type and male-rare or male-absent types was suggested as well. Finally, habitat segregation between the male-numerous and male-absent types in a local field, and differences in host preference were demonstrated.

At this time of reporting the study, the author would like to express his sincere thanks to Prof. Dr. Hans Mori of Hokkaido University and Dr. Minoru Ichinone, Department Head of Plant Pathology and Entomology, Hokkaido Agricultural Experiment Station for their warm instruction given from the start of study and detailed review of the manuscript. In the days of the National Institute of Agricultural Sciences (Nishigahara in Tokyo), many research leaders and colleagues gave good criticism and advice as well as encouragement to the author, especially Prof. Dr. Shin'ichi Takagi of Meijou University; Dr. Takeshi Yushima, Department Head of the First Environmental Sciences, Kyushu Agricultural Experiment Station; Dr. Shun'ichi Iwata, Department Head of Plant Pathology and Entomology, National Institute of Agricultural Sciences; Dr. Kenji Umeya, Department Head of Cultivation and Environment, Agricultural Research Center; and Dr. Tsutomu Nishizawa, Chief Researcher of the Phytonemic Research Laboratory, National Institute of Agricultural Sciences. Much gratitude is also expressed to those persons mentioned above. Many thanks are due to Dr. Masae Shiyomi, Chief Researcher of Ecological Systems Analysis Laboratory, Pasture Field Experiment Station, for his kind advice in statistical analysis of data. Thanks are also extended to Dr. Yasushi Mitsui, Chief Researcher of the Pest Research Laboratory, Hokkaido Agricultural Experiment Station and (late) Mr. Koichi Okamoto of Phytonemic Research Laboratory, National Institute of Agricultural Sciences for their useful criticism as well as assistance in the same laboratory during the course of study. And last but not least, all related workers in the prefecture organizations are gratefully remembered for their kind guidance and useful information on surveying and collecting nematodes in local fields.

I. Materials and Methods

Here, principal materials and methods, which were commonly applied to respective subjects, are described, and additional details for each section and study are presented later.

Nematode species studied: *Rotylenchulus reniformis* Linford and Oliveira, 1940, called "nisefukuro sentyu" in Japanese.*[1] This species includes three biological types as follows: (1) "the male-numerous type" (abbreviated as MNT hereafter), in which male occurrence is usual and numerous with females; (2) "the male-rare type" (abbreviated as MRT hereafter), in which male occurrence is rare or at very low frequency, (3) "the male-absent type" (abbreviated as MAT hereafter), in which no male occurrence is usual.

Original locality of isolated populations used for experiment: (called "laboratory populations" with respective abbreviation hereafter) (1) ASH-a and ASH-b populations: Both originated from single females of the MRT population, collected in a sweet potato (*Ipomoea batatus* Lam., cv. Kanto No. 14) field, Kaijo Branch of Agricultural Experiment Station, located in Asahi city, Chiba prefecture, in October 1971 and continually propagated on sweet potato (cv. Norin No. 2) in pots as mentioned below. (2) MIZ population: Originated from 15 egg masses of the MNT population collected in a sweet potato field (cv. unknown) of Ogaguchi district, Mizuho town, Nagasaki prefecture, in September 1962 and propagated on sweet potato by the same method as others. (3) SIB population: Originated from infested soil containing a MAT population collected in a burdock (cv. unknown)*[2] field, Shibi district, Tsuruda town, Kagoshima prefecture, in December 1971 and propagated by the same method as the others. (4) AKN population: Originated from soil containing a MAT population on sweet potato, collected in the type locality of the synonymized *R. nicotiana* Yokoo and Tanaka, 1954, Wakimoto district, Akune city, Kagoshima prefecture, in December 1971 and propagated by the same method as the others. (5) HAW population*[3]: Originated from infested soil with an MNT population on cowpea (unknown cv.), collected in the type locality of *R. reniformis* Linford and Oliveira, 1940, Oafu Island, Hawaii, United States, in October 1965 and propagated by the same method as the others. (6) TEX population*[4]: Originated from infested soil with an MNT population on cotton (*Gossypium hirsutum* L.), collected in a Texas field*[5] and provided by Texas A&M University, Texas, United States, in October 1971 and propagated by the same method as the others. (7) M×H population: Originally crossed between 100 young females of the MIZ population and 110 males of the HAW population and propagated on potted tomato for first 4 months and thereafter on sweet potato by the same method as the others.

Propagation and maintenance of the laboratory populations: Wooden boxes (width × length × depth = 25 × 40 × 15 cm) were used as soil containers for

[1] This name is said to originally be for the genus *Rotylenchulus,* but now it means *R. reniformis* in practice.

[2] "*Arctium lappa* L., producing an edible root," noted by the translator (the author).

[3] Introduced with necessary legal permission.

[4] Ibid.

[5] The correct record of the locality is missing (noted by the translator = the author).

growing host plants (sweet potato or tomato) with nematodes until the year 1970 and, after that, wooden containers were exchanged for plastic ones (26 × 32 × 16 cm). Each container was filled 70% deep with the Nishigahara soil (sandy loam), which was steam-sterilized, and 300 to 600 ml of original soils collected in respective localities, mentioned above, or an egg mass produced by a single female was incorporated or inoculated after two sweet potato vine cuttings were planted. A dose of 2 g ammonium sulfate, 2 g calcium superphosphate, and 2 g potassium chloride per container was applied as plant nutrients. Containers received host plants and nematode inocula were placed on the beds in the greenhouse and maintained with careful watering. To avoid nematode contamination, spaces of 50-cm distance were maintained between containers until the year 1970 and, after that, L-shaped transparent plastic plates (35 × 28 × 35 cm) were substituted for the spaces. In April every year, one half of the nematode-infested soil in the containers was renewed with fresh steam-sterilized soil (called "sterilized-mixed soil" hereafter), which was prepared by mixing two parts of the Nishigahara soil with one part of river sand (each screened with a 5-mm-pore sieve) in volume. At the same time a 2-g dose of each fertilizer, mentioned above, was applied.*[6] Positions of containers in the greenhouse were carefully changed in randomized design every 4 or 6 months. Soil temperature in the container, monitored by an auto-recording thermometer, fluctuated between 15 ˚C in winter and 34 ˚C in summer during the course of the study.

Mass production of nematode materials used for experiments: Three parts of sterilized-mixed soil and 1 part of infested soil taken from respective laboratory populations (containers) were carefully incorporated in the container, which was the same as mentioned above. Two or three sweet potato vine cuttings (cv. Norin No. 2) were planted and supplied with a dose of three basic nutrients, 2 g each, mentioned above. Then, the containers were carefully maintained with watering in a greenhouse having temperature fluctuation of 22 to 28 ˚C for a 4 to 5-month period before use. Using this method ample fresh nematode materials [second-stage juvenile (J2), young female (Yf), adult female (Af) and (or) male] were easily obtained at the time of experiments.

Nematode extraction methods: In order to examine soil populations of nematodes in both fields and experimental pots and (or) to recover nematode materials from the container soils, one of following three extraction methods was used. Abbreviation of the methods will be indicated hereafter in each section and study.

(1) SSB. An amount of 100 to 400 ml soil was washed with Seinhorst's elutriator (Seinhorst, 1956) for 15 to 20 minutes, and extracts were poured onto a 325-mesh metal screen. Then, all materials on the screen were placed on the Baermann funnel sieve fitted with a sheet of Japanese paper (traditional thin paper made from *Broussonetia* plant skin) at room temperature. After 48 hours nematodes in clean water were recovered.

(2) SCF. A given amount of soil (100 to 400 ml) was suspended in a plastic bucket with a volume of tap water equal to eight times that of the soil and sieved with 325-mesh metal screen. Each soil was sieved three times repeatedly. Extracts on the screen were then processed by the double-layer centrifugal-flotation technique,*[7] using a Kubota centrifuge with eight tubes of 50 ml in volume. Centrifugation was at 500 rpm for 5 minutes and repeated twice per sample. The extracting solution to suspend nematodes within the tube was adjusted to 1.32 specific gravity with sodium sulfate. After centrifugation, liquid layers in the tube were decanted into 1.5 liters tap water in a glass container (10 cm in diam. and 35 cm in length), and extracted nematodes were allowed to settle down for 48 hours. Nematodes on the bottom in the container were taken out with about 100 ml water for experimental use or for examining numbers.

(3) SVB. A given amount of soil was sieved with 325- or 400-mesh metal screens by the same method as in SCF, and extracts on the screen were processed to Baermann funnels and nematodes allowed to settle to the bottom at room temperature for 48 hours.

Size of clay pots used for nematode culture tests: Pots denoted "small clay pots" were 5 cm in diam., filled with 75 ml sterilized-mixed soil. "Big clay pots," were 11 cm in diam. and 13 cm in height, filled with sterilized-mixed soil until 90% full.

II. Geographical and Local Distributions

Although the geographical distribution of *R. reniformis* in Japan was partly known (Chikaoka, 1964; Nakasono et al., 1960; Saegusa and Matsumoto, 1960; Yokoo and Tanka, 1954; Yoshida, 1965), the nationwide distribution was not yet clear. Here for the first time, the distribution of the nematode was surveyed nearly throughout entire areas of Japan to examine the relationship between differences in male occurrence among populations and any associated factors. Additionally, in Nagasaki prefecture, the local distribution of the different biological types was examined in a restricted area (fields) for habitat segregation, as shown later.

[6] Two cuttings of the host vine were newly planted (Ibid).

[7] Originally developed by Takagi (1970), Japanese Journal of Applied Entomology and Zoology 14:108-110 (in Japanese), noted by the translator.

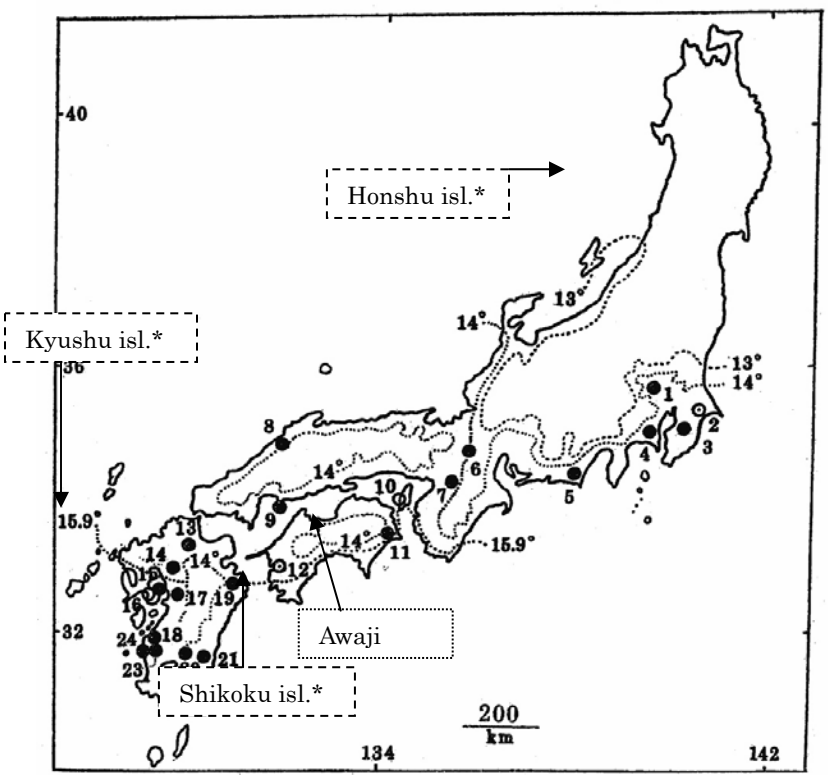

FIG. 1. A map of geographical distribution of *Rotylenchulus reniformis* in Japan. 1: Tokorozawa, 2: Asahi, 3: Kimizu, 4: Hiratuka, 5: Omaezaki, 6: Kusatu, 7: Kashiwabara, 8: Ohta, 9: Mukouhigashi, 10: Minamiawaji, 11: Tokushima, 12: Uwajima, 13: Mitsui, 14: Chikugo, 15: Mizuho -1, 16: Mizuho-2, 17: Nishigoshi, 18: Minamata, 19: Usa, 20: Miyakonojo, 21: Kyoutsuka, 22: Kanoya, 23: Tsuruda, 24: Akune. Dark spot: Females without males,.Dark spot in white circle: Mostly females with rare occurrence of males. White spot: Both females and males are usual. Note that 13 ˚C, 14 ˚C, and 15.9 ˚C stand for isothermal lines of annual average air temperatures. *Names added by the translator.

1. Geographical distribution in Japan

A survey was conducted through the northernmost areas in Fukushima prefecture of Honshu, Shikoku, and the southernmost areas of Kyushu islands. Information on records of the nematode in the literature was also studied for discussion.

Materials and methods

Numerous samples of soil*[8] were collected by prefectural workers (soil examiners and technical researchers for plant pathology and pest management in each prefecture) in their local fields and sent to the author's laboratory (in Tokyo). Those soils were examined for nematode genus and species. The following areas were visited by the author to take soil samples: Souma district of Fukushima prefecture; Gozenyama, Toyobamidasaki, and Nakaminato districts of Ibaraki prefecture; Tokorozawa district (city) of Saitama prefecture; Asahi and Kimitsu districts of Chiba prefecture; Suzaka city of Nagano prefecture; Makinohara-daichi and Omaezaki districts of Shizuoka prefecture; Ohta city of Shimane prefecture; Tokushima city; Awaji island; Uwajima city of Ehime prefecture; Mizuho district of Nagasaki prefecture; Miyakonojo city of Miyazaki prefecture; Tsuruda district and Akune city of Kagoshima prefecture. Target fields to collect soil samples for *R. reniformis* examination were mainly those cultivated to sweet potato and great burdock, but other fields of vegetables, such as potato and radish, apple, mulberry, and tea plants were also checked. Soil samples sent by prefecture workers contained volumes of 300 to 1,000 ml in most cases and were packed in vinyl or polyethylene bags. Soil sampling by the author was done as follows: Soil samples of 600 to 1,000 ml were collected from the 5- to 15-cm depth at six to nine sites within a field and packed in polyethylene bags. Collected soil was kept in a refrigerator at 5 ºC until nematode extraction. Sub-samples of 150 to 200 ml soil taken from each evenly mixed soil sample were processed to extract nematodes by means of SSB or SVB until 1968 and, after that, by SCF. Nematodes were examined under Nikon stereo and compound microscopes.

[8] More than 300 in number, noted by the translator (Author).

Results

Rotylenchulus reniformis was ascertained to inhabit soils in the following districts or areas as shown in Figure 1: in Honshu island, Asahi and Kimitsu districts of Chiba prefecture, Omaezaki district of Shizuoka prefecture, Kashiwabara district of Nara prefecture, Kusatsu district of Shiga prefecture, Ohta district of Shimane prefecture, and Mukaushima island of Hiroshima prefecture; in Shikoku island, Minami Awaji island and Tokushima city of Tokushima prefecture, and Uwajima district of Ehime prefecture; in Kyushu island, Mii and Chikugo districts of Fukuoka prefecture, Mizuho district of Nagasaki prefecture, Nishigoshi and Minamata districts of Kumamoto prefecture, Usa district Oh'ita prefecture, Miyakonojo and Kyoutsuka districts of Miyazaki, Kanoya, Tsuruda, and Akune district (city) of Kagoshima prefecture. In these localities, nematodes of MNT, in which both females and males (adult, also the same hereafter) were detected together, were recorded only in Awaji island and the Mizuho-2 field (Fig. 1) although nematodes of MNT in Awaji island could not be confirmed by the field examination made in 1969. Nematodes of MRT were obtained only in Asahi district of Chiba prefecture and Uwajima district of Ehime prefecture. All other localities examined had only females with no males. Host plants of those nematode populations detected were recorded as follows: great burdock in Tokorozawa, Mii, Mizuho-1, Minamata, and Tsuruda; sweet potato in Asahi, Kimitsu, Hiratsuka, Omaezaki, Kashiwabara, Mukauhigashi, Tokushima, Uwajima, Chikugo, Mizuho-2, Nishigoshi, Usa, Miyakonojo, Kanoya, and Akune; onion in Minamiawaji; tomato in Kusatsu and Kyotsuka; leguminous crops in Ohta. In Souma district (vegetables growing) of Fukushima and great burdock cropping areas along Naka river in Ibaraki prefecture, more than 20 field samples were examined each but no *Rotylenchulus* nematodes were detected. In Suzaka city of Nagano prefecture apple and vegetable fields did not show *R. reniformis* to be present. Results obtained here suggested that a northernmost habitat of *R. reniformis* in Japan was around Tokorozawa district (city) of Saitama prefecture and northern limit of the nematode distribution was on the 14 ℃ isothermal line of annual average air temperature (Fig. 1).

2. Local distribution in fields of Mizuho town in Nagasaki prefecture

Considerable variation in rates of male occurrence (sex ratio) of *R. reniformis* was frequently observed in field surveys of Nagasaki prefecture in the early 1960s (Ohshima, pers. comm., 1963). In order to determine the reason for this variation, the horizontal distribution of the nematode was examined in restricted fields.

Materials and methods

Fields examined and soil sampling: Two neighboring fields separated by a stone wall of about 50 cm in height, each occupying a similar area (12 × 15 m), in Ogaguchi district of Mizuho town, Nagasaki prefecture, were examined for the horizontal distribution of different biological types (MNT and MAT). One of the fields, on the higher side of the slope, was designated as Field A and the other on the lower side, Field B (Fig. 2). Both fields were cropped to sweet potato (unknown cultivar) in summer 1963, and a border zone of about 1 m in width was placed in Field A along the border between Field A and B, where burdock was growing in late January 1964, at which time samples of soil were collected. Field A was divided into nine rectangular plots (numbered from 1 to 9) and one belt zone (no. 10, Fig. 2). Each plot had an area of 3.7 × 5 m. A soil sample of about 800 ml was collected from several spots in the central part of each plot and the belt zone. Collected soils packed in polyethylene bags were brought to the laboratory and stored in the refrigerator at about 5 ℃ until nematode extraction and investigations in April 1964. Prior to this examination, Mr. Ohshima (technical officer in the former Nagasaki prefecture Research Center of Agriculture and Forestry) took soil samples from plot no. 5 (designated as no. 11 in circle) and the belt zone (no. 13) of Field A and in Field B (nos. 12 and 14) and provided them to the author in September 1962, and October 1963.

Nematode extraction: Three 150-g sub-samples were taken from each soil sample and processed to extract nematodes by SSB.

Results

As shown in Figure 2, total numbers of extracted nematodes (J2, Yf, and male) and the ratio of males to males plus Yf expressed as percentage varied according to sampling plots. Nematode numbers per 150 g soil ranged from 2 to 318 and male percentages fluctuated between 0 and 50% among plots in Field A. It was particularly interesting that zero percentage males was detected only in plot no. 3 and belt zone no. 10 along the border between the two fields. As for Ohshima's soil samples, abundant males and females (Yf) were extracted at spot no. 11 (in plot no. 5) and no. 12 (in Field B) collected in 1962, but no males and just a small number of males (0.2%) were found at spot no. 13 (in the belt zone in Field A) and 14 (in Field B), respectively, in 1963. The results appeared to be similar between the author's sample soils and those of Ohshima, although spot no. 12 was

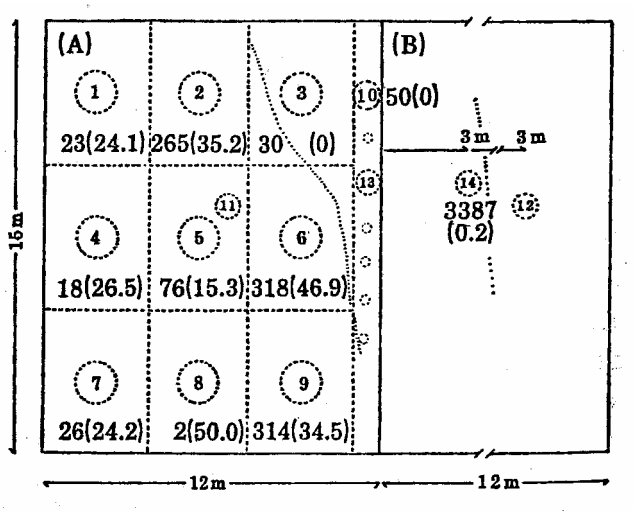

FIG. 2. Local distribution of *Rotylenchulus reniformis* in two neighboring fields, located at Ogaguchi district of Mizuho town, Nagasaki prefecture. Note: Number in circle shows the plot number. Those under the circle indicate extracted nematode numbers (J2, Yf, and male) in total and those in parentheses mean male % to the sum of Yf and males; no. 11 in circle of the plot no. 5 and no. 12 in Field B were sampling spots by Ohshima in September 1962. Furthermore, nos. 13 and 14 in Field B* were also Ohshima's collection spots in October 1963. Dotted lines show a postulated border between populations of biological types (MNT and MAT). *Note that there was a distance of 3 m between the sampling spot nos. 14 and 12 (added by the translator).

not checked by the author in January 1964. A difference in male numbers was evident between spot no. 14 and no. 12 in Field B. "There was a distance of 3 m between the two spots."*[9]

Results obtained here suggested that there were two biological types of populations of *R. reniformis* in both Field A and B, and at the same time, their habitats were segregated from each other although they partly overlapped on the border.

3. Discussion

There were found to be two characteristic facts regarding *R. reniformis* in Japan. The first one was that the nematode in Japan was distributed in relatively cool and warm areas with the northernmost border near the 14 ˚C isothermal line of annual average air temperature, and the second one was that there were three biological types, distinguished by i) males and females usually occurring at about same frequency (MNT), ii) frequent females but of very rare males (MRT), and iii) only females observed with males absent (MAT).

In order to clarify the distribution of the three types, information on the nematode in Nagasaki prefecture and the Southwestern islands of Japan wase studied through literature published by Ohshima et al. (1963), Gotoh (1965, 1968, 1976), and Gotoh and Ohshima (1963). Localities of the nematode distribution were reported as plotted on the map shown in Figure 3. In Nagasaki prefecture, Nishisonogi and Higasisonogi areas surrounding the Ohmura bay showed to have populations of MNT, while areas extending eastward onto Isahaya (6 in Fig. 3), Shimabara peninsula, including Moriyama (12 to 14), Aino (20, 21), Azuma (16, 19), Mizuho (15, 18) districts and so forth often indicated coexistence of both types (MNT and MAT). In the southwestern (subtropical) islands, especially in many islands including Amami, Okinawa isles, and others, both MNT and MAT populations had been demonstrated to have segregated distributions according to Gotoh and Ohshima (stated above). An MRT population in Tanegashima and many independent MAT populations in the remaining islands of the area were also recorded in their reports. Findings and information on geographical and local distributions of *R. reniformis* in Japan, obtained here, show that populations of MNT have their habitat in geographically more limited or more southern areas than populations of MAT and MRT, because the northernmost localities of MNT are recorded in Awaji island situated between coasts of Tokushima of Shikoku island and Kobe city of Honshu island, and all other habitats occur in areas of Nagasaki in Kyushu or areas in the southwestern (subtropical) islands (Amami and Okinawa islands), while MAT and MRT, particularly MAT populations, are detected from wider areas covering Tokorozawa in Saitama prefecture, north to Tokyo and all other southern (warmer) parts of the country, including southern Hoshu as well as remaining areas (Kyushu island and southwestern islands). The MRT population is localized in limited areas (Asahi in Chiba, Uwajima in Ehime, and Tanegashima in Kagoshima prefecture).

It appears that MNT populations are adapted to warmer conditions than are MAT and MRT populations, explaining why the northernmost habitat of the MNT is farther south than the MAT and MRT, especially the MAT.

To analyze this problem more clearly, the relationship between the geographical distribution of *R. reniformis* and climatic factors world-wide, especially rainfall and annual average air temperature, were studied again through published literature (Artero et al., 1977; Ayala, 1961; Brunei Department of Agriculture, 1972; Bustillo, 1972; Cohn, 1973; Dasgupta et al., 1968; Decker et al., 1966; East African Agriculture and Forestry Research Organization, 1972; Edmunds, 1970, 1971; Fassuliotis and Rau, 1967; Germani, 1978; Guerout, 1975; Huang, 1972; Khair, 1978; Linford and Oliveira, 1940; Muralidharan and Sivakumar, 1975; Nath et al., 1969; Neal, 1954; Oteifa and Osman, 1974; Peacock, 1956; Rebois et al., 1978; Sayre, 1964; Sasser et al., 1962; Shepherd, 1977; Smith and Taylor, 1941; Taylor et al., 1970; Thames et al., 1971; Van den Berg, 1978; Winoto and Lim, 1972) and "Rikanenpyo" (an annual

[9] Communicated by Mr. Ohshima at surveying time in 1964 (noted by the translator).

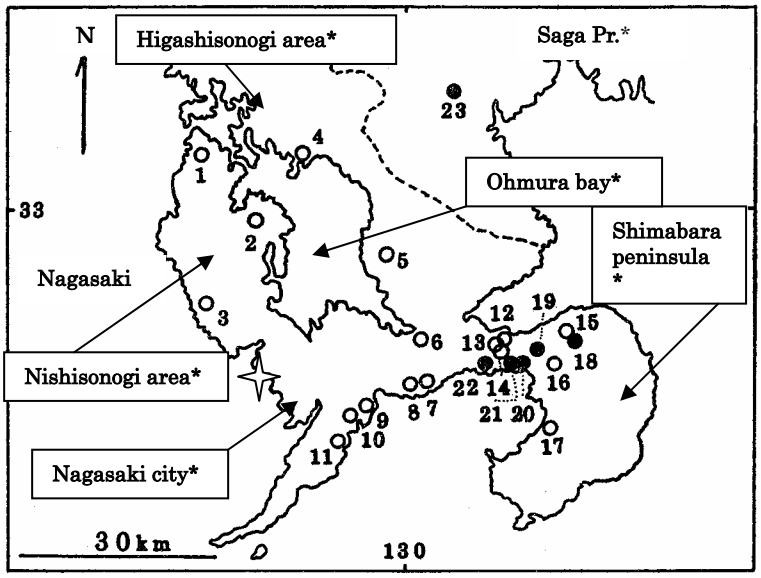

FIG. 3. A map of distribution of *Rotylenchulus reniformis* in Nagasaki and Saga prefectures. Numbers under circle spots, where nematodes were collected, stand for local names as follows. In Nagasaki prefecture, 1: Saikai; 2: Nishisonogi; 3: Sotome; 4: Kawatana; 5: Isahaya; 7: Iimori-1; 8: Iimori-2; 9: Mogi-1; 10: Mogi-2; 11: Mogi-3; 12: Moriyama-1; 13: Moriyama-2; 14: Moriyama-3; 15: Mizuho-1; 16: Azuma-1; 17: Obama; 18: Mizuho-2; 19: Azuma-2; 20: Aino-1; 21: Aino-2; 22: Moriyama-4. In Saga prefecture, 23: Shiota. Note that empty circles show populations of MNT and the dark one, MAT. * The local name was newly added by the translator for better understanding.

book for scientific data, Tokyo Astronomical Observatory, 1976). World-wide distribution of the nematode from the temperate to tropical zones was documented, and thermal optima for the nematode appeared to be comparatively high as follows: It lives widely in the temperate, sub-tropical and tropical zones in North and South America, such as the Hawaiian islands in the Pacific Ocean, South Carolina, Georgia, Alabama, Louisiana, Texas, Florida, and Mexico in North America, many islands of the Caribbean Sea, and Colombia as well as Brazil in South America. In areas surrounding the Mediterranean, this nematode is recorded from Spain in the western rim of the sea, Israel and Lebanon in the eastern, and Egypt in the southern (North Africa). In Central and South Africa, representative locations recorded for the nematode distribution are Senegal, Gambia, Ghana, Central Africa, Somaliland, Kenya, Zambia, South Africa, and Madagascar. In Asian areas, Iraq, Iran, Pakistan, India, Sri Lanka, Malaysia, Indonesia, the Philippines, and Oceania (especially northern parts of Australia) are also recorded as habitats of this nematode.

Among these localities of the nematode's distribution, the northernmost habitat in the Northern Hemisphere is Málaga in Spain, which is located at the latitude of 36° 09' N and has an annual average air temperature (A'T, hereafter) of 18.2 °C, and approximately similar to the A'T of Charleston (32° 54' N) in South Carolina, U.S.A., while the southernmost reported occurrence in the Southern Hemisphere is Cape Town in South Africa, located at 33° 58' S with A'T of 15.9 °C. Localities that have A'T lower than 20 °C are Atlanta (33° 39' N and 16.4 °C) in Georgia of North America, Nairobi (01° 18' S and 17.5 °C) in Kenya, Neghell (05° 17' S and 19.5 °C) in Ethiopia, and Lima (12° 01' S and 18.4 °C) in Peru, while almost all of other geographical distributions are spread over areas with A'Ts that are higher than 20 °C, ranging from 20.8 °C for Cape Verde islands in the Atlantic Ocean to 29.4 °C for Somaliland in Africa. Annual rainfall in those localities varies widely from 31 mm in Lima (lowest value) to 5,550 mm in Burunei (highest). So, rainfall appears to have no relation to the geographical distribution of *R. reniformis* in the world. In conclusion, information obtained here showed that the geographical distribution of *R. reniformis* is within areas in the world with A'Ts between 15.9 °C at Cape Town and 29.4 °C in Somaliland.

The lower A'T (15.9 °C) as a thermal index of the limit of the nematodes distribution around the world was plotted on the map of Japan as in Figure 1. An isothermal line of 15.9 °C A'T commenced with the area around Shionomisaki (16.8 °C) and ran through Wakayama (15.8 °C) to the southern part of Awaji island and after that, it came down to the southern sea shores (Tokushima with 15.5 °C) of Shikoku island and reached the lower areas (Oh'ita with 15.3 °C) in the eastern area of Kyushu island. On the western side of Kyushu, the line started again from the lower

areas north to Kumamoto, by crossing the Ariake sea, south to Saga with 15.8 ℃, and went over Sasebo to reach the northern sea shores of Nagasaki prefecture. The line of 15.9 ℃ A'T on the map suggested that all areas on the southern side of the line would provide possible habitats for MNT populations of *R. reniformis*.

In fact, MNT populations in Japan were detected only from areas on the southern side of the line. So, it was considered that the northernmost limit of habitat for MNT populations in Japan agreed with the isothermal line of the temperature (15.9 ℃ A'T) that was observed in the localities outside Japan. However, it could not be explained why MNT populations were found only from restricted areas (Awaji island, Nagasaki prefecture, and southwestern islands) in southern parts of Japan, and not from other regions, especially on Kyushu island, where suitable temperatures also occur. It was postulated that MNT populations happened to be introduced to those areas from tropical or sub-tropical areas some time in the past but dissemination mechanisms of the nematode, especially by artificial means, did not work after the introduction.

It was distinctive that the northern limit of the habitat for MAT and MRT populations was on the isothermal line of 14 ℃ A'T, which was lower than that of MNT populations (15.9 ℃ A'T). The reason why there was a difference in northern limit of habitat between biological types (MAT, MRT, and MNT) was not entirely clear but a discussion was attempted as follows. A difference in thermal tolerance between the nematode populations in the low temperature season (January and February) is considered to be a factor affecting the geographical distribution of *R. reniformis*. Another is the physiological difference in reaction to day length as observed in many kinds of insects (Itoh, 1975). In the nematode *Globodera* the day length was postulated as one of the limiting factors of its geographical distribution. In the Andes mountains, *G. pallida* and *G. rostochiensis* showed different habitat locations segregated by a border of latitude 15.6° S. There was no relation between nematode habitats and elevation above sea level. The difference in day length was considered to be the primary factor limiting the geographical distribution of this genus (Evans et al., 1975). Greater reproduction of *G. rostochiensis* occurred under longer day-length conditions (Ellenby, 1958). There is the possibility that the geographical distribution of these two *R. reniformis* populations in Japan is also affected by day length. However, these *R. reniformis* populations are sympatric and coexist in areas south of the 15.9 ℃ isotherm. This is different from the two Andes *Globodera* spp. that have an allopatric distribution. The differences in the characteristics of *Rotylenchulus* and *Globodera* make it difficult to draw any conclusions from these distribution relationships. If day length were related to the distribution of *R. reniformis*, MNT populations would be more likely affected by it. Experimental analysis on the topic, however, could not be done here.

Factors affecting the distribution of MAT populations, which were observed more broadly than MRT populations (Fig. 1), would include kind of foods, soils, rainfall, natural enemies, interspecific competition, and temperature. However, differences in the former five factors between northern and southern parts separated by the isothermal line of 14 ℃ were small; for example, distributions of nematode trapping fungi in Japan studied by Mitsui (1975) and of such competitive nematodes as *Meloidogyne* and *Pratylenchus* (Gotoh, 1974, 1976). More attention, here, was also paid to temperatures rather than others as follows.

In the past, it was well known that the northernmost habitat of an insect pest (*Triporyza incertullas* Walker: Lepidoptera, attacking rice) was on the isothermal line of –3.5 ℃, which was the annual lowest temperature in winter and passed through Awaji island. Since the same temperature (–3.5 ℃) was proven to be the super-cooling point of this insect by experiments, this temperature was considered the determinant of the northernmost limit of its habitat in Japan (Kisita and Yagi, 1930). Additionally, this isothermal line (–3.5 ℃) overlapped the line of 14 ℃ A'T in almost entire parts and it agreed with the northernmost limit of habitats in many other insect species. The term "Honshu-nangansen" (Southern sea shore line of Honshu) was coined to indicate the border between northern and southern insect distributions (Kisita and Kawada, 1933a,b). The super-cooling point or lethal low temperature has not been determined for *R. reniformis* (MAT). Thirty-five percent of individuals of *Aphelenchoides ritzemabosi* adults and J2 survived exposure to –70 ℃ for 48 hours (Asahina, 1959). J2 of *Meloidogyne incognita* died at temperatures of –5 to –7 ℃ and death of eggs was 100% after 10 days at –4 ℃ (Vrain, 1978). The northernmost limit of habitats of the southern root-knot nematode *M. incognita* in Japan is known to be on the isothermal line of 12 ℃ A'T, which extends into more northern areas than *R. reniformis* (MAT) (Gotoh, 1976), suggesting that the former would be more tolerant to cold climatic conditions than the latter. Therefore, it is considered that *R. reniformis* (MAT) would have a lethal point by cooling that is higher than that of *M. incognita* and rather similar to that of southern insects. Furthermore, the low temperature in the winter season would work as a major factor for determining the northern limit of habitats of

R. reniformis MAT populations through the same mechanism as in many other species of southern insects. Watanabe, however, criticized explanation by "low temperature mechanism" of the northern limit of habitats in insects and stressed the importance of temperatures during the growing season, especially the effective accumulative temperature in summer (Watanabe, 1952). It is clear for *Etiella zinckenella* (Lepidoptera: lima bean podborer) with the northernmost limit of habitats in Japan regulated by foods and temperatures in summer rather than low temperatures in winter (Naitoh and Masaki, 1961).

Temperature is also known to be the main factor affecting the distribution of *Meloidogyne* nematodes in the Netherlands and Venezuela (Dao, 1970), and of *Pratylenchus* in Japan (Gotoh, 1974).

As mentioned above, it was elucidated that there were three biological types of *R. reniformis*, distributions of which were geographically different in Japan. If frequency of male occurrence between those types was determined by non-genetic factors such as physical environment, nutrient condition of hosts, and population densities (Ellenby, 1964; Triantaphyllou, 1960, 1973; Trudgill, 1967), differences in the distribution of those types would be facultative, as affected by environmental variation. If so, research was needed on which environmental factors were involved in those phenomena, such as sex determination, male differentiation, and so forth. On the other hand, if the differences were not determined by environmental factors but by genetic ones, habitat segregation between MNT and MAT populations as observed in Mizuho town, Nagasaki prefecture would have an ecological significance. In this case those types could not inhabit the same soils and would not be able to occupy the same ecological niche, and thus could be considered to be independent biological units or taxa. Therefore, geographical differences in distribution of those types would not be facultative and temporary but obligatory, determined by physiological as well as ecological mechanisms based on genetic variation.

In the later sections, the subjects discussed above will be expanded by results of experimental analysis. The next section will present results of morphological analysis of biological types with a discussion of the polymorphism in *R. reniformis* from a morphological perspective.

III. Morphological Comparison of Young Females from Nine Field Populations

Specimens of *R. reniformis* were collected from several localities in Japan, which were selected according to biological types, and nematode samples were also provided from the Hawaii Pineapple Institute, where an MNT population was distributed. Young females from those collections were used for morphological measurements. First, measurements

Table 1. Comparison of measurements and De Man's values of young females among nine populations of *Rotylenchulus reniformis*.

Character	Location of populations in descending order									LSD*
Body length	Asa	Ehi	Aku	Hir	Ain	Tok	Miz	Haw	Miy	
(μm)	439.2	419.7	415.1	393.8	383.2	376.9	375.5	372.9	367.7	17.6
Greatest body	Miy	Aku	Asa	Tok	Ehi	Hir	Ain	Haw	Miz	
width (μm)	16.9	16.7	16.5	16.4	16.3	16.3	15.9	14.8	14.3	0.43
Stylet length	Ehi	Asa	Tok	Aku	Ain	Hir	Miy	Miz	Haw	
(μm)	19.0	18.9	18.7	18.6	18.6	18.5	18.1	16.7	16.4	0.34
Orifice	Asa	Aku	Ehi	Mizu	Miy	Hir	Ain	Tok	Haw	
(μm)	20.5	15.8	14.9	14.1	14.1	13.8	12.9	12.8	12.5	0.57
A	Asa	Miz	Ehi	Aku	Haw	Hir	Ain	Tok	Miy	
(ratio)	26.7	26.3	25.7	24.9	24.9	24.2	24.1	22.9	21.7	1.06
B	Ehi	Aku	Miy	Asa	Hir	Miz	Ain	Tok	Haw	
(ratio)	4.9	4.9	4.9	4.7	4.7	4.7	4.6	4.6	3.9	0.22
C	Haw	Miz	Ain	Tok	Ehi	Asa	Hir	Aku	Miy	
(ratio)	18.9	16.3	15.0	14.3	14.3	13.8	13.7	13.2	13.0	0.91
O	Asa	Miz	Aku	Miy	Ehi	Haw	Hir	Ain	Tok	
(ratio)	1.08	0.89	0.85	0.78	0.78	0.77	0.74	0.69	0.68	0.06
H	Ehi	Asa	Tok	Ain	Miy	Aku	Hir	Haw	Miz	
(%)	40.1	34.5	34.5	31.9	28.5	27.8	27.6	25.3	23.3	(2.16)**
V	Miz	Haw	Asa	Ehi	Ain	Aku	Tok	Hir	Miy	
(%)	71.4	70.8	70.0	69.8	69.5	69.3	68.6	68.4	67.5	(3.07)**

* Least significant difference at 95% level. **Data were tested after converted by the formula $\sin^{-1} x$. Remarks for the population abbreviation: Asa = the Asahi ; Ehi = the Ehime; Aku = the Akune; Hir = the Hiroshima; Ain = the Aino; Tok = the Tokorozawa; Miz = the Mizuho; Haw = the Hawaii; Miy = the Miyazaki population.

were compared by De Man's formula (allometric growth). Second, principal components analysis was applied to data for comparison. Then, the relationship between morphological variations and biological types was examined.

1. Morphometric comparison of allometric growth and values from De Man's formulae

Direct measurements and values from De Man's formulae, which are commonly applied in the description of nematode species, were used here for comparison.

Materials and methods

Nematodes used: Nematode specimens were extracted by SSB from sample soils collected in the following nine locations. MNT population: Mizuho town (sweet potato field) of Nagasaki prefecture; Oafu island (Cowpea field) of Hawaii. MRT population: Asahi city (sweet potato field) of Chiba prefecture; Uwajima city (sweet potato field) of Ehime prefecture. MAT population: Tokorozawa city (great burdock field) of Saitama prefecture; Mukoushima town (sweet potato field) of Hiroshima prefecture; Aino town (sweet potato field) of Nagasaki prefecture; Kyozuka town (tomato field) of Miyazaki city; Akune city (sweet potato field, type locality of syn. *R. nicotiana*) of Kagoshima prefecture. Twenty young females were randomly picked from each population and killed by holding in hot water (about 55 ℃) for 15-20 minutes. Then, specimens were fixed by 5% formalin solution for 3 to 5 days and mounted on slides in 5% formalin (Seinhorst, 1966). Microscopic observations and morphological measurements were made under a Nikon stereo microscope at 30 to 60 × magnification and a compound microscope at 200 to 1,000 (oil immersion) × within 3 to 5 days after fixation. A camera lucida and ocular micrometer were used for sketching and measuring nematode body (length and size) as well as details of body parts (stylet, body widths, and so forth) according to Thorne.*[10] The following 25 characters were measured and used for comparison: body length, stylet length, tail length, greatest body width, position of dorsal esophageal gland opening (orifice, distance from stylet knobs), length of hyaline portion in tail, De Man's values (a, b, c), O value (ratio of opening position of dorsal esophageal gland per stylet length), H value (ratio of hyaline portion length per tail length), and V value (percentage of vulval position from anterior end of body per body length) because of their comparative constancy. The mean value of each measurement and De Man's value were compared with LSD (least significant difference) at the 95% level.

Results

Variation in each value was considerably large, as shown in Table 1. Body length and stylet length were relatively larger in MRT (Asahi and Ehime) and MAT (Akune, Hiroshima, Aino, and Tokorowawa) populations than in MNT (Mizuho and Hawaii) except for the Miyazaki population (MAT). Largest mean body length was 439.2 μm in the Asahi population of MRT, and followed by 419.7 μm in the Ehime (the same type) and 415.1 μm in the Akune (MAT) population. The greatest mean body width was shown by the Miyazaki population (MAT) and followed by Akune and Asahi populations, and the smallest value was shown by Hawaii and Mizuho populations (MNT). Stylet length had the same tendency as body length with remarkably shortest stylets in Hawaii and Mizuho populations. Position of the orifice in the Asahi population was at the longest distance and the Mizuho showed a medium position, but the Aino, Tokorozawa, and Hawaii had it a significantly shorter distance from the knobs than the Asahi population.

The De Man's values demonstrated a different tendency from that of body length and greatest width. The "a" value was greatest in the Asahi (MRT), Mizuho (MNT), and Ehime (MRT); medium in Hawaii (MNT); and smallest in the Tokorozawa as well as Miyazaki (MAT) populations. The "b" value was greatest in the Ehime and smallest in the Hawaii population. The Asahi and Hiroshima showed an intermediate value. The "c" value of both Hawaii and Mizuho populations (MNT) was the greatest, and the smallest one was for Miyazaki and Akune populations (MAT). The greatest "O" value of the Asahi was striking and the "H" was greatest in the Ehime population, followed by the Asahi and Tokorozawa. MNT populaions (Hawaii and Mizuho) were smallest in this value. The "V" value was greater in Mizuho and Hawaii populations, but there was no significant difference among populations.

As shown above, morphological variation in the nine populations tested was large. Generally speaking, nematodes of MRT and MAT populations were larger than those of MNT populations with the exception of the Miyazaki population, which had a shorter body length and greater body width. There was a possibility that an uncertain factor affected the condition of Miyazaki specimens during slide preparation but re-examination of the specimens could not be done.

As shown in Figure 4, the features of tail were characteristic according to populations. Each figure presented here was representative of more than 90%

[10] Thorne (1961) Principles of Nematology, 553 pp. McGraw-Hill Book Co. USA. (added by the translator)

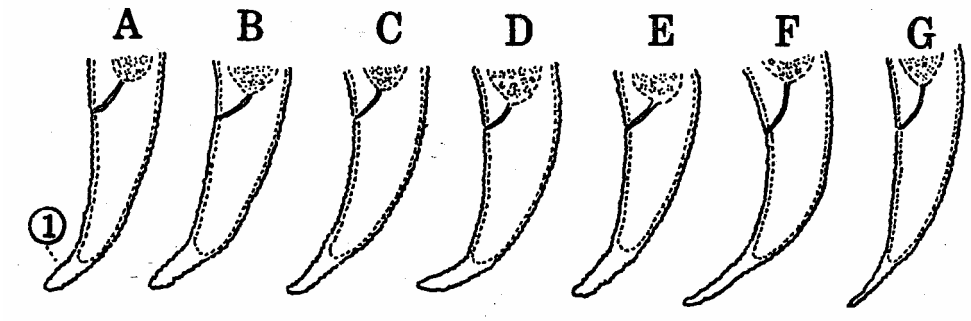

FIG. 4. Feature of tail parts of young females in *Rotylenchulus reniformis* collected from different locations in Japan and Hawaii. A) Mizuho and Hawaii. B) Aino. C) Miyazaki and Akune. D) Hiroshima. E) Tokorozawa. F) Ehime. G) Asahi. ①: Hyaline portion of tail.

of the individuals of each population investigated, but a small number of nematodes showed variable similarity to other populations. For example, a low frequency of individuals having tail features of the Asahi or Ehime were observed in the Akune field population, and there were some individuals with tail shape similar to that of Hiroshima or Tokorozawa populations in the Mizuho as well as Hawaii populations. However, it was certain that no nematodes with Asahi or Ehime tail shape were observed in either Mizuho or Hawaii population. Body length and H value are presented in Table 1, whereas mean length of hyaline portion with 95% confidence limit is plotted against mean body length to show the relationship between the two characters in Figure 5. Results showed that the longer body length was related to the longer hyaline portion. The Asahi and Ehime were similar to each other and formed a larger group of both characters.

The Mizuho and Hawaii (MNT) belonged to the smallest group, and other populations (MAT) such as Miyazaki, Aino, Hiroshima, Tokorozawa, and Akune were grouped into an intermediate position. The results would not suggest a correlation between geographical cline and morphological characters of *R. reniformis*.

2. Statistical comparison by principal components analysis

Morphological variation of *R. reniformis* was analyzed by applying principal components analysis for comparison.

Materials and methods

Eleven characters out of 25 measurements were used for analysis here as follows: body length, position of esophageal gland orifice (from stylet knobs), excretory pore position (distance from head end), length of esophagus (distance of esophageal junction from head end), tail length, phasmid position (distance from tail end), width of head sclerotization, stylet length, greatest body width, length of hyaline portion, and annulation number in ventral side of tail from anus to the edge of hyaline portion. Eight populations were used except for the Hawaii. Principal components analysis was studied with Okuno's text (Okuno, 1967a;b).

Results

Principal component analysis (P.C.A.) is a multivariate analysis, and more than three variables (measurements of nematodes) can be analyzed to obtain highly integrated information for comparison. The mathematical principle underlying the method is not discussed here. Data obtained from 20 young female specimens from each of eight populations, which were the same as in the previous section, were analyzed using a computer program provided by the computing

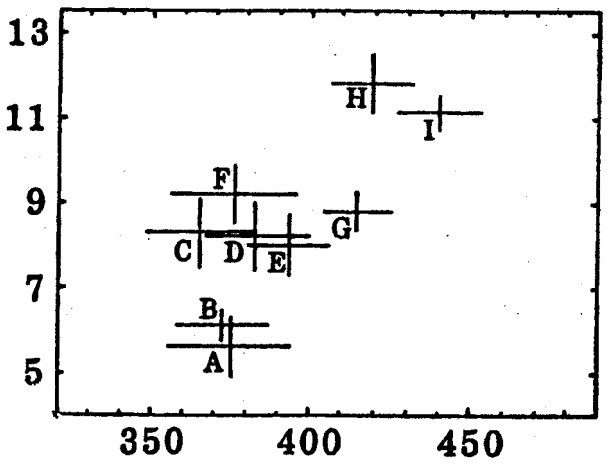

FIG. 5. Relationship between length of hyaline portion of tail and body length in young females of *R. reniformis* collected from different localities in Japan and Hawaii. A) Mizuho. B) Hawaii. C) Miyazaki. D) Aino. E) Hiroshima. F) Tokorozawa. G) Akune. H) Ehime. I) Asahi. Mean and 95% confidence limit.

center in the Ministry of Agriculture, Forestry and Fishery, Japan.

The average and correlation coefficients of each variable (measurement) are given in Table 2. No variable showed a negative correlation with any other. Body length had a small coefficient of correlation with body width and annulation number of tail but relatively large ones with other variables. Relatively large coefficients were also observed between width of head sclerotization and other characters, especially body length, stylet length, and length of hyaline portion. Tail length had a small coefficient of correlation with esophagus length but it showed relatively high values with other characters, particularly phasmid position. Annulation number of tail had a high value with tail length but a low one with all other characters. From these statistical data based on 160 individuals it was not possible to classify those nematodes, but P.C.A. provided additional meaningful information as follows.

In order to summarize variabilities of 11 variables into three principal components (integrated characteristic values), Z1, Z2 and Z3, coefficients and contribution values (what percentage of information [variability] in the first 11 variables could be explained) on the three principal components were calculated as shown in Table 3.

The first principal component (Z1) was described by loading 11 variables with the coefficients shown in Table 3 as follows:

$$Z1 = 0.349x1 + 0.242x2 + \bullet\bullet\bullet + 0.158x11 \qquad (1)$$

(Here, x1 was a standardized value with original data as mean = 0 and variance = 1)

Loadings for the first principal component were all positive numbers, ranging from 0.158 (annulation number of tail) to 0.365 (tail length), meaning that the larger value of 11 variables would result in the greater amount for Z1. In other words, if Z1 was large on each individual, the nematode had a large body size, but a small Z1 would mean a small body size. Therefore, this component can be defined as nematode size.

The second principal component (Z2) was written as follows:

$$Z2 = 0.210x1 + 0.336x2 + \bullet\bullet\bullet + -0.523x11 \qquad (2)$$

Loadings for Z2 were positive number on x1, x2, x3, x4, and x7 but negative on other variables (x5, x6, x8, x9, x10, and x11). The greatest positive loading was on esophagus length (x4 = 0.428), and the greatest negative one was on the annulation number of tail (x11 = –0.510). Loadings on size or length of body parts (organs) were larger than that of total body length for the second principal component (Z2). A larger tail annulation number and greater body width would result in a smaller value for Z2. In contrast, a longer esophagus and more posterior position of the excretory pore would increase the value for Z2. This second principal component is related to the part characters or shape factors rather than size. There were some relationships between the second principal component and De Man's values ("a" and "c"): Nematodes with relatively larger Z2 values have larger "a" and "c" (Table 1, Figs. 6 and 7).

TABLE 2. Average, standard deviation, and correlation coefficient matrix among 11 characters on eight populations of *Rotylenchulus reniformis* in Japan (n = 160).

Varble	Cod	Av. (µm)	SD (µm)	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Body l.	X1	396.2	36.84	1.00										
Egor	X2	15.0	2.88	0.55	1.00									
Excrp	X3	83.6	7.74	0.66	0.49	1.00								
Eso l	X4	110.7	10.67	0.55	0.36	0.60	1.00							
Tail l	X5	28.4	4.04	0.67	0.43	0.47	0.29	1.00						
Phas	X6	22.3	3.76	0.61	0.40	0.46	0.38	0.82	1.00					
Hd sc	X7	7.1	0.39	0.63	0.44	0.51	0.44	0.59	0.56	1.00				
Styl l	X8	18.4	0.83	0.48	0.18	0.43	0.42	0.53	0.48	0.69	1.00			
Grbw	X9	16.2	1.00	0.32	0.11	0.24	0.11	0.54	0.44	0.52	0.62	1.00		
Hyal p	X10	8.9	2.40	0.50	0.35	0.35	0.34	0.62	0.62	0.60	0.57	0.42	1.00	
Ann t	X11	19.9	3.14	0.21	0.07	0.10	0.01	0.56	0.41	0.18	0.21	0.37	0.05	1.00

Varble = Variable; Cod = Code; Av = Average; SD = Standard Deviation; Body l = Body length; Egor = Position of esophageal grand orifice; Excrp = Distance of excretory pore from head end; Eso l = Length of esophagus; Tail l = Tail length; Phas = Position of phasmid from tail end; Hd sc = Width of head sclerotization; Styl l = Stylet length; Grb w = Greatest body width; Hyal p = Length of hyaline portion; Ann t = Annulation number on ventral side of tail.

TABLE 3. Principal component matrix with loadings and contribution rate of the three components.

Variable	Symbol	1st Principal (Z1)	2nd Principal (Z2)	3rd Principal (Z3)	Contribution rate (%)
Body l	x1	0.349	0.210	0.168	76.4
Egor	x2	0.242	0.336	0.361	62.9
Excrp	x3	0.296	0.351	0.113	68.2
Eso l	x4	0.248	0.428	- 0.052	61.9
Tail l	x5	0.365	- 0.231	0.250	87.7
Phas	x6	0.351	- 0.150	0.185	74.6
Hd sc	x7	0.350	0.025	- 0.253	74.0
Styl l	x8	0.317	- 0.125	- 0.470	80.5
Grb w	x9	0.262	- 0.428	- 0.284	73.9
Hyal p	x10	0.312	- 0.015	- 0.300	62.8
Ann t	x11	0.158	- 0.510	0.523	81.8
Eigen valueλK		5.484	1.523	1.043	
$\sqrt{\lambda K}$		2.342	1.234	1.021	
$\sum\lambda K$		5.484	7.007	8.050	Av
Contribution rate(%)		49.85	63.70	73.18	73.2

The third principal component (Z3) was described as follows:

$$Z3 = 0.168x1 + 0.361x2 + \bullet\bullet\bullet + 0.523x11 \qquad (3)$$

Both positive and negative loadings were also included on variables for this component (Z3). Positive and largest loading (0.523) on tail annulation number was contrasted with that for the second principal component. The third principal component would be defined as details of body structures or organs.

The percentage of variability explained by the three principal components was 73%, and variability in tail length was the best explained among 11 variables, ranging from 62% to 88% (contribution rate).

The score of each individual in eight populations (n = 160) used was calculated by formulas (1), (2), and (3), and then the Z2 component (ordinate) for each was plotted against the Z1 component (abscissa) in two dimensional coordinates (Figs. 6 and 7) to test grouping of the nematodes. At a glance, scores scatter over all areas on the Z1 and Z2 -axis, and there are about three groups of scores that can be discriminated by the localities from where the nematodes were collected. The first group was defined as large scores of both Z1 and Z2 components, and the second group was characterized by small Z1 but large Z2 scores. The third group had small scores, scattering over a range from about +0.5 to -1.5 or -2.5 for both Z1 and Z2 components. Almost all individuals of Asahi and Ehime populations were included in the first group and all nematodes of the Mizuho population and a part of the Aino belonged to the second group. Nematodes of the Tokorozawa, Hiroshima, Miyazaki, Akune, and most of the Aino populations were in the third group. Nematodes of the Miyazaki population appeared to have smaller scores for both components, compared with other populations.

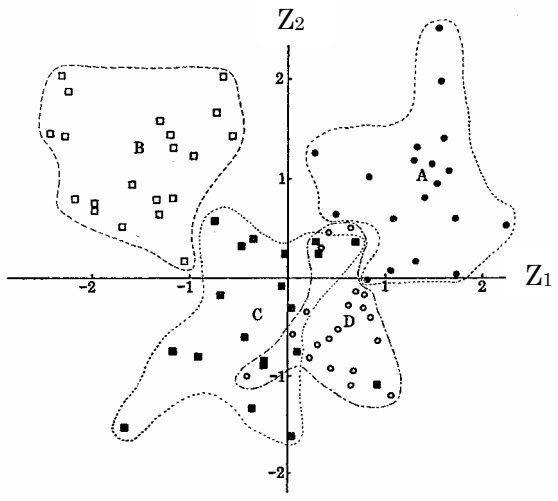

FIG. 6. Scattering map of scores on the first principal component (Z_1) and the second principal component (Z_2) calculated with the Asahi, Tokorozawa, Mizuho and Akune populations. A) Asahi. B) Mizuho. C) Tokorozawa. D) Akune.

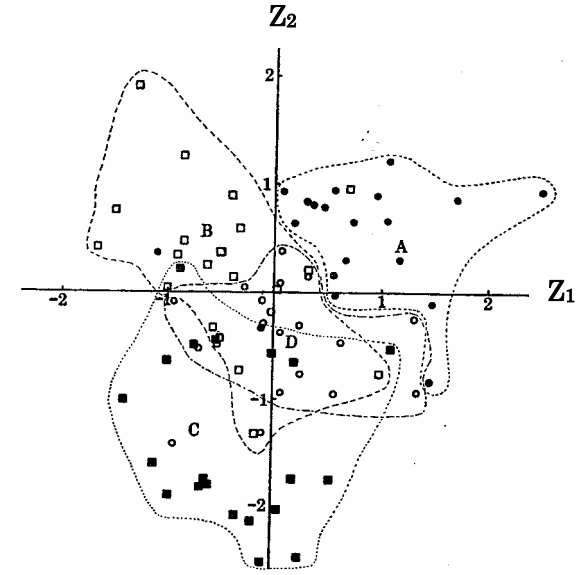

FIG. 7. Scattering map of scores on the first principal component (Z1) and the second principal component (Z2) with the Hiroshima, Ehime, Aino and Miyazaki populations of *Rotylenchulus reniformis*. A) Ehime. B) Aino. C) Miyazaki. D) Hiroshima.

3. Discussion

Although considerable variations of measurements on young females from nine populations of *R. reniformis* were investigated, there were some characteristic differences in the average values of characters, such as body length, greatest body width, stylet length, orifice position of esophageal gland, and tail portion. The Asahi and Ehime populations showed the largest values in body length and certain other characters, which contrasted with smallest or shortest values in the Mizuho, Hawaii, and Miyazaki populations. The remaining populations such as Tokorozawa, Hiroshima, Aino, and Akune were intermediate.

Comparison of characters was complicated by De Man's allometric values ("a", "b", and "c") as above-mentioned. The reason for this complication is considered to be that the extent of differences in body size and in other morphological characters is decreased by applying De Man's formula when allometric growth of nematode is the same among populations with a similar range of morphological variation. In Figure 8, the relationship between body length and greatest body width in the Asahi, Ehime, and Mizuho populations, which showed similar values in De Man's "a" (body length divided by the greatest body width), is presented. Body length and greatest body width were considerably different between the Asahi and Mizuho populations, but the slope of the regression of body length on the greatest body width appeared to be similar between the two, while slope of the regression in the Ehime population was different from that of the former two while scattering of the data was similar to that of Asahi population. This probably means that similarity in variability of measurements of characters results in a similar value by De Man's formula even when the slope of the regression between those characters is different among populations or species of nematodes. The relationship between "a" value by De Man's formula and the regression coefficient between the greatest body width and body length in the nine populations is illustrated in Figure 9. Here, De Man's "a" values and the regression coefficients showed a generally linear relation, with the exception of the Ehime population (I in Fig. 9), which had about the same "a" value as the Asahi and Mizuho populations.

According to Geraert (1968), De Man's (allometric) values, which are commonly used for species description of nematodes, have almost no meaning for morphological comparison in many cases because of wide variations of those characters. Three sources of individual variation in morphological characters are known as follows: first, allometric growth of characters does not fit a linear regression through the origin in most nematode species (Wu, 1960), secondly, nematode growth and morphological characters are apt to be affected by many kinds of environmental factors such as temperature, foods (kind, quality, quantity, or hunger), aging, and population density (Bird and Mai, 1967; Cayrol and Legay, 1967; Coomans, 1963; De Grisse and Loof, 1970; El-Sherif, 1972; Evans and Fisher, 1970a; 1970b; Fisher, 1966a; b; Geraert, 1978; Goodey, 1952; Gysels and Bracke, 1964; Kline, 1976; Monoson, 1971; Taylor and Jenkins, 1957), and thirdly, some geographical factors cause variation in nematode morphology (Bird and Mai, 1967). Variation in V value (length of body from head end to the position of vulva divided by entire body length expressed as a

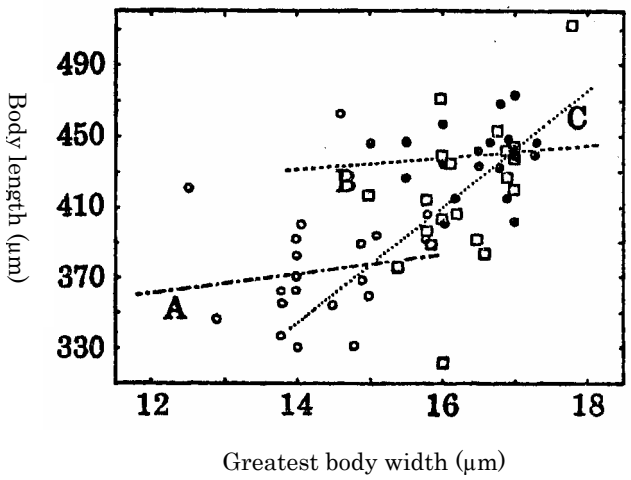

FIG. 8. Regression of the body length on the body greatest width of young females in Asahi, Ehime and Mizuho populations. Regression line, A) Mizuho (empty circle). B) Asahi (black). C) Ehime (rectangle). $n = 20$ in each population.

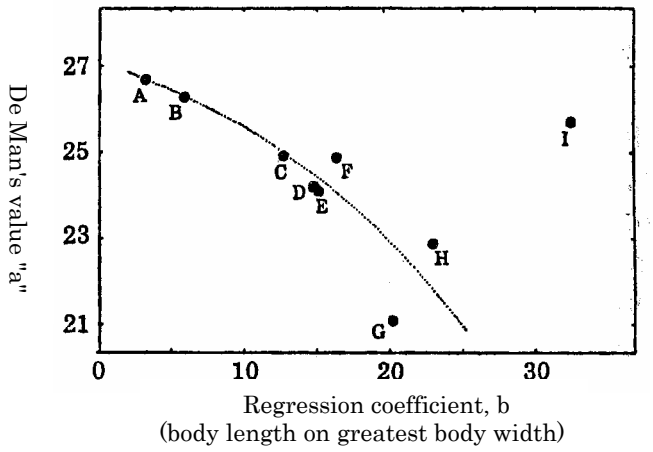

FIG. 9. Relationship of deMan's "a" and regression of the body length on the greatest body width in 9 populations of *Rotylenchulus reniformis*. A) Asahi. B) Mizuho. C) Akune. D) Hiroshima. E) Aino. F) Hawaii. G) Miyazaki. H) Tokorozawa. I) Ehime.

percentage) is commonly recognized to be narrow in many nematode species. It is evident that morphological variations investigated in nine populations of *R. reniformis* involved the first source of variation mentioned above, i.e. allometric non-linearity, as seen in Figure 8. As to the second source, air temperatures appear to vary with geographical locations, and soil temperatures in nematode habitats will be affected by environmental conditions, such as, for example, differences in kinds of plants growing around the habitat. From a global perspective, soil temperature in the nematode habitat can be considered to be correlated with air temperature. Therefore, it is reasonable to examine air temperature instead of soil temperature in relation to morphological variation of nematodes. If temperature, which varies with geographical change, influenced morphological variation of this nematode, a cline was expected to be seen in the populations measured but no such cline was observed. Thus temperature seems to contribute little to the morphological variation of this nematode. Population densities in soils were commonly moderate when nematodes were extracted in the laboratory, except for that of the Mizuho population, which was considerably higher than the others. Aging of nematodes appeared to be about the same. This is likely because nematode specimens of each population were collected during September to October, except for the Hawaii population, which was extracted in July. Thus, specimens collected likely were newly hatched during the growing season, rather than old nematodes, that had over-wintered.

As seen above, wide variations of De Man's values observed in nine populations of *R. reniformis* appear to have resulted mainly from the first source of variation. This probably means that this nematode species is too morphologically variable to apply De Man's formula to examine intra-specific variation.

Principal components analysis demonstrated that 64% of original information of 11 characters in the young female was summarized with the first and second principal components (Z1 and Z2). Scores of the two components for eight geographically different populations were scattered on the Z1 and Z2 axes but partitioned into three characteristically defined groups. Nematodes in the first group were comparatively large and (or) long in entire body length as well as other detailed body parts or organs, such as stylet length, orifice position, and excretory pore position, but were characterized by a relatively narrow body width, longer hyaline portion, and smaller number of annulations of tail. The second group was characterized by small values for an entire body length, stylet length, body width, and tail annulation number but with a relatively long esophagus and high values for distance of orifice and excretory pore position. The third group exhibited a feature that contrasted with the former two groups, namely, lengths of entire body and esophagus were not great, and the orifice of esophageal gland was a shorter distance from stylet knobs than in other groups, while relatively greater values were seen for body width and number of tail annulations.

The first group included most individuals of Asahi and Ehime populations, which were referred to as the MRT in terms of male occurrence as mentioned before, and the second group included all individuals of the Mizuho population, which were recognized as the MNT and some of the Aino population. Probably the Hawaii population would be placed in this group if it were combined with the other eight populations for computing. These two groups are distinguished from each other by entire body length (Table 1) and particularly with different tail shapes as shown in Figure 4, suggesting a possibility to be distinguishable biological units (taxa). The third group consisted of individuals, mostly of the Aino, Tokorozawa, Akune, Hiroshima, and Miyazaki populations, and their biological type was the MAT. Most individuals of this group appeared to have morphological characteristics intermediate between the first and the second groups as described by Dasgupta et al. (1968), but it was noteworthy that their body shape was characterized by a comparatively broad body with a high number of tail annulations. Further study must be made on details of morphological characters in males, mature females, and J2 with scanning electron microscopy.

Morphological comparison by De Man's values did not give clear results due to complicated variations in characters, but application of the principal compo-

TABLE 4. Males extracted from sweet potato field soils in Kaijou Branch, Asahi town, Chiba prefecture and in Wakimoto district, Akune city, Kagoshima prefecture.

Location	Young female	Male	J2[a]	Total	Ratio (♂/♀)
Asahi[b]	25,607	8	6,733	32,348	0.00031
Akune[c]	4,050	0	211	4,261	0

[a] J2 = second-stage juvenile.
[b] sample size was 3.8 liters.
[c] Sample size was 3.0 liters.

nents analysis to morphological information for eight populations of *R. reniformis* as illustrated here clearly showed three distinct morphological groups.

IV. Rates of Male Frequency on Field and Laboratory Populations

Three morphological groups were distinguished among geographical populations of *Rotylenchulus reniformis* in Japan, and three biological types based on differences in rates of male occurrence were noted. In this section, the difference or variation in frequency of males among the geographical populations was studied to determine whether the rate of male occurrence in a population is controlled only by environmental factors or by genetic factors through experiments on nematodes collected in fields and from laboratory populations (isolates).

1. Male frequency in field populations

Both fields, where males were rarely encountered and not encountered, were examined for rates of male occurrence.

Materials and methods

Fields examined: Sweet potato (cv. Norin No. 1) field in Kaijou Branch of the former National Agricultural Experiment Station, Asahi town, Chiba prefecture and sweet potato (cv. unknown) field of a farmer in Wakimoto district, Akune city, Kagoshima prefecture (Type locality of the syn. *R. nicotiana* Yokoo and Tanaka, 1954).

Soil sampling and nematode extraction: 3.8 liters soil was collected from the 3 to 15-cm deep soil layer at several sites in the Kaijou Branch field (designated as the Asahi) on September 5 1966, and 3 liters soil was from the field of Wakimoto district (designated as the Akune) by the same method as that in the Asahi on May 6, 1966. All soil of each sample collected was processed to extract nematodes in the laboratory by SSB 2 days after soil sampling for the Asahi, and 3 days after collection for the Akune population.

Results

As shown in Table 4, 25,607 young females were extracted along with eight males from the Asahi soil, yielding a ratio of males to young females of 0.00031, whereas 4,050 young females were detected along with 200 J2 from the Akune soil and no males were encountered as when sampled previously. Sampling time was in autumn for the Asahi and early summer for the Akune population. It was not known whether sampling time (season) affected the difference in male occurrence between the two localities or not. If the biological potential for male occurrence were the same between the two nematode populations, at least one male would be expected to appear in the nematode population extracted from the Akune soil but no males were recognized. The results obtained here seem to support the biological types described in section II, in which the Asahi population was defined as MRT and the Akune as MAT.

2. Male frequency in three populations of Mizuho town, Nagasaki prefecture under laboratory conditions

Isolated nematode populations (designated as "laboratory population"), which were originally collected in the fields of Mizuho town, Nagasaki prefecture, and described in section II, were tested for male frequency under greenhouse and laboratory conditions. Male occurrence was examined successively during a period spanning the first three generations and, after that, the laboratory populations were carefully maintained with potted sweet potato as previously mentioned to confirm stability of the biological characters in terms of male occurrence after 12 or 14 years of culturing.

TABLE 5. Sex ratio in the second generation of population MIZ-1 (experiment 1).

Code of vial (egg-mass)	Male	Female*	Sex ratio (♂/♀)
1	30	22	1.4
2	32	36	0.9
3	58	46	1.3
4	69	50	1.4
5	25	26	1.0
6	13	14	0.9
7	17	19	0.9
Average	34.9	30.4	1.1

*Young female.

Materials and methods

Nematode populations tested: Three populations were collected in the fields of Ogaguchi district, Mizuho town, Nagasaki prefecture (described in section II) as follows. (1) Population MIZ-1: Soil samples were collected from the central part in the plot no. 5 (host: sweet potato) of Field A (Fig. 2) in January 1964. (2) Population MIZ-2: Soils were collected from the plot 10 (great burdock) in Field A (Fig. 2) in January 1964. (3) Population MIZ-3: Soil was sampled from the central area in Field B (sweet potato) in September 1962.*[11] Volume of soil samples was about 800 ml each, and part of the soil was used for examining the nematode population as described in section II. Biological type was the MNT for population MIZ-1 and MIZ-3, and MAT for population MIZ-2.

Experiment 1: Soils collected were put in big clay pots (see section I) and planted to great burdock (cv. Takinogawa) in a greenhouse with temperature of 25-28 ˚C. After 2 months of cultivation, roots with parasitizing females of *R. reniformis* were removed from pot soils and washed gently with tap water. Egg-masses enveloping female bodies on roots were picked up under a stereo microscope and kept separately in a small amount of distilled water in 6-ml vials, which were covered with screw caps that had small pores for air exchange. The vials were stored in an incubator at 25 ˚C for 40 days. After that, young females and adult males, which hatched from eggs and developed in the vial water without feeding, were examined for sex ratio. (The hatched second-stage juveniles of *R. reniformis* can undergo three molts to develop to young females or adult males in distilled water without any uptake of food from outside of body, as described in section VII.)

Experiment 2: Effect of host plants for the second generation's nematodes obtained in experiment 1 (population MIZ-1) on the sex ratio in the third generation was tested here. Steam-sterilized "Nishigahara soil" (section I) was put in big clay pots (section I) and tomato (cv. Fukuju No. 2), or great burdock (same as above) were planted. Nematodes developed in each vial numbered 3, 4, 5, and 7 (Table 5) were separately inoculated onto the potted plants and cultured in a greenhouse as in experiment 1. About 2 months later, egg-masses obtained from each pot were separately incubated in vials as in the previous test and nematode sexes were examined again. Soil populations in each pot were also examined by extracting nematodes from the entire soil content of pots with SSB.

Experiment 3: Nematodes developed in the no. 5 vial (egg-mass), in which males and females emerged in experiment 1 (population MIZ-2, Table 6) were used to inoculate potted tomato plants and cultured as in the previous test. The sex ratio of the next generation also was examined similarly.

Experiment 4: Remaining soils infested with population MIZ-2 or MIZ-3 were inoculated to sweet potato (cv. Norin No. 1) planted in sterilized-mixed soils in wooden and plastic containers, and carefully cultured in a greenhouse for 12 (MIZ-2) or 14 (MIZ-3) years (see section I for details). At the termination of culturing in 1976, egg-masses on roots of sweet potato were picked up under a stereo microscope, and sex ratios of nematodes were examined after having developed in vials, as in experiment 1. At the same time, 100 ml aliquots of soil were sampled from culturing containers and processed to extract nematodes by SCF (see section I) in order to examine nematodes for sex ratio. Three replications of 100 g soil were extracted.

Nutrients: Host plants in this series of experiments were supplied with 0.5 g ammonium sulfate, 0.5 g calcium superphosphate, and 0.5 g potassium chloride per pot.

[11] Erratum: year 1963 in the original paper, corrected with 1962 as in section II, p. 6 (by the translator = the author).

TABLE 6. Sex ratio in the second generation of population MIZ-2 (experiment 1).

Code of vial	Number of egg-mass / vial	Male	Female*	Sex ratio (♂/♀)
1	4	0	53	0
2	4	0	37	0
3	4	0	62	0
4	5	0	174	0
5	3	43	62	0.7
6	3	0	92	0
7	2	0	47	0
Average	3.6	6.1	75.3	0.1

*Young female.

Results

Experiment 1: Results are shown in Tables 5 and 6. In population MIZ-1, all seven egg-masses tested gave both males and young females, and sex ratios ranged from 0.9 to 1.4 with an average of 1.1. On the other hand, more than one egg-mass were incubated together in a vial for population MIZ-2 and six vials out of seven contained only young females without males; the remaining vial, no. 5, showed occurrence of males with young females with a sex ratio of 0.7. Thus, most nematodes in population MIZ-2 were of the MAT, but a few nematodes of the MNT coexisted with them.

Experiment 2: The ratio of males to females in the third generation after inoculation of *R. reniformis* males and females to great burdock or tomato was not significantly different on the two hosts (Table 7). This suggests that there was no affect of these hosts on the sex ratio of *R. reniformis* in subsequent generations of the nematode.

Experiment 3: Only two egg-masses were available to measure the sex ratio in this experiment and no males emerged from them, as shown in Table 8. Despite apparent coexistence of MAT and MNT populations in vial no. 5, in which three egg-masses were incubated (Table 6), only young females developed. It was not known why nematodes of the concomitant MNT population failed to parasitize potted tomato roots.

Experiment 4: Male frequencies of populations MIZ-2 and MIZ-3 in the egg-mass vial incubation test and in the soil extraction survey were significantly different for the two populations even after 12 or 14 years of culture in a greenhouse (Tables 9 and 10). No males of the MIZ-2 population were detected in either the vial incubation test of egg-masses or in extractions of nematodes in soil from the culturing containers. In contrast, population MIZ-3 had almost the same numbers of males and young females in egg-mass incubations and in extracted soil populations. The original MIZ-3 population had abundant males and females present when collected in September 1962. The production of abundant numbers of males and females appears to have remained stable over the 12–14 years of culture under greenhouse conditions. The original MIZ-2 population was contaminated with a few individuals of the MNT population that were responsible for the occasional presence of egg-masses with males prior to the 12–14 years of culture. After 12–14 years of culture there were no males detected in the vial incubation test or from soil indicating that the MNT individuals present may have died out altogether.

3. Male frequency in male-numerous type populations under laboratory conditions

Male occurrence in three MNT populations (MIZ, HAW, and TEX, see section I for details) maintained in the greenhouse was tested to confirm their stability.

Materials and methods

"Steam-sterilized-mixed soil" was put in "big clay pots" (see section I) and nematode-free tomato seedlings (cv. Fukuju No. 2, with four leaves) were planted in each pot. After 24 hours, potted tomatoes were inoculated separately with nematodes extracted from the propagation container soils of the MIZ, HAW, and TEX populations as follows: MIZ with 2,012 nematodes per pot and a sex ratio (male/female) of 0.5, HAW with 2,790 nematodes per pot and 0.9 sex ratio, and TEX with 1,582 nematodes per pot and 0.9 sex ratio. Four inoculated pots per population were maintained in a greenhouse from late August to late September. Two milliliters of "Hoagland nutrient solution" (Hoagland and Arnon, 1950) was supplied to pots once a week. After 18 days of culturing, two pots from each population were randomly selected to

TABLE 7. Sex ratio in the third generation of population MIZ-1 as affected by host plants for the second generation (experiment 2).

Inoculum		Host plant	Material examined	Male	Female	Sex ratio (♂ / ♀)
Vial code in experiment 1	Nematode number/pot ♂ : ♀					
3	58 : 46	Great burdock	Soil	4	12	0.3
4	69 : 50	Great burdock	Egg-mass 1	35	25	1.4
		Great burdock	Egg-mass 2	55	51	1.1
		Great burdock	Egg-mass 3	31	12	2.9
		Great burdock	Soil	11	6	1.8
5	25 : 26	Tomato	Egg-mass 1	39	22	1.8
		Tomato	Egg-mass 2	1	3	0.3
		Tomato	Egg-mass 3*	1	0	-
		Tomato	Egg-mass 4	0	0	-
		Tomato	Egg-mass 5	16	13	1.2
		Tomato	Egg-mass 6	5	5	1.0
		Tomato	Egg-mass 7	0	0	-
		Tomato	Egg-mass 8	50	57	0.9
		Tomato	Soil	6	2	3.0
7		Tomato	Egg-mass 1	24	24	1.0
Average		Tomato		-	-	1.5
		Great burdock		-	-	1.3

This included 61 eggs, remaining unhatched in the incubation vial.

extract nematodes from pot soils by SCF in order to examine sexes. Egg-masses on roots were also collected and incubated in petri dishes of 7 cm diam. with two replications, in which 60 egg-masses per dish were arranged on a layer (3-mm depth) of 0.6% water agar. The dishes with egg-masses were maintained in an incubator with constant temperature of 28 ℃ for 30 days, and sexes of developed nematodes in dishes were examined under a stereo microscope (40×). Remaining pot cultures were maintained further in the greenhouse until 33 days after inoculation and processed to extract nematodes from soils, and egg-masses from roots were picked up for egg-mass incubation tests as in the previous 18-day treatment. Soil temperature in the greenhouse fluctuated between 18 and 32 ℃ during the culturing period.

Results

As shown in Tables 11 and 12, some survivors from the primary inoculum (starved males and starved young females with translucent intestines) still remained in soil 18 days after inoculation. Many egg-masses were already produced by adult females on tomato roots, and newly hatched J2s and developing juveniles (molting juveniles of J2, J3, and J4) were also extracted from pot soils, but newly developed males and young females were not yet present. Egg-masses harvested 18 days after inoculation hatched well and developed to males and young females on water agar in petri dishes during 30 days of incubation at 28 ℃. Males that developed in the dishes were about the same in number as females in

TABLE 8. Sex ratio in the third generation of the vial no. 5, with male occurrence of population MIZ-2 in experiment 1 (experiment 3).

Egg-mass code	Male	Female	J2	Sex ratio (♂ / ♀)
1	0	4	0	0
2	0	51	47	0

TABLE 9. Differences in male occurrence with the vial test of eggs between population MIZ-2 and MIZ-3 after 12 years (MIZ-2) or 14 years (MIZ-3) of culture in greenhouse.

Egg-mass code	MIZ-2				MIZ-3				
	Male	Female	J2	Eggs*	Male	Female	♂/♀	J2	Eggs*
1	0	28	0	34	30	30	1.0	2	17
2	0	22	0	13	39	42	0.9	6	21
3	0	43	4	22	15	18	0.8	0	43
4	0	6	0	0	12	16	0.8	1	26
5	0	85	4	28	22	18	1.2	0	23
6	0	124	1	28	30	36	0.8	3	5
7	0	70	4	23	10	13	0.8	2	3
8	0	26	1	20	18	15	1.2	4	5
9	0	64	0	24	50	35	1.1	0	12
10	0	95	3	44	51	49	1.0	5	64
11	0	80	0	71	47	49	1.0	2	71
12	0	61	0	29	50	55	0.9	6	29
13	0	101	2	13	35	45	0.8	1	34
14	0	117	2	25	20	31	0.6	5	0
Average	0	66.5	1.5	26.7	30.6	33.0	0.9	2.6	25.2
S.E.**	0	9.6	0.4	4.4	4.0	4.0	0.1	0.9	5.9

* Unhatched eggs. ** Standard error.

TABLE 10. Differences in male occurrence of soil populations between population MIZ-2 and MIZ-3 after 12 years (MIZ-2) or 14 years (MIZ-3) of culture in greenhouse.

	MIZ-2			MIZ-3			
	Male	Female	J2	Male	Female	Sex ratio (♂/♀)	J2
Average	0	78.0	184.3	1,286.5	1,270.3	1.0	1,250.4
S.E.	0	7.4	21.5	52.0	84.1	0.1	75.0

Remarks: Nematode numbers per 100 g soil, with 3 replications; S.E.: Standard error.

TABLE 11. Male frequency in pot soils of the MIZ, HAW, and TEX populations 18 days and 33 days after inoculation.

Population code	Culturing duration (day)	Pot code	Egg-mass on roots	J2-J4 juvenile	Male	Female	Inoculum (nematode)		Sex ratio (♂ / ♀) ***
							Male*	Female*	
MIZ	18	1	+++ **	3,765	0	0	27	0	-
		2	+++	6,301	0	0	161	80	-
	33	3	+++	5,930	3,455	3,989	539	60	0.9(1.0)
		4	+++	14,206	3,298	3,944	680	34	0.8(1.0)
HAW	18	1	+++	12,683	0	0	80	0	-
		2	+++	10,333	0	0	27	27	-
	33	3	+++	29,571	5,861	5,461	1,166	100	1.1(1.3)
		4	+++	17,450	2,764	3,996	599	133	0.7(0.8)
TEX	18	1	+++	5,341	0	0	0	0	-
		2	+++	6,087	0	0	0	27	-
	33	3	+++	10,257	966	1,499	699	0	0.6(1.1)
		4	+++	11,622	3,230	4,362	666	167	0.7(0.9)

*Identified by extent of exhausted intestine of nematodes; ** numerous; ***numbers in parentheses include the exhausted nematodes (= inoculum individuals).

TABLE 12. Male and young female occurrence from the egg-masses harvested 18 days after inoculation with the MIZ, HAW, and TEX populations (average of two replications).*

Population code	J2 - J4	Male	Young female	Sex ratio (♂ / ♀)	Average juvenile number hatched / egg-mass
MIZ	87.5	937.0	1,133.5	0.8	36.0
HAW	146.5	813.5	978.5	0.8	32.3
TEX	286.0	670.0	670.0	1.0	27.1

* Egg-masses were incubated on agar layer in petri dishes at 28 ˚C for 30 days.

all three MNT (MIZ, HAW, and TEX) populations (Table 12). After 33 days' culture, pot soils harbored newly developed males and young females in each population, and sex ratio was 0.9 on the average for the MIZ and HAW but 0.7 for the TEX populations (Fig. 11).

As demonstrated above, although there was a tendency for male frequencies to be lower than female frequencies in the three populations tested, the occurrence of males was a stable characteristic for these MNT populations. The fewer numbers of males than females may be due to differing rates of development for males and females in those instances where large numbers of juveniles and eggs were present. It may also be an artifact of different extraction efficiencies for the various nematode stages when extracted with the Baermann funnel method (SVB).

4. Male frequency in laboratory populations under different soil temperatures

Effect of soil temperature, which is one of the important environmental factors affecting nematode growth and development, on male frequency was tested.

Materials and methods

Measurements of effects of temperature on male occurrence were made in the experiments on effects of soil temperatures on nematode growth described in section VII and are presented here. Summarized materials and methods were as follows-- Nematode populations tested: MNT population, MIZ, HAW, TEX, and M×H (see section I); MRT population, ASH-a, and ASH-b; MAT population, SIB and AKN. Host plant was tomato (cv. Fukuju No. 2) planted in small clay pots. Thirteen soil temperature treatments ranged from 15.9 to 33.4 ℃. Pot soils were processed to extract nematodes by SCF. Nematode numbers were averaged over four to six successive extractions conducted during the late period of culturing (see section VII- 5 for details) to compare frequency in male occurrence among biological types.*[12]

Results

As shown in Table 13, sex ratios in MNT populations ranged from 1.0 to 3.0 in all temperature treatments. The MIZ population had a maximum value of 2.1 at 16.9 ℃ and a minimum of 1.1 at 33.4 ℃, so that sex ratio increased as the soil temperature decreased. In the HAW population, the maximum value was 1.9 at 26.4 ℃, while the TEX and M×H populations showed maximum ratios of 3.0 and 1.4 at 25.2 ℃, respectively. The ASA-a and -b of MRT population produced a low frequency of males at 29.2 ℃ and 19.9 ℃ (sex ratios of which were 0.0004 or less), but the SIB and AKN of the MAT population did not produce any males in any temperature treatment.

Results obtained here suggest that soil temperature does not significantly alter male occurrence for any biological type of *R. reniformis.* Apparently, there are no special temperatures that can change or reduce male frequency in MNT populations and there are no temperatures that induce or increase male emergence in MRT and MAT populations. A sex ratio of 3.0 in the TEX population at 25.2 ℃ appears to be caused by factors other than temperature. In this population, some males and females infected with *Pasteuria* sp., a bacterial parasite of nematodes, were observed late in the experiment.

5. Male frequency in laboratory populations with different host plants

Effect of host plants on male occurrence was tested by using laboratory populations.

Materials and methods

Host plants tested: Cowpea (cv. unknown), great burdock (cv. Takinogawa), tomato (cv. Fukuju No. 2), and sweet potato (cv. Norin No. 2). Three seeds of

[12] This sentence was dropped in the original paper (the translator = the author).

TABLE 13. Sex ratio (male/female) of eight laboratory populations as affected by soil temperatures (average of four to six replications).

Soil temperature (°C)	Laboratory population tested*							
	MIZ	HAW	TEX	M×H	ASH-a**	ASH-b**	SIB**	AKN**
33.4	1.1	1.3	-	-	-	-	-	-
33.0	1.1	1.2	1.0	-	-	-	-	-
30.5	1.3	1.6	-	-	-	-	-	-
29.8	-	-	1.1	1.0	-	-	-	0
29.2	-	-	-	-	0.0004	0.00004	0	-
26.4	1.8	1.9	-	-	-	-	-	-
25.8	-	-	-	-	0	0	0	-
25.2	-	-	3.0	1.4	-	-	-	0
23.0	1.2	1.8	-	-	-	-	-	-
19.9	-	-	1.0	1.1	0.0005	0	0	0
16.9	2.1	1.6	-	-	-	-	-	-
16.6	-	-	-	-	0	0	0	-
15.9	-	-	1.1	1.0	-	-	-	0

* No treatment was applied. ** Lack of treatment at more than 30°C was due to difficulty of development of nematodes in MRT and MAT populations (see section VII for details).

each of the first three crops or one vine cutting of sweet potato were placed in big clay pots (see section I) with "steam-sterilized mixed soil" and plants were carefully grown in the greenhouse with soil temperatures ranging from 22 to 30 ℃ for a month. After that, nematodes of six laboratory populations, which were extracted by SVB from the propagation containers, were inoculated as follows: MIZ population with 1,753 individuals per pot (sex ratio: 0.8); HAW with 3,436 (0.8); TEX with 1,956 (0.9); ASH-a with 1,948 (0); SIB with 2,071 (0); AKN with 2,088 (0). The test had one replication. Diluted Hoagland nutrient solution (1/2 strength) was applied to pots with 2 ml once a week. After 37 days of culturing, pot soils were processed to extract nematodes by SCF and nematodes were examined for male occurrence under a stereo microscope.

Results

As shown in Table 14, each of three MNT populations (MIZ, HAW, and TEX) produced males on all four test plants with some variation in sex ratio. In contrast, none of the MRT (ASH-a) or MAT (SIB and AKN) populations had males on any plant. The ratio (1.9) of the HAW population on great burdock was greater than on other plants, and the ratio (0.4) of the TEX population on cowpea was the lowest among the four plants.

From these results it can be concluded that the host plant may have some effect on the occurrence of males in MNT populations. However, there were no host plants that prevented the occurrence of males.

6. Male frequency in laboratory populations with host plants cultured at different nutrient levels

In this study, a test was conducted to determine whether male occurrence is affected by the level of nutrients supplied to the host plant.

Materials and methods

Nematode populations tested: The same as in the previous study, namely, the six populations, MIZ, HAW, TEX, ASH-a, SIB, and AKN.

Host plant: Tomato (cv. Fukuju No. 2) seeds were planted to small clay pots with "steam-sterilized mixed soil," and a set of pots for each population tested was separately embedded in steam-sterilized sand within large plastic containers (the same as for nematode propagation, see section I) to protect pots from severe desiccation. Tomato plants in pots were thinned to one plant per pot and grown in a growth cabinet under sunlight, in which soil temperature was maintained at 29 ℃ when light and 25 ℃ in the dark. All pots were supplied with 2 ml diluted Hoagland's solution (1/2 strength) per pot at the beginning of plant growth and the pot set for each nematode population was divided into two parts, one of which was for sufficient nutrient treatment (SNT) and the other for insufficient nutrient treatment (INT). SNT was given the same amount of the diluted Hoagland's solution each week thereafter when

TABLE 14. Differences in male occurrence of six laboratory populations as affected by host plants.

Popul	Host plant tested **											
	Cowpea			Great burdock			Sweet potato			Tomato		
	Mal	Fem	S.ra	Mal	Fem	S.ra	Mal	Fem	S.ra	Mal	Fem	S.ra
MIZ	5,688	7,238	0.8	1,575	1,450	1.1	10,2 37	11,651	0.9	3,809	4,926	0.8
HAW	862	920	0.9	633	340	1.9*	533	534	1.0	6,327	6,127	1.0
TEX	240	644	0.4*	733	801	0.9	3,402	3,064	1.1	8,823	7,638	1.2
ASH-a	0	509	0	0	1,203	0	0	160	0	0	209	0
SIB	0	1,022	0	0	717	0	0	4,465	0	0	185	0
AKN	0	1,185	0	0	1,790	0	0	912	0	0	801	0

* $P \leq 0.001$(χ2 test). ** Popul = Population; Mal = Male; Fem = Female; S.ra = Sex ratio.

INT received only tap water. Isolation of each nematode population with plastic plates (see section I) and watering were also carefully done.

Thirty-three days after germination, tomato plants grew to have three or four leaves and those plants in pots of INT seemed to be pale green compared with those in SNT pots. Thirty-five days after seeding, potted plants in both SNP and INP were inoculated with nematodes as follows. MNT populations: MIZ with 1,295 individuals per pot (sex ratio = 0.7), HAW with 1,296 (0.6), TEX with 1,024 (0.4); MRT population: ASH-a with 800 (0); MAT populations: SIB and AKN with 800 (0) each. Seventy days after inoculation, tomato roots were removed from pots and washed gently with tap water. Fresh root weights were measured and pot soils were processed to extract nematodes by SCF. Nematode numbers per pot and sexes were determined under a stereo microscope. Five replications were included for each combination of nutrient treatment and nematode population.

Results

As shown in Table 15, root weights were significantly greater in the SNT than that in the INT. Average nematode numbers per pot (five replications) showed different tendencies among the nematode populations. Nematodes in the younger stages (J2 to J4) were more numerous in the SNT than in the INT treatment, but male and female numbers appeared unrelated to host nutrient treatments. Sex ratio in each nematode population of MNT was not significantly different between the SNT and INT. It was interesting that a very small number of males occurred in the INT of ASH-a (MRT) and of AKN (MAT). The 0.0001 sex ratio for the AKN was calculated based on one male and this was the first example of male occurrence in this population.

The results obtained here suggest that the occurrence of males in the six laboratory populations including MNT, MRT, and MAT was not affected by poor growth due to nutrient deficiencies of the host plant. This was true for both the MNT populations with abundant males as well as MRT and MAT with few or no males. ASH-a and AKN populations produced no males under sufficient nutrient conditions, but a few were produced under insufficient nutrient conditions. However, the low numbers of male nematodes present made it difficult to attribute the difference to plant nutrient levels.

TABLE 15. Differences in male occurrence of six laboratory populations as affected by host plant nutrients (soil populations on the average with standard error, five replications).

Popu*	Sufficient nutrient treatment					Insufficient nutrient treatment				
	Root (g)	Mal	Fem	Sex rat	J2 - J4	Root (g)	Mal	Fem	Sex rat	J2 - J4
MIZ	0.61±0.06	1,320	1.340	0.9±0.1	4,783	0.34±0.06	1,588	1,420	1.1±0.1	2,316
HAW	0.68±0.14	1,096	1,090	1.2±0.1	2,504	0.39±0.09	660	773	1.3±0.1	1,273
TEX	0.64±0.13	600	1,008	0.6±0.1	2,016	0.40±0.09	436	1,056	0.5±0.1	536
ASH-a	0.94±0.14	0	1,940	0	2,268	0.47±0.10	0.2	2,247	.0003**± .0003	998
SIB	0.49±0.06	0	477	0	564	0.33±0.11	0	56	0	59
AKN	0.63±0.09	0	1,277	0	2,808	0.34±0.07	0.2	504	.0001**± .0001	567

* Popu = Population, Mal = Male, Fem = Female, Sex rat = Sex ratio. J2 - J4 = 2nd stage - 4th stage juvenile. **Zero was omitted.

7. Discussion

Results obtained with the field examination and experimental analysis of laboratory populations revealed that frequency of male occurrence of *R. reniformis* is not easily influenced by environmental changes, viz., soil temperatures, kind of host plant, and (or) nutrient condition of the host plant, suggesting that even juveniles from egg-masses laid by females parasitizing poor host plants suffering insufficient nutrients or other environmental factors can undergo normal development and sexual differentiation.

According to Badra and Ismail (1981), sex ratios of *R. reniformis* (MNT) in soils increased to 8.8 when the host plant (cowpea) was grown with an excessive supply of phospholic acid. This was about 10 times as much as usual and correlated positively with content of phenylalanine in tissues of the host plant. Phenylalanine is a precursor at phenol biosynthesis in plants and is thought to induce male differentiation of nematodes at high concentrations. The nutrient status of host plants in this case would have to influence the development of juveniles indirectly through effects on their adult female mothers, which ingested food from the host, because of the peculiar development of second-stage juveniles of this nematode into immature females or males without feeding, as mentioned above. Therefore, it is possible that the increased sex ratio (male/female) observed by Badra and Ismail resulted from greater death rate of females than males in the soil. It would have been interesting to do a similar experiment with phospholic acid on MNT and MRT populations in Japan as part of this study but it could not be done.

Variations in male occurrence within a species are widely known in other plant and animal parasitic nematodes (Christie, 1929). For example, rate of male occurrence in *Globodera rostochiensis* increases with a rise in the number of parasitizing individuals per unit of host root (Ellenby, 1964; Trudgill, 1967). This is explained by increase of induction into males by deficient food for female development. A similar phenomenon is known for *Meloidogyne incognita* and *M. arenaria* (Triantaphyllou, 1960). Sex reversal of female juveniles to males in the second and third stages is a principal mechanism for male increase in *Meloidogyne*. Males are also increased in resistant host plants (Dropkin, 1959). According to McClure and Viglerchio (1966), sex ratio of *M. incognita* is dependent on the nutrient condition of the host plant but not on nematode densities in roots. Additionally, a parthenogenetic population of *Aphelenchus avenae* induces male development by all individuals when they are exposed to a temperature of 30 ˚C at the early second stage (Hansen et al., 1972). In *Heterodera avenae*, on the other hand, increased nematode density on host roots and (or) poor host plant nutrition results in a higher death rate of females so that males appear to rise in number more than females (Bridgeman and Kerry, 1980). A similar situation is known in *H. schachtii* as well (Kerstan 1969; Sengbusch, 1977).

As mentioned above, nematode sex determination or sex differentiation occurs in two ways, namely genetic and non-genetic (environmental). Even in cases where environmental factors seem to affect sex ratio, the direct reason sometimes is not increased males but rather a relative decrease in females resulting from an increased female death rate. Sex determination of *R. reniformis* might be based on a genetic mechanism. Sex chromosomes are known in animal parasitic but not in plant-parasitic nematodes although an electron microscope study on sex chromatin in a plant nematode is underway (Goldstein, 1981). The haploid chromosome number in NMT populations of *R. reniformis* is nine (Nakasono, 1966). Cytogenetic and electron microscope studies will add further information on the mechanism of sex determination in this nematode.

V. Role of Males in Reproduction of Laboratory Populations

In the previous section, it was demonstrated that about the same number of both sexes of the MNT population in *R. reniformis* emerged under any condition, (e.g., in the field and under laboratory conditions) without significant variation. Likewise, consistently very few or no males in MRT and MAT populations were observed to occur over the same wide range of conditions. In this section, the role of males in reproduction was studied with those populations of *R. reniformis*.

1. Role of males on three laboratory populations from Mizuho town of Nagasaki prefecture

Mizuho populations tested in the previous section were examined here first.

Materials and methods

Nematode populations tested: The same as used in section IV-2, namely, MIZ-1, MIZ-2 and MIZ-3.

Culturing: Nematodes were cultured in the greenhouse at soil temperatures of 25 to 27 ℃ following inoculation either with females only (FOT) or with both sexes (BST).

Experiment 1: MIZ-1 (MNT) was tested after 3 years of culture in the greenhouse. One week after

TABLE 16. Numbers of produced and hatched eggs, and percentage frequency of males and females in the culture with both sexes of MIZ-1 population (experiment 1).

Egg-mass Code	Total eggs / egg-mass	Hatched juvenile	Unhatched egg	Percent hatch	Percent individual developed	Percent male	Percent female
1	121	110	11	90.9	84.5	52.7	47.3
2	170	115	55	67.6	73.5	56.0	44.0
3	115	97	18	84.3	100.0	60.8	39.2
4	148	132	16	89.2	86.4	55.3	44.7
5	122	101	21	82.8	87.1	54.5	45.5
6	98	93	5	94.9	92.5	44.2	55.8
7	77	69	8	89.6	89.9	46.8	53.2
8	145	111	34	76.6	88.3	41.8	58.2
9	101	95	23	94.1	86.3	50.0	50.0
10	138	127	18	92.0	92.9	60.2	39.8
11	164	160	4	70.7	60.0	50.0	50.0
12	69	65	4	94.2	87.7	49.1	50.9
13	92	88	4	95.7	79.5	48.6	51.4
14	124	113	11	91.1	72.6	45.1	54.9
15	155	150	5	97.8	84.7	46.5	43.5
Average	122.5	108.4	15.8	87.4	84.4	50.8	49.2
S.E.	8.0	6.8	3.6	2.4	2.5	1.5	1.5

tomato seedlings (cv. Fukuju No. 2, four-leaf stage) were transplanted separately to "big clay pots" filled with "steam-sterilized mixed soil," each potted tomato was inoculated with both 130 males and 100 females (young female) in BST, and with 100 females in FOT, nematodes of which were extracted from the propagation containers by SVB. Nematodes for inoculum were carefully picked up with a thin needle and placed in small Syracuse watch glasses with tap water.

TABLE 17. Numbers of produced and hatched eggs, and percentage frequency of males and females in the culture with only females of MIZ-1 population (experiment 1).

Egg-mass Code	Total eggs/ egg-mass	Hatched juvenile	Unhatched eggs	Percent hatch	Egg in 2-16 cell stages	1 cell egg	Percent egg in cell division
1	15	0	15	0	4	11	26.7
2	42	0	42	0	25	17	59.5
3	31	0	31	0	9	22	29.0
4	69	0	69	0	-	-	-
5	57	0	57	0	13	44	22.8
6	54	0	54	0	20	34	37.0
7	145	0	145	0	68	77	46.9
8	23	0	23	0	9	14	39.1
9	14	0	14	0	0	14	0
10	17	0	17	0	0	17	0
11	34	0	34	0	18	16	22.9
12	36	0	36	0	-	-	-
13	0	0	0	0	0	0	0
14	93	0	93	0	24	67	25.8
15	69	0	69	0	26	44	36.2
Average	46.6	0	46.6	0	16.5	13.0	26.6
S.E.	9.6	0	9.6	0	4.5	3.9	3.2

Sexes of nematodes used as inoculum were then checked to avoid contamination, especially in FOT inoculum. Inoculated tomatoes were grown in the greenhouse with regular watering and Hoagland's solution application (not diluted, 2 ml a week) for 30 days. After that, tomato roots were removed from pot soils and washed gently with tap water. Egg-masses with adult females on roots were carefully collected and placed separately in small Syracuse watch glasses with distilled water. The watch glasses, each with an egg-mass and adult female, were again placed in inverted petri dishes (25-cm diam.) with a little tap water and stored in an incubator of 27 ℃ for 40 days. During the incubation period, hatched juveniles in each watch glass were counted every 2 days and transferred to other watch glasses for examining their sex differentiation during another period of 30 days of incubation in the same way as described for egg-masses. At the end of egg-mass incubation (40 days), eggs remaining in the watch glasses were examined to determine numbers unhatched, extent of egg development, and numbers dead. Tomato culture treatments included three replications (three pots) each, and for the incubation test 15 egg-masses were randomly picked from each of the three replicates (pots).

Experiment 2: MIZ-1 (MNT), MIZ-2 (MAT), and MIZ-3 (MNT) populations were used after 12 years (MIZ-1 and MIZ-2) or 14 years (MIZ-3) of culture in the greenhouse. "Steam-sterilized mixed soil" was placed in plastic cups (7-cm diam. 200 ml in volume), the walls of which were perforated with a needle to make two pores at about 3-cm height for water escape. Tomato seedlings were transplanted to those cups as in the previous experiment and nematodes were inoculated in the following way to test possibility of crossing between MIZ-1 males and MIZ-2 females as well as test the male role in reproduction of MIZ-3 population. In the crossing test, 61 males of MIZ-1 and 60 young females of MIZ-2 were inoculated together into a cup 30 days after transplanting of tomato, and as a control, 90 females of MIZ-2 without males were inoculated to other cups. In the male role test on MIZ-3 (experiment 3), only 42 females were inoculated to a cup, and as a control, 37 males with 35 young females were inoculated to other cups. All treatments were replicated five times (five pots) and maintained in the greenhouse in the same way as in the previous test. After 32 days of culture, egg-masses were removed from pot soils to examine egg numbers produced. At the same time, pot soils were processed to extract nematodes by SCF and examined to count hatched young juveniles as well as developed males and young females. Tomato plants were supplied with 2 ml diluted Hoagland solution (1/2 strength) once a week during cultivation.

Results

Experiment 1: Each of three replications of egg-mass incubation showed similar results and data on one representative replication were presented in Tables 16 and 17. In BST (both sexes) of MIZ-1, egg numbers per egg-mass were 122.5 on the average and 87.4% of those hatched; 84.4% of hatched juveniles developed to males or females, and percentages of the two sexes were almost equal to each other (Table 16). In contrast, in FOT, eggs per egg-mass

TABLE 18. Egg numbers per egg-mass produced by females in the inoculation with MIZ-2 females plus MIZ-1 males and that of MIZ-2 females only (experiment 2).

Egg-mass code	MIZ-1 male and MIZ-2 female inoculation treatment		MIZ-2 female inoculation treatment
	Egg / egg-mass	Male staying within egg-masses*	Egg / egg-mass
1	94	0	80
2	93	0	107
3	106	0	73
4	69	0	34
5	73	0	83
6	55	0	66
7	106	0	75
8	57	0	70
9	57	0	-
Average	78.9	0	73.5
S.E.	7.0	-	6.8

* If males are attracted by females on roots, they usually stay within egg-mass around female body for a long time as described in section VI.

TABLE 19. Male occurrence in soils of the inoculation with MIZ-2 females plus MIZ-1 males and that of MIZ-2 females only (experiment 2).

Inoculation treatment	Pot code	Population in second generation		
		Male	Young female	J2
MIZ-2 female + MIZ-1 male	1	0	117	810
	2	0	13	47
	3	0	37	398
	4	0	1	68
	5	0	4	641
	Average	0	34	392.8
	S.E.	-	21.6	151.8
MIZ-2 female only	1	0	10	386
	2	0	24	510
	3	0	6	149
	4	0	55	458
	5	0	20	577
	Average	0	23.0	416.0
	S.E.	-	7.7	73.7

averaged 46.6 in number; eclosion of these eggs was not observed. No eggs at morula or more developed stages were recognized, although 26.6% of these unhatched eggs were observed to have undergone cell division up to 2- to 16-cell stages.

Results obtained here show that females cultured without males can produce some eggs but their eggs cannot develop to the J2 and hatch. Thus, the male appears to have an important role and its copulation with females on roots is prerequisite for reproduction of MIZ-1 population (MNT). Oviposition might be stimulated by copulation between sexes.

Experiment 2: Results on MIZ-2 population were presented in Tables 18 and 19. There was no difference in egg numbers produced by females when inoculation with MIZ-2 females plus MIZ-1 males was compared with MIZ-2 females without any males (Table 18). No males of the MIZ-1 population inoculated were observed

TABLE 20. Difference in egg numbers of MIZ-3 population cultured from inoculations with males plus females and with females only (experiment 3).

Egg-mass code	Male and female inoculation treatment		Female-only inoculation treatment
	Egg / egg-mass	Male remaining within egg-mass	Egg / egg-mass
1	186	2	6
2	139	8	1
3	135	3	1
4	162	4	5
5	142	2	5
6	107	0	3
7	89	1	1
8	243	3	0
9	103	8	0
10	105	1	16
11	109	2	29
12	79	2	16
13	109	5	4
14	-	-	6
Average	131.4	3.2	6.6
S.E.	3.5	0.2	0.6

TABLE 21. Nematode numbers and male occurrence in soils of MIZ-3 population cultured from inoculations with males plus females and with females only (experiment 3).

Inoculation treatment	Pot code	Nematode number in second generation			
		Male	Female	J2 - J4	Sex ratio (♂ / ♀)
Male plus Female	1	94	69	183	1.4
	2	31	20	158	1.6
	3	24	16	140	1.5
	4	120	144	280	0.8
	5	88	57	265	1.5
	Average	71.4	61.2	205.2	1.3
	S.E.	18.7	23.1	28.4	0.1
Female only	1	0	0	0	-
	2	0	0	0	-
	3	0	0	0	-
	4	0	0	0	-
	5	0	0	0	-
	Average	0	0	0	-
	S.E.	-	-	-	-

within egg-masses produced by MIZ-2 females. Males of the next generation were not observed in soil populations extracted from either inoculum treatment (MIZ-2 females plus MIZ-1 males, or with MIZ-2 females without males); only young females and J2 were observed in both treatments (Table 19).

Results obtained here suggest that neither attraction of males by females nor crossing occurred between MIZ-1 (male) and MIZ-2 (female) in the treatment. So, males are not essential for reproduction of the MIZ-2 population.

Experiment 3: As shown in Tables 20 and 21, results on MIZ-3 population were about the same as that on MIZ-1 population as mentioned above (experiment 1). Male numbers remaining in egg-masses averaged 3.2 in the both sexes inoculation. No second-generation nematodes were produced unless males were present in the first generation (Table 21). Thus, it is evident that MIZ-3 population cannot produce the next generation without males, because the male is essential for reproduction.

2. Effect of males of the Mizuho population (male-numerous type) on reproduction of the Akune population (male-absent type)

The possibility of crossing between geographically isolated two populations, the Mizuho (MIZ), which belongs to MNT, and the Akune (AKN) belonging to MAT, was tested.

Materials and methods

Nematode populations tested: The Akune population (AKN) of MAT and the Mizuho population (MIZ) of MNT were used (see section I for details). Tomato seedlings (the same as in the previous test) were transplanted to small pots with "steam-sterilized mixed soil" and grown in a growth chamber for 30 days, in which temperature was maintained at 29 ˚C with 8,000 lux illumination for 12 hours and at 26 ˚C during 12 hours dark. After 30 days of culture, each potted tomato plant was inoculated with a nematode mixture containing 500 young MIZ females, 350 MIZ males, and 500 AKN females, and cultured in the same chamber for 19 more days. Culture of inoculated tomatoes was replicated six times (six pots) and supplied with Hoagland solution (not diluted) once a fortnight. Then, roots of each potted tomato were removed and 18 randomly selected egg-masses on roots were separately incubated in small Seinhorst fixing dishes (Seinhorst, 1956) with 1.5 ml distilled water. The dishes, which were placed in large inverted petri dishes (same as mentioned above) with a little tap water, were kept in an incubator maintained at 27 ˚C for 30 days. After that, hatched and developed nematodes in dishes, which were designated as the second generation, were examined for male occurrence and, additionally, some nematodes of the second generation, which were isolated with dishes, were cultured again in the same way as the first culture for 33 days. Finally, nematodes in the third generation were extracted from the second culture pot set by SCF and examined for male occurrence. Here, the egg-mass incubation test was omitted.

TABLE 22. Male occurrence in the second generation cultured with mixed nematodes of the Mizuho population (MIZ, males and females) and the Akune population (AKN, females).

Male emergence in 2nd gen.	Examination item	Replication (pot code) 1	2	3	4	5	6
Yes	Egg-mass number*A	16	13	13	12	15	10
	Sex ratio (♂/♀) in 2nd gen**	1.0	1.0	0.9	0.9	0.9	1.0
	Male staying within egg-mass	3.1	2.2	2.0	1.0	1.3	1.2
No	Egg-mass number*B	2	5	5	6	3	8
	Sex ratio (♂/♀) in 2nd gen**	0	0	0	0	0	0
	Male staying within egg-mass	1.5	0	0.4	0.3	0	0.3

* A plus B in respective replication = 18. ** 2nd gen stands for the second generation.

Results

Table 22 presents results in the first culture inoculated with mixed nematodes from two biological types, MNT (MIZ population, males and females) and MAT (AKN population, females only). Ten to 16 of the 18 egg-masses tested produced both sexes and the remaining two to eight egg-masses did not produce males in the second generation.

Sex ratio of the former group of egg-masses in the second generation was 1.0 or 0.9, contrasted to 0 in the latter. Numbers of male within the egg-mass were 1.0 to 3.1 on the average in the former and 0 to 1.5 in the latter. These males were all inoculated individuals. Rates of male occurrence in third generation from individual isolates of the first culture were similar to those in the second generation as shown in Table 23. Isolates that did not produce anymales in the second generation did not produce any males in the third one either; and similar constancy was observed for the other isolates, in which both sexes occurred in the second and the third generation.

Results obtained here suggest that the egg-masses

TABLE 23. Male occurrence in the second and the third generation after mixed culture of two biological types, MNT (MIZ, males and females) and MAT (AKN, females only) on tomato plants.

Pot code in 1st culture	Egg-mass code in 1st culture	Second generation (egg-mass incubation test) Male	Female	Sex rat*.	Third generation (soil population extracted from pots) Male	Female	Juvenile	Sex rat.*
3	1	0	44	0	0	58	88	0
	2	0	39	0	0	0	0	0
4	1	0	44	0	0	149	149	0
	2	0	32	0	0	4	13	0
	3	0	32	0	0	73	115	0
5	1	39	31	1.3	8	6	13	1.3
	2	32	21	1.5	50	16	102	3.1
	3	0	18	0	0	27	16	0
6	1	40	30	1.3	45	45	123	1.0
	2	28	28	1.0	69	31	64	2.2
	3	19	15	1.3	0	4	0	0
	4	27	31	0.9	27	30	52	0.9
	5	0	37	0	0	102	101	0
	6	0	57	0	0	183	231	0
	7	0	42	0	0	198	95	0

* Sex rat. = Sex ratio

that produced both sexes for two generations originated from the MIZ population (MNT)while those with no male emergence in the second or the third generation came from the AKN population (MAT). Therefore, it was postulated that no hybrids were produced in mixed culture as a result of crossing between the inoculated biological types, MNT and MAT.

3. Role of males in reproduction of the male-numerous, male-rare, and male-absent type populations

Six laboratory populations, which were different in biological type, were tested by culturing females of them on tomato to determine the role of males in reproduction and their affinity for crossing with males of MIZ population.

Materials and Methods

Nematode populations tested: MNT including MIZ, HAW, and TEX populations; MRT including ASH-a; MAT including SIB and AKN (see section I). Tomato plants (cv. Fukuju No. 2), which were grown in "big pots" with "steam-sterilized mixed soil" (see section I) in the greenhouse (26 to 28 ˚C) for 25 days, were each inoculated with 100 females of one population and either 105 MIZ males or no males. Tomato plants with nematodes were grown in the same greenhouse for an additional 33 days. Then, roots were removed and washed gently with tap water. Egg-masses with adult females on roots were picked up carefully to incubate in petri dishes (9.5 cm diam.) with 0.6% water agar (15 ml/dish), which were kept in an incubator at 27 to 28 ˚C for 40 days. After the end of incubation, dishes were examined under the microscope to evaluate larval hatch and sexes of sexually differentiated nematodes. Egg-mass numbers incubated differed according to pots (three replications). Pot soil was processed to extract nematodes by SCF and extracted nematodes were sexed under the microscope. Tomato plants were supplied with 2 ml Hoagland solution once every 10 days during the culturing period.

Results

As shown in Tables 24 and 25, no nematodes of the next generation were extracted from soil of pots that were inoculated only with females of MNT populations, including MIZ, HAW, and TEX, but many progeny were produced in pots co-inoculated with females of one of the three populations and males of the MIZ population (Table 24).

In contrast, many females and juveniles but no males of the next generation were extracted from pot soil inoculated with MRT or MAT populations (ASH-a, SIB and AKN) regardless of male inoculation. Similar results were obtained in the egg-mass incubation test (Table 25). No nematodes emerged in petri dishes, in which egg-masses from the inoculation with only females of MNT population were incubated,

TABLE 24. Differences in soil populations of next generation after 33 days of culture on tomato inoculated with different combination of males and females of MNT, MRT, and MAT populations (average of three pot replications).

Biological type	Popul. tested	Inoculation treatment*	Male	Female	J2 - J4	Sex ratio (♂/♀)	Remarks
MNT	MIZ	♂+♀	44	19	111	2.3	
		♀	0	0	0	-	
	HAW	♂+♀	228	273	714	0.8	
		♀	0	0	0	-	
	TEX	♂+♀	219	246	1,136	0.9	
		♀	0	0	0	-	
MRT	ASH-a	♂+♀	0	27	128	0	11.1**
		♀	0	2	116	0	
MAT	SIB	♂+♀	0	13	354	0	5.9**
		♀	0	20	958	0	
	AKN	♂+♀	0	16	392	0	8.8**
		♀	0	2	474	0	

* Males of the MIZ were inoculated in all "♂+♀" inoculation treatment. ** Old individuals of males, which were inoculated.

TABLE 25. Differences in nematodes of the second generation hatched from egg-masses incubated in petri dishes, of which eggs were produced with different combination of males and females of MNT, MRT, and MAT populations.

Biol. Type	Popul code	Inoculation treatment***	Egg incubat*	Unhatched egg**			Hatched individual			
				No cl	2-26	J2	J2-J4	Male	Female	♂/♀
MNT	MIZ	♂+♀	2	5	14	5	0	8	8	1.0
		♀	24	101	19	0	0	0	0	-
	HAW	♂+♀	28	52	505	132	34	1,037	1,210	1.2
		♀	24	212	105	0	0	0	0	-
	TEX	♂+♀	31	8	511	176	123	1,164	1,274	1.1
		♀	24	186	75	0	0	0	0	-
MRT	ASH-a	♂+♀	11	0	396	6	37	0	257	0
		♀	20	0	320	13	-	0	951	0
MAT	SIB	♂+♀	26	0	676	60	0	0	501	0
		♀	7	0	153	1	62	0	187	0
	AKN	♂+♀	34	0	706	66	12	0	518	0
		♀	24	0	464	13	8	0	803	0

Biol. type = Biological type, * Egg incubat = Egg mass incubated, ** No cl = Egg with no cell division and J2 = second-stage juvenile within eggshell. ***Males of MIZ population were used for pairing.

while many nematodes emerged from eggs laid by females of MRT and MAT populations in both inoculation treatments; no males were found among the latter. As mentioned above, it is obvious that the male is essential for reproduction by the MNT population and pairing of males of the MIZ population with females of other MNT populations (HAW and TEX), which originated from geographically isolated locations, can produce progeny without any abnormality, but there is no sexual interaction between MNT males and MRT or MAT females. The male appears not to be necessary for reproduction of the latter two under natural conditions.

4. Discussion

The MIZ-1, MIZ-2, and MIZ-3 populations were originally collected from two neighboring fields in Mizuho district, Nagasaki prefecture and maintained as isolates in the greenhouse for years (sections II and IV). The MIZ-1, and MIZ-3 produced about equal numbers of males and females in both field and laboratory conditions and they required males to reproduce, a character which was not altered even after 12 or 14 years of culture in the greenhouse. In the MIZ-2 population, males were not essential for reproduction and only female progeny were produced for many generations. Similarly, three laboratory populations (MIZ, HAW, and TEX) of MNT produced males and females under all experimental conditions when inoculated with both sexes but they could not reproduce whenever males were absent. The MIZ laboratory population originated from 15 egg-masses laid by females of the MIZ-1 population while the other two, HAW and TEX, have their origins in geographically isolated in Hawaii and Texas respectively in the United States. The successful reproduction demonstrated by culturing females of HAW and TEX with males of the MIZ population proves that these three populations are the same biological species (Mayr, 1965; Mayr et al., 1953).

Oviposition might be stimulated by copulation between sexes because females in MNT populations produced greater numbers of eggs, although the phenomenon was not apparent in MRT and MAT populations when they were cultured with MIZ males. In Australian *Aphelenchus avenae*, there are known to be two biological population types, amphimictic and parthenogenetic (Fisher, 1972). Oviposition in the former is stimulated by copulation between males and females, and delayed by postponed copulation. Interestingly, the oviposition by females of the latter is reduced when they are placed with males of the former. Oviposition by females of MRT and MAT was not investigated in relation to the presence of MNT *R. reniformis* males. In the culture begun with AKN females and MIZ males, some males were observed in AKN egg-masses, suggesting copulation. However, it seems unlikely male occurrence in MRT or MAT populations would be significantly changed by the presence of MNT males. *Meloidogyne hapla* can reproduce amphimictically when males are numerous in the population yet reproduce parthenogenetically when males are sparse or absent (Triantaphyllou, 1966). In *R. reniformis* populations such as ASH-a or ASH-b that produce only few males, it is still possible that these males can copulate with females. More cytogenetic analysis is needed on this topic.

TABLE 26. Numbers of parasitizing females on roots of sweet-potato examined.

Root	Root number	Length in total (mm)	Length / root (mm)	Female number on root	Female / 1 mm root
Tap	6	340	56.7	52	0.15
Feeder	79	944	11.9	130	0.14
				182	

According to Triantaphyllou and Hirschmann (1964), the MNT population of *R. reniformis* reproduces amphimictically and karyogamy between egg and sperm nuclei is normal. The seminal receptacle of females of MNT was ascertained to contain many sperm when males and females were cultured together (Nakasono, 1966). In contrast, nematodes of MRT and MAT populations are assumed to reproduce parthenogenetically as in many other nematode species of Tylenchida lacking males (Triantaphyllou, 1971), instead of by self-fertilization. In fact, the hermaphroditic or male organ was not found in the adult females of MRT or MAT populations, when examined by the author. Sivakumar and Seshadri (1971a) reported the interesting result that females of a MNT population of *R. reniformis* in India produced almost normally both male and female progenies in equal numbers without males in the culture. This would suggest possible distribution of MNT populations in the world that can reproduce parthenogenetically without males. Many males occurred in the second generation of their experiment without males, distinguishing their population from Japanese populations of MRT and MAT, in which few or no males emerge in any case.

Finally, both Japanese and North American populations of MNT essentially need males for reproduction and both sexes are produced in about equal numbers on most occasions, while MRT and MAT populations can reproduce without males and rare or virtually zero male occurrence in populations is usual. In the next section, sexual attraction between those laboratory populations will be tested to clarify differences in intensity of attractiveness among them.

VI. Sexual Attraction of Males by Females in Laboratory Populations and Differences in Rates of Attractiveness among Populations

A MNT population of *R. reniformis* needed males for reproduction, and copulation between sexes appeared prerequisite for normal propagation. Meeting of males, which move freely in soil, with females that sedentarily parasitize host roots, so that the posterior body part is mostly out of root tissues and enveloped by eggs laid within a gelatinous matrix, has not ever been studied in this nematode. Sexual attraction of males by females in *Panagrolaimus* nematodes was first clearly analyzed by Greet (1964). If a similar phenomenon in *R. reniformis* is involved in copulation, biological relationships and sexual isolation could be experimentally studied by comparing intensity of the attractiveness not only among MNT populations but also between MNT and MRT or MAT populations. With this in view, sexual attraction by *R. reniformis* in soil and on water agar plates was studied.

1. Male attraction by females on host plant roots in pot soil

Numbers of males within egg-masses laid by females on potted sweet potato roots were examined to analyze sexual attraction.

Materials and methods

Nematode population tested: The MIZ population was employed. Two vine cuts of sweet potato (cv. Norin No. 2) were planted to soil infested with the nematode in a propagation container, in which population density was evaluated as 400 per 100 g soil, and grown in a greenhouse at a soil temperature of 22 to 31 ˚C for 70 days. Then, roots were removed and washed gently with tap water. Removed roots were separated into two groups, tap roots and feeder roots. Egg-masses laid by females on each root group were counted and dissected with fine forceps to examine males within them under a stereo microscope. Fertilizers were supplied to the container in the way as described in section I.

Results

Six tap and 79 feeder roots were collected and examined. Numbers of parasitizing females and root lengths were as shown in Table 26. One hundred and eighty-two females were counted on roots and most laid egg-masses; 116 females were randomly selected to examine associated males and determine egg numbers per egg-mass. A total of 420 males were found within the egg-masses of those females and the number of males per egg-mass (= female) was 3.6 on average, ranging from 0 to 17. Males within egg-masses were observed to coil themselves around

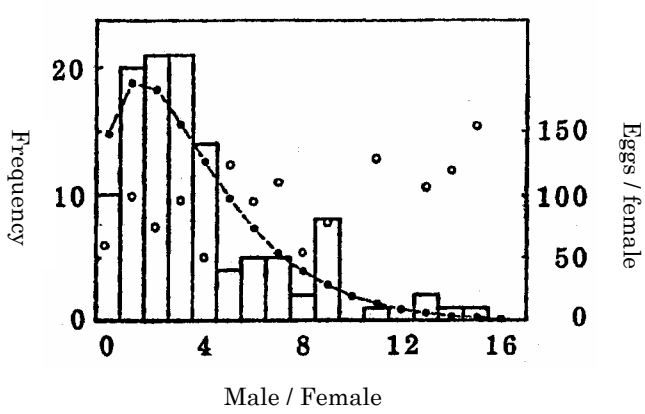

FIG. 10. Frequency distribution of males per female (males staying within egg-mass). The curve shows "theoretical value of negative binomial distribution" calculated, in which $\overline{X}$ = 3.62, s^2 = 10.24, k^ = 1.981, χ^2 = 10.153, 0.05 < *P* < 0.10, empty circle = eggs.

the female body at the vulval position in most cases and some males were evidently copulating with females. An exhausted intestine was a typical feature in those males and their behavior in water appeared to be almost static or sluggish when prodded by a needle. Additionally, some males were dead within egg-masses. About 90% of females examined appeared to be ending their oviposition, 2% were in the middle stage of egg-laying, and remaining 8% were already dead.

Frequency distribution of male number per female (or egg-mass) was drawn as in Figure 10. It showed a negative binomial distribution, suggesting a contagious distribution of male frequency.

Egg numbers per female (egg-mass) showed a trend to increase with rise of male number per female. Empty eggs, which already hatched, could not be counted exactly and those females with egg-masses containing more eggs seemed to be in an earlier stage of oviposition so that most of their eggs were still in developing stages before hatch. Egg-masses of those younger females were associated with more males than those of older females. Ten females were not accompanied by any males but their egg-masses were observed to have some J2 stage eggs (Fig. 10). Probably, males had left these females due to reduced attractiveness by aging of females. Results obtained here would suggest that two factors influence male frequency distribution per female: The first factor is intensity of attractiveness by females and the second, retention of males as affected by qualitative or quantitative change of some attractant, possibly emitted by the female. Accordingly, these observations indicate that male attraction by females on host roots is evident in *R. reniformis* and a female attracts more than one male. Likewise, it must be true that more males are attracted by young females on roots than by old ones.

2. Sexual attraction of males by females between the male-absent type and the male-numerous type populations on agar plates

Male attraction by females was tested on water agar in vitro to develop an attraction assay.

Materials and methods

Nematode population used: The MIZ (MNT) and AKN (MAT) populations were used. In order to provide virgin females for attraction tests, tomato seeds (cv. Fukuju No. 2) were placed to small clay pots filled with "steam-sterilized mixed soil" and grown in a growth cabinet, maintained at 29 ˚C for 10 hours light and 20 ˚C for 14 hours dark. Plants with four leaves were inoculated with 100 to 150 young females, carefully hand picked from mixed nematodes that had been extracted from the propagation container of each population by SVB. Then, the plants were grown for three more weeks and roots were removed from pots. Mature virgin females were carefully collected from roots with fine forceps and a needle, and washed with distilled water three times before use. Males, which were extracted from the propagation container of the MIZ population by SVB and placed in tap water in a Syracuse watch glass with a few soil particles, were stored in a dark incubator regulated at 15 ˚C for 4 weeks before use.

Attraction test: A thin plate (about 3 mm thick and 35 mm diam.) of 0.7% water agar was prepared in a 58 mm-diam. watch glass (Fig. 11). Four mature virgin females were embedded in agar at the corners of a ca. 0.7 mm square at the watch glass center so as to keep females in a vertical position facing the agar surface. Each watch glass containing the test females in agar was placed in an inverted petri dish (9 cm diam.) with 20 ml of 0.5% water agar to protect the agar and females from drying. After 12 hour incubation of the females within the dish at 26 ˚C in the dark, four males held in the incubator were introduced to the center of a quadrangle of four females on the agar, and attraction was tested at 26 ˚C in the dark. After the introduction of males, the distribution of males on the agar was examined in the dark room under a stereo microscope at intervals of 1, 8, 12, 24, and 48 hours. Tests were done with four replications. As a control treatment, virgin females of both MIZ and AKN populations were killed by dipping them in hot water at 62 ˚C for 6 minutes and tested in the same way as living females.

The attractiveness of evaluation method of Greet (Greet et al., 1968) was partly modified here as follows. The agar plate in the watch glass was divided into four zones, designated as zone a, b, c, and d, as shown in Figure 11 and in Table 27. For evaluating

TABLE 27. Distribution zones on agar plate for males introduced and loadings for evaluation of attractiveness.

Zone code	Definition	Area (mm2)	Loading
a	Female areas: about 0.4-mm-diam. concentric circle surrounding the respective embedded females	0.126 / ♀	82.1
b	Female quadrangle area: 4-mm-diam. concentric circle surrounding the center of quadrangle of females, excluding zone a	12.06	8.4
c	Annular area: 4-mm width surrounding zone b	100.48	2.9
d	Outer annular area: all the remaining outer area	848.59	1.0

attractiveness, male numbers distributed in respective zones were weighted by loadings for zones, which were calculated as a relative value of the inverse square root of area (mm^2) of respective zones and total value (designated as "index of attractiveness" : "At" for convenience) was calculated as follows:

$$At = 82.1xa + 8.4xb + 2.9xc + xd$$

Here, x is numbers of males distributed in each zone. "At" was converted into logarithmic value (log_{10}) and analysis of variance was applied to test significance of difference in male distribution (attraction).

Results

Male distribution on the plate changed with time as indicated by percentage frequency in Figure 12, and it showed a pattern that differed between females of MIZ and AKN populations. As for females of MIZ, there was a clear difference in male percentage distribution between living and killed females. In the living female plate of MIZ 1 hour after male introduction, male percentages were 69% in zone a, 25% in zone b, and only 6% in both zone c and d, while in the killed female plate no males were in zone a, and the greatest percentage was observed in zone c, followed by zone b (25%). On the other hand, in the living plate of the AKN population, only a small percentage of males were observed in zone a and most males were distributed in the outer zones, especially zone d with 45% after 1 hour; the killed female plate showed a similar tendency.

After 8 and 12 hours, zone a in the living female plate of MIZ had 93% of the males, while in the killed female plate 50% of the males had moved to zone d (data at the 8-hour observation were not presented because results were almost the same as at 12 hours) but some 20% were also in zone a. At this time, in the AKN population, 70% males had moved to zone d in both living and killed female plates. After 24 hours and 48 hours, zone a in the living female plate of the MIZ population still maintained the highest percentage of males but some gradual migration to outer zones was observed, while in the killed female plate, an increase of male percentage in zone a occurred

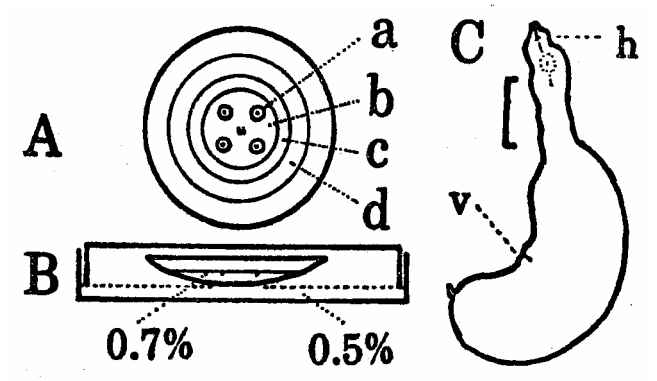

FIG. 11 Illustration of the agar plate/watch glass arrangement used for sexual attraction tests and shape of virgin female A: Distribution zones on the agar plate (0.7% water agar) in watch glass; B: Inverted petri dish, in which the watch glass was placed with 0.5% water agar; C: Mature virgin female; a, b, c, and d stand for zone a, b, c and d, respectively; h: head region of female; v: vulva; Scale for female: 100 μm

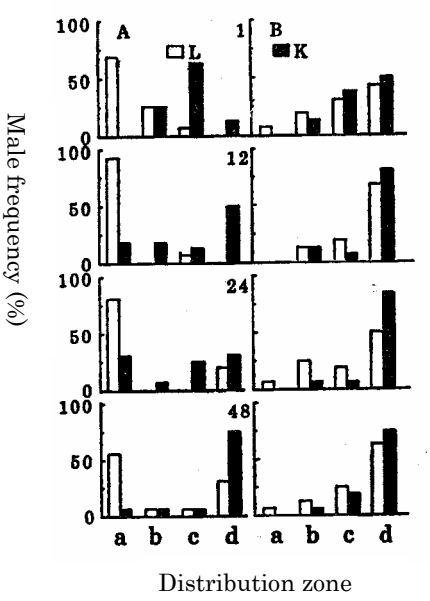

FIG.12. Changes in percentage distribution of M0IZ males on 4 zones around females of mature virgin females of MIZ and AKN populations embedded in agar plates. A: MIZ population; B: AKN population; L: Living female; K: Killed female; 1, 12, 24, and 48 are the time in hours after male introduction to the agar plate, at which time male distribution was examined. See text for distribution zones.

TABLE 28. Analysis of variance on logarithmic values of indices of attractiveness ("At") obtained with sexual attraction test on the agar plate.

Source of variation	Symbol	Degree of freedom	Sum of squares	Mean square	Ratio
Total	T	19	6.8507	-	
Hour	H	4	0.0694	0.01735	1.1208
Population	P	1	3.9964	3.9964	＞258.1654**
Female's state (living or killed)	L	1	1.8540	1.8540	＞119.7674**
Interaction					
H × P		4	0.2649	0.0662	4.2781
H × L		4	0.1029	0.0266	1.6558
P × L		1	0.5016	0.5016	＞32.4031**
Error	E	4	0.0619	0.0155	

after 24 hours but 75% of the males moved to the outer zone after 48 hours. In the AKN population, most males moved to the outer zone and stayed there during the test time regardless of whether living or killed females were embedded. In order to test the significance of differences observed in male percentage distributions between living and killed females or between two populations, analysis of variance was applied to "At" values with the results that differences in male distribution were significant between living and killed females and between the two biological populations ($P < 0.01$) as shown in Table 28. Differences among hours after male introduction were not significant. Interaction between time and population or time and female's state (living or killed) was not significant but between population and female's state was significant ($P < 0.01$).

Differences between populations and between female states were compared by LSD (least significant difference) as shown in Table 29. "At" of the MIZ population was significantly greater than that of AKN at the 0.01 level, and living females of both populations had a significantly greater "At" value than killed females at the 0.01 level as well. Killed females of the MIZ population showed a greater "At" value than living females of AKN (significant at the 0.05 level), thus females of MIZ appeared to maintain some effect on males' behavior even after killed by hot water at 62 ˚C for 6 minutes.

Results obtained here suggest that differences in the distribution pattern of males on the agar plates resulted from sexual attraction of males by females, which were embedded in the agar. It was clear that males of the MIZ population were more strongly attracted by females of the same biological population than by females of the AKN population. The sexual attraction assay adopted here might be applicable to other nematode populations of *R. reniformis*.

TABLE 29. Comparison of "At" (logarithmic value) between populations and between female' state (living or killed).

Population	Female' state		Total
	Living	Killed	
MIZ	2.3361	1.4104	3.7465
AKN	1.1253	0.8331	1.9584
LSD (0.05) :	0.2188		
(0.01) :	0.3619		
Total	3.461	2.2435	
LSD (0.05) :	0.1547		
(0.01)	0.2559		

TABLE 30. Differences in sexual attraction expressed as index of attractiveness ("At" as logarithmic value) among different duration of male storage at 15 ˚C after extracted from soils (MIZ population).

Duration of male storage at 15 ˚C after extracted				
3 weeks	2 weeks	0 week	1 week	Control * (killed female)
2.3143	2.2813	1.9761	1.4864	1.2398
LSD (0.05)	0.7737			

* 3-week storage males were applied.

3. Storage duration of males after recovered from pot soils and male reaction to females

Since males were often observed to move vigorously in water when newly extracted from soil, and since occasionally, those males moved away quickly on agar plates without any reaction to the embedded females, the effect of duration of male storage on sexual attraction by females was tested on agar plates.

Materials and methods

Nematode population tested: The MIZ population was tested. Mature virgin females were obtained as in the previous test and males also were extracted from the propagation container as previously. Recovered males were stored in Syracuse watch glasses with tap water and a few soil particles in an incubator regulated at 15 ˚C in the dark. Male storage durations after extracted were for 0 (used the day when extracted), 1, 2, and 3 weeks before the test. The attraction test was also done in the same way as in the previous study and with five replications. Male distribution on agar plates was examined 16 hours after male introduction. As a control, killed females were embedded in the agar plate and males of the 3-week storage treatment were used.

Results

As shown in Table 30, the greatest index of attractiveness ("At" in logarithmic value) was 2.3143 with males stored 3 weeks, followed by 2.2813 with males stored 2 weeks. Males of 0-week storage showed 1.9761. Some attraction of males was also induced by killed females in this experiment.

Although differences were not significant, there was a tendency for males to respond better to females when they were stored for a longer time after extracted from soil.

4. Comparison of sexual attraction rates among laboratory populations

Sexual attraction was compared between populations of the same and different biological types (MNT, MRT, and MAT) to evaluate the possibility of intercrossing and sexual isolation.

Materials and methods

Nematodes used: Males of each of the MNT populations (MIZ, HAW, and TEX) were tested in response to females of MNT, MRT (ASH-a), and MAT (SIB and AKN) populations. Mature virgin females and males were prepared as in the previous experiment. The attraction test also was the same as before except that females were embedded at the corners of a 1.3-mm square. Male distribution was examined 16 hours after being introduced to the center of the female quadrangle. Females of the same population as that of males were killed in hot water as mentioned above and used as a control.

Experiment 1: Males of the MIZ population were introduced to the test agar plates, where females of each population tested were embedded. Males were preserved at 15 ˚C in the dark for 2 weeks before the test.

TABLE 31. Differences in sexual attraction among six laboratory populations (MIZ, HAW, TEX, ASH-a, SIB, and AKN) as MIZ males were attracted ("At" in logarithmic value).

Population						
HAW	MIZ	TEX	SIB	ASH-a	AKN	(KIL)*
2.2172	2.1477	2.0370	1.3360	0.9764	0.9468	0.7034
LSD (0.05) :	0.4221					

* Control: MIZ females were embedded after killed by hot water of 60 ˚C for 10 minutes.

TABLE 32. Differences in sexual attraction among six laboratory populations (MIZ, HAW, TEX, ASH-a, SIB, and AKN) as HAW males were attracted (Same as before).

Population						
TEX	HAW	MIZ	SIB	(KIL)*	ASH-a	AKN
2.0080	1.8580	1.5996	1.5242	1.1879	0.9522	0.7703
LSD (0.05):	0.6046					

* Control : HAW females were embedded after killed in hot water of 60 ˚C for 10 minutes.

Experiment 2: Males of the HAW population were introduced to the test agar plates, where females of each population tested were embedded. Male storage was at 15 ˚C in the dark for 5 days after extracted from soil.

Experiment 3: TEX males were introduced to the test agar plates, where females of each population tested were embedded at the corners. Male storage was for 5 days after extracted as in the previous test.

Results

Experiment 1: As shown in Table 31, males of the MIZ population were attracted to females of all three MNT populations (MIZ, HAW, and TEX) with significantly greater "At" values (logarithmic), ranging from 2.0370 to 2.2172, while attraction was significantly weaker when they were introduced to the agar plates of MRT (ASH-a) or MAT (SIB and AKN) females, with lower values of "At," ranging from 0.9468 to 1.3360. Control (killed females) showed the lowest value (0.7034).

Experiment 2: As shown in Table 32, as in the previous test, greater "At" values of HAW males were produced by MNT females (TEX, HAW, and MIZ), ranging from 1.5998 to 2.0080, but these values were not different significantly from 1.5242 by SIB females (MAT). Attractiveness was more varied here. AKN females had the least value, 0.7703.

Experiment 3: As shown in Table 33, males of the TEX population were attracted well by females of all MNT populations (MIZ, HAW, and TEX) with greater "At" values, ranging from 1.96276 to 2.2571, and they were followed by 1.5276 of AKN' s females (MAT) with no significant difference. The least value (0.7840) was for the control. As demonstrated above, it was established that males of each population of MNT were more strongly attracted by females of the same biological type (MNT) than by those of the other types (MRT and MAT). Additionally, females of the ASH-a population (MRT) showed a relatively low intensity of attractiveness for males of three populations of MNT when compared with females from populations of MAT, especially the SIB population.

5. Behavior of males attracted by females on agar plate

Tracks made by males on the test agar plate were observed to determine their behavioral characteristics after introduced and (or) after each test was finished.

Materials and methods

Nematode populations used: The same nematodes that were tested in the above experiments were used for the track observation. The male behavior or residence in zones a and b were investigated immediately after male introduction to the center of the female quadrangle agar plate under a compound microscope, and similarly, tracks made by males on the agar plate were traced with a camera-lucida after the end of experiment. Character-

TABLE 33. Differences in sexual attraction among six laboratory populations (MIZ, HAW, TEX, ASH-a, SIB, and AKN) as TEX males were attracted (same as before).

Population						
TEX	MIZ	HAW	AKN	SIB	ASH-a	(KIL)*
2.2571	2.1303	1.9652	1.5276	1.3047	1.1832	0.7840
LSD (0.05) :	0.6263					

* Control : TEX females were embedded after killed in hot water of 60 ˚C for 10 minutes.

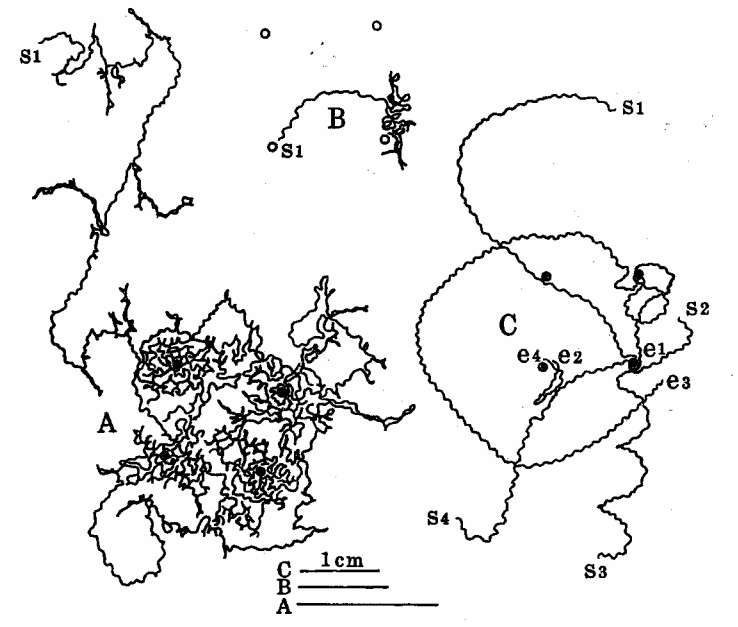

FIG. 13. Tracks left by males on the agar test plate. A: Track made by a male attracted by living females (four black circles) of MIZ population; B: Track by a male on the agar plate with killed MIZ females (four empty circles); Tracks made by males of MIZ on the agar plate with living AKN females (four black circles); s_1, s_2, s_3, and s_4 stand for starting point for attraction of different individuals, respectively, and e_1, e_2, e_3, and e_4 are the end of attraction of respective males. In A, male's track was not clear at the end.

istic patterns of malebehavior were compared among populations and between living and killed females.

Results

In a preliminary test, males did not respond to the females when they were placed more than 3 mm away from females of the MNT population embedded in agar, while most males commenced their response to females immediately or in about 1 minute when they were introduced to the center of the females embedded as shown in the above test. Males in a few cases stayed motionless at the center point for 30 minutes or more and then started moving, and their behavior patterns showed differences related to the biological types of female (MNT, MRT, and MAT) embedded on the agar plate and related to the female's state, living or killed. When males were introduced to the MNT female's plate, some of them almost directly approached the female, and some others first moved away out of the female quadrangle area and made a return at a distance of about 3 mm from the female and approached the female, by making a circuitous or zigzag pattern of migration with many sudden changes of direction (Fig. 13A). Near the female, their track was traced as spiral and counterclockwise direction. Males, which reached the embedded living female (of the MNT), stayed for a prolonged time and showed three behavioral patterns: (i) remaining motionless near the female, (ii) attempting copulation with the female, and (iii) moving slowly around the female. Tracks made by these males were often observed to include much sharp turning and backward movement while processing toward females (Fig. 13A, but these types of movement were rare and limited in the area close to females when males were placed onto plates of killed MNT females or living females of MRT and MAT populations (Fig. 13B, C). In most cases their stay with the latter kinds of females was not prolonged and they left the female after a short period.

In sum, responses of MNT males to living MNT females clearly differed from responses to MRT and MAT females and dead MNT females.

6. Discussion

Egg-masses produced by females on sweet potato roots were observed to contain 3.6 males on the average, and male frequency distribution per egg-mass (female) was evidently contagious. This fact suggests that the egg-mass or female enclosed within it induced male attraction. Male attraction tests with the agar plate showed that males aggregated close to females embedded in agar for a prolonged time and some appeared to attempt copulation with females. According to Greet (1964), males and females of *Panagrolaimus rigidus* are attracted to each other by sex attractants emitted by both sexes, which are water soluble and permeable through cellophane. Similar sexual attraction was reported for other free-living and plant-parasitic nematodes recently (Ahmad and Jairajpuri, 1980; Balakanich and Samoiloff, 1974; Chin and

Taylor, 1969; Green, 1966; Jones, 1966). Since egg-masses without females of *R. reniformis* did not elicit male attraction when tested on similar agar plates in the preliminary experiment (not presented here), the retention of males in egg-masses and male attraction by females on agar plates could be explained by the presence of a water-soluble substance, such as a sex attractant emitted by females, in this nematode as well.

Females of *Globodera rostochiensis* are known to attract males from a distance of 15 cm in soil (Evans, 1970), while females of *R. reniformis* appeared only to be able to attract males placed within 3-mm distance of females on agar plates here. So, the distance from which males of this nematode in soil are attracted by females might be shorter than in *Globodera*.

Most males were observed to have an optically refractive intestine and to move very actively in water and on agar plates just after they were extracted from soils, but their response to females was not positive. Perhaps these active males are too young to respond sexually to females on roots and need more time to mature and acquire sexual sensitivity. During this maturing period they may disperse randomly in soil and then later mature and encounter females. This interpretation would be partly supported by better attraction being obtained with males after they were stored longer at 15 ˚C. Addition of a few soil particles controlled fungal growth well in the male storage dishes.

Measuring the "At" value (logarithmic) of male attractiveness to females as described here is a modified method after Greet et al. (1968). It gives a theoretically greatest value of 2.5164 when all four males aggregate in zone a (very close to the embedded females) at the time of examination (here 16 hours after male introduction, for example) and gives a value of about 0.9 or less when males behave randomly on the test agar plate, in which case most males distribute in zones c and d.

Indices of attractiveness, "At," were greater than 1.6 when females of three MNT populations (MIZ, HAW, and TEX) were tested, and they were also greater than that when females of MRT and MAT populations were tested. These results show that MNT populations are very close to each other biologically despite their geographical isolation. On the other hand, "At" values of MRT and MAT females were relatively small without exception in all tests, and sometimes very close to the value expected for random behavior. In some cases, however, they showed "At" values up to 1.3 or 1.5, such as in SIB and AKN females. This probably suggests that these populations would have some sexual affinity for the MNT population and it agrees with the results in the previous section, where some males of the MIZ population were observed to remain within AKN females' egg-masses when they were cultured together.

Species-specificity or strain-specificity in sexual attraction of nematodes has been demonstrated in free living nematodes. On the other hand, there is evidence that cyst nematodes including *Globodera* and *Heterodera* exhibit vigorous attraction of males by females not only within species but also between species (Green and Plumb, 1970). According to Green and Plum, for example, sex attractants emitted by females of *G. rostochiensis* attract not only males of the same species but also males of *G. mexicana, G. tabacum*, and *H. avenae*, and they classified cyst nematode species tested into three biological groups by the intensity and range of inter-specific attraction of males (Green and Plumb, 1970). They considered that more than six kinds of sex attractants emitted by females are involved in such inter-specific attractions, some attractants were lost to a large extent in some species, and other kinds of attractants were lost to a small extent in other species during the evolutionary process. The present biological groups or relationships were formed as a result. Relationships among MNT, MRT, and MAT populations of *R. reniformis* seem to have resulted from such evolutionary process, wherein intensity of sex attraction may have changed and production of common sex attractants may have diminished and (or) increased differently among populations.

Males of MRT and MAT populations could not be tested for sexual attraction because they are only rarely produced. It is likely based on the results of the experiments conducted in this study that MNT populations of *R. reniformis* are reproductively isolated from populations of MRT or MAT.

VII. Environmental Factors and Differences in Nematode Development in Laboratory Populations

Morphological changes in molting and developing juveniles were investigated under different conditions, such as soil temperature and pH of the juvenile incubation medium, to determine differences in molting and development of juveniles and (or) duration of life cycle among laboratory populations.

1. Morphological changes in juveniles during molting development

In order to determine morphological criteria for comparing the length of time for juvenile development and life-cycle completion under controlled conditions, morphological changes in juveniles during molting and development in distilled water were studied.

Materials and methods

Nematodes used: The MIZ population of the MNT and the ASH-a of the MRT were used. Second-stage juveniles (J2) of each population were prepared by incubating many egg-masses (50 to 100 in number) in Syracuse watch glasses with distilled water, which were placed in large petri dishes containing a little tap water, at 25 to 26 ˚C in the dark. Juveniles hatched during the first 24 hours were all discarded and fresh juveniles within 12 hours of hatching were used for the investigation. For microscope observations of molting and juvenile development, juveniles were separately placed in hanging drops of distilled water (pH = 7) on depressed slide glasses and then, those slide glasses were stored in big petri dishes with a small amount of tap water. Petri dishes holding those juveniles with hanging drops were kept in an incubator regulated at 27 to 28 ˚C in the dark. Water in hanging drops was replaced with fresh water every 4 days, and observation of the juveniles was done at intervals varying from 0.5 to 24 hours, under a compound microscope (400 to 1,000 × magnification). Morphological changes in molting and developing juveniles were recorded by taking photographs, and body length and width were measured by sketching them with a camera lucida as necessary.

Results

Morphological changes and movement in molting and developing juveniles were investigated during the period spanning the second, third, fourth, and pre-adult (immature) stages and recorded as in Table 34. The total history from active movement of juveniles in the second stage just after hatch to the pre-adult stage of females or males could be divided into 21 characteristic steps of development, which were coded by numbers from 0 to 20. There was no difference in the 21 steps of development between biological types (MNT and MRT), but some differences in morphological features were observed between sexes after the beginning of molting in the second stage. No feeding was needed by juveniles during this developmental period. A brief description on the 21 steps in development of juveniles can be given as follows.

At step 0, juveniles just after hatch, showed very active movement in water and then gradually reduced their activity with sluggish movement at step 1, and finally stopped moving to be almost quiescent at step 2, but their movement could be activated again by some stimulation (Fig. 14A). After this step, they went to step 3, where their quiescent state was maintained without any reaction to stimulation and outer edges of stylet knobs gradually became inconspicuous (Fig. 14B). Following this the stylet knobs and shaft also became inconspicuous and, finally, invisible (step 4, Fig. 14C). After this, spaces appeared between the old (J2) cuticle and body in the lip region at first (step 5) and then at the tail end, to start molting (step 6), which was completed in step 7 (Fig. 14E). The J3 cuticle was shed in steps 10, 11, and 12, and the J4 cuticle in steps 15, 16, and 17, respectively.

Morphological features of the J3 head region were seen as in Figure 13F, and shedding of the J3 cuticle (third molt) began as illustrated in Figure 15A. The head region of the J4 was similar to that of J3 as shown in Figure 15, B and the fourth molt began as shown in Figure 15C, with space appearing first between sclerotized structures at the stylet position and surrounding tissues. Figure 16 shows shedding of the J4 cuticle with formation of the new stylet (a cone-like structure at first) and structures of the esophageal region as well as the tail end after the fourth molt was finished in the female. Young females and males were still enclosed by old cuticles after the fourth molt in water. The third- and fourth-stage juveniles did not have a functional stylet but had some sclerotized structures at the position of the conical part of the stylet, which were cast off when shedding the old cuticle. The disappearance and regeneration of stylet (knobs and shaft) regularly coincided with other changes in morphological characters, such as development of sex organs, which occurred during molting. At step 19, all organs or morphological characters were completed to yield a young female or male at step 20, although the reproductive system in the female was still immature and the male did not show sexual attraction as shown in the previous section. At the same time, both sexes showed active movement and young females appeared ready to invade host roots, while males did not exhibit such feeding behavior.

Shedding of the old cuticle began with separation of the old cuticle from the body in the anterior end and then in the posterior end, and it seemed to be accompanied by shrinkage and twisting movements of the body at each molt. After the molt was finished, body length and width recovered to some extent. Figure 17 shows relationships between changes in percentage body lengths and molts in MIZ males and females, as well as ASH-a females. Rates of shrinkage in body length were greater in ASH-a females than in MIZ males and females, and the final body length recovered after the completion of 3 molts was 88% of the J2 in ASH-a females, while the recovery of body length was 98% of the J2 in females and 110% in males of MIZ, where males acquired greater body length than before molting. On the other hand, body width after completion of 3 molts was 97% of the J2 in ASH-a females, 95% in MIZ females, and 90% in MIZ males; thus males of MIZ became the most slender in shape.

TABLE 34. Developmental process of juveniles of *Rotylenchulus reniformis* as divided into 21 steps with characteristic changes during the period of development from the second stage to the pre-adult.

Step	Movement	Esphagous & intestine	Lip region & stylet	Cuticle & molting	Sexual character	Time (h)
0	Active	Full contents	Stylet clear	—	—	24 – 192
1	Less active /sluggish	Full / lots of granules in pseudocoelom	Ditto	—	—	4 – 24
2	Quiescent (reactive, possible)	Dark in intestine	Ditto	—	—	12 – 24
3	Quies. (reactive, impossible)	Ditto	Outer margins of knobs faded	—	—	
4	Quies.	Ditto /eso.** valve faded	Knobs/stylet shaft faded, many granules appear and conical part visible	—	—	3
5 (2nd mlt)	Quies.	Ditto/ditto	Cavity around conical part	Space between cephalic framework & anterior end of body with constriction of body		
6	Quies. but twist in ant./ posterior parts*	Ditto/ditto	Cephalic framework & conical part shed	J2 cuticle & cephalic framework separated from body in anterior & posterior ends	Vulva (vul) region becoming transparent in♀	4 – 6
7	Quies./ rarely twist.	Ditto/ditto	Concave of lip region just after shed	J2 cuticle separated in middle of body	Cloaca region in ♂& vul. region Swelling	
8	Quies.	Darkness of intestine reducing/ditto	Lig region getting round but lacking stylet & cephalic framework	New cuticle not refractive (invisible)	♂&♀ditto	1
9	Quies.	Ditto/ditto	Some sclerotized (scl) structure instead of stylet	New cuticle getting re- fraction (slightly visible)	♂&♀ditto	30 – 72
10 (3rd mlt)	Quies.	Ditto/ditto	Space around scl structures	New cuticle clear, beginning of constriction in ant. end of body	♂&♀ditto	4 – 6
11	Quies./twist slowly	Ditto/ditto	Shedding of scl structures	J3 cuticle separated in both ends of body	♂&♀ditto	
12	Quies./twist slowly	Ditto/granules reducing	Concave of lip region, again	J3 cuticle separated in middle of body	♂&♀ditto	
13	Quies.	Granules reducing	Recovering of concaved lip	New cuticle invisible yet (not refractive)	♂&♀ditto	1
14	Quies.	Ditto	Lacking stylet & cephalic framework but scl structures again appear	New cuticle getting refractive (slightly visible)	♂&♀ditto	30 – 72
15 (4th mlt)	Quies.	Ditto	Space around scl structure & tip of conical part of stylet appearing	Beginning of constricr- tion of both ends of body	Vul in ♀/ spicules in ♂developing	
16	Quies./twist.	Ditto	Whole conical part formed & scl structure shed	J4 cuticle shedding in both ends of body with constriction	Ditto	4 – 6
17	Quies./twist.slowly & occasionally.	Eso. valve regenerated / eso.glands developing longer	Stylet shaft & knobs being regenerated	Completion of J4 cuticle shedding, new cuticle not yet visible (not refractive)	80% of vul & spicules formed	
18	Quies.	Complete eso. valve & glands regenerated	New stylet regenerated with complete & strong shape	Cephalic framework regenerating, new cuticle getting refraction (visible)	Sex organ in ♀& ♂formed completely, hyaline portion formed in ♀tail	3 – 6
19	Recovery of activity (slow movement)	Completion of eso. structure regeneration	Thrusting movement of stylet begins	Cephalic framework formed completely, three shed cuticles enclosing preadult female or male body	Sex organ in both sexes very clear	24 – 48
20	Moving as usual/ female ready to parasitize host, male needs more time to mature	Female's structure in esophagus seems stronger than in J2	Thrusting of stylet actively repeated	Female and male still enclosed by old shed cuticles in water	Ditto	Until root invasion in ♀

* Old cuticle already shed in anterior and posterior ends. ** Eso. stands for esophagous.

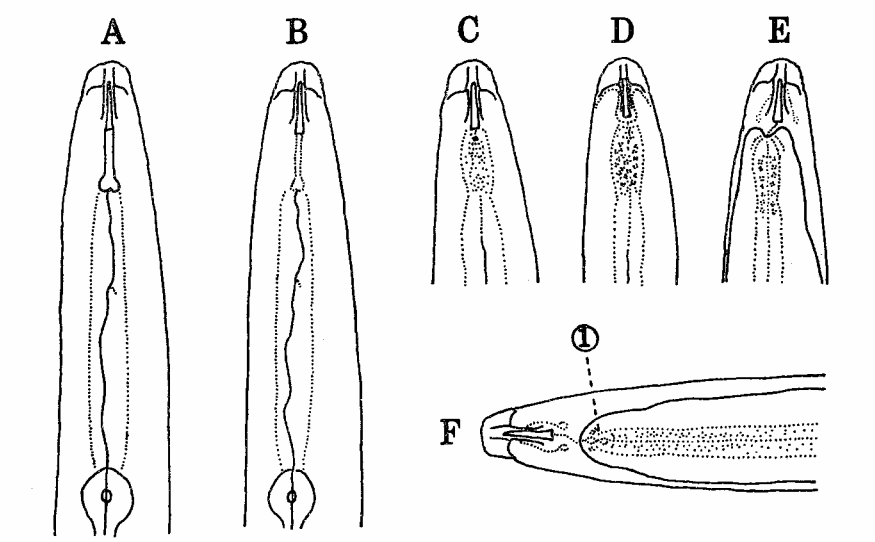

FIG. 14. Morphological changes in developing second-stage juvenile (J2) and the third stage (J3). A: J2 in quiescent state (step 2); B: Stylet knobs gradually faded (step 3); C: Stylet knobs and shaft completely lost structures (step 4); D: Space between conical part of stylet and surrounding tissues (starting of second molt, step 5); E: Depressed lip region just after completion of shedding J2 cuticle (step 7); F: Head region of J3 (step 9); ① Sclerotized structure at the position of conical part of stylet.

As mentioned above, the developmental process from juvenile to vermiform adult was defined by 21 steps, which were identified with morphological changes and movement of the body. It is noteworthy that there are differences in rate of body shrinkage and recovery between males and females of MIZ and also between the MIZ and ASH-a populations.

2. Juvenile developmental rate and comparisons of development

In order to find a useful way to compare developmental rates of juveniles among different populations, developmental steps, which were elucidated above, were applied to compare juvenile development here.

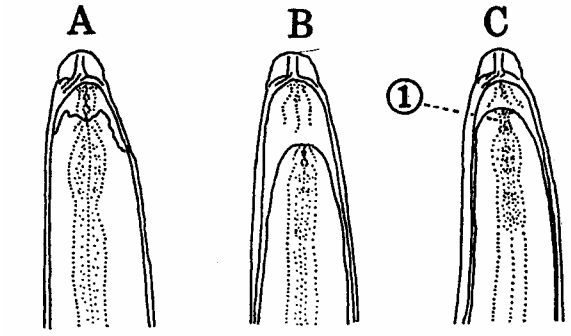

FIG. 15. Morphological changes in developing third- (J3) and fourth-stage (J4) juvenile A: Separation of J3 cuticle in early time (step 11); B: Head region of J4 after completion of third molt (step 14); C: Beginning of fourth molt with space around the sclerotized structure (step 15); ① Space around the sclerotized structure.

Materials and methods

Nematode populations used: The MIZ population of MNT and the AKN population of the MAT were used. Preparation and incubation methods of J2

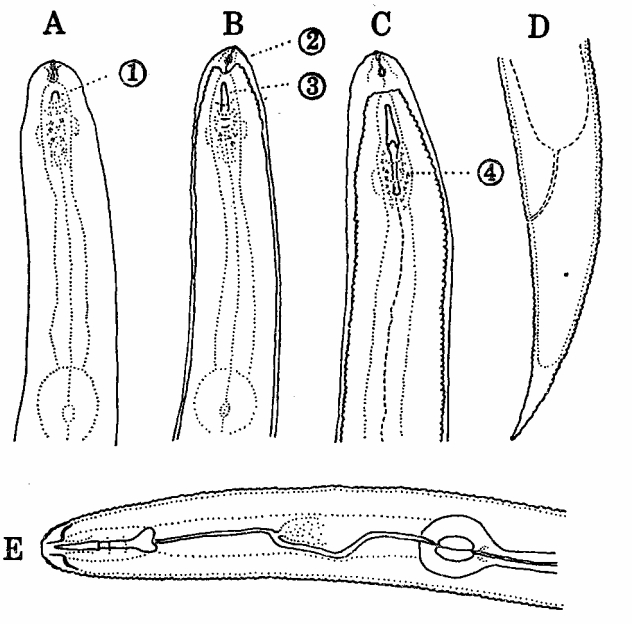

FIG. 16. Morphological changes in molting and development of fourth-stage juvenile (J4) A: Early stage of fourth molt, in which stylet cone is started to develop (step 15); B: Conical part of stylet is formed and shedding of J4 cuticle is proceeding in anterior part of body (step 16); C: Shedding of J4 cuticle is completed along entire body length, and stylet shaft and knobs are under being organized (step 17); D: Tail part of young female just after completion of the fourth molt (ASh-a population, step 20); E: Anterior portion of young female with strong stylet and esophageal organs (step 20).

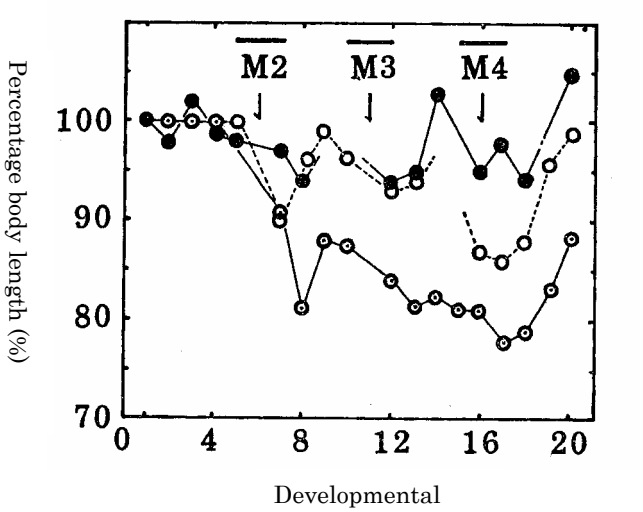

FIG. 17. Relationships between changes in percentage body lengths and molts in juveniles. Black circle: Male of MIZ population (partly lacking data); Empty circle with dotted line: Female of MIZ (partly lacking data); Empty circle with a dot in it: Female of ASH-a population; M2: The second molt; M3: The third molt; M4: The fourth molt, Average of 5 to 7 individuals examined.

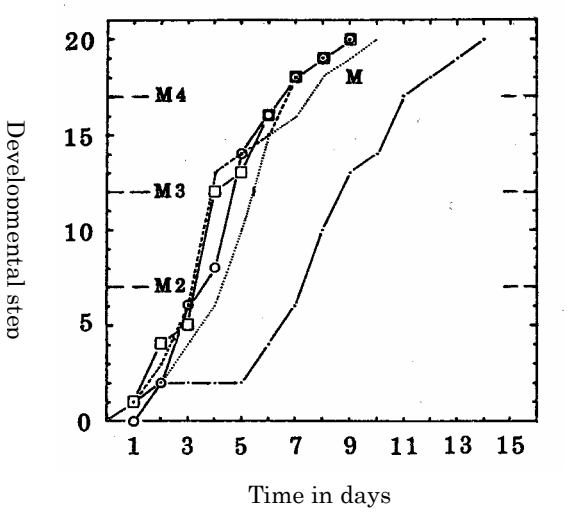

FIG. 19. Relationship between developmental steps of juveniles and time in MIZ females. Dotted line (M): Average of nine individuals; Other lines: Both extremes and moderate individuals. M2, M3, and M4: The same as in Fig. 18.

for investigation of development were the same as in the previous study. Numbers of nematode used were 16 for the MIZ and 12 for the AKN population. Observations were done at 24-hour intervals, under a compound microscope.

Results

Fourteen out of 16 juveniles were used (five males and nine females) in the MIZ and 10 females out of 12 populations (Figs. 19 and 20). Differences in time required for completion of development through the juvenile stages mainly depended on the length of time from steps 0 to 2. It varied from 1 to 11 days for AKN females, while it was from 1 to 6 days for females juveniles in the AKN population successfully completed development in hanging drop incubation at 27 to 28 ˚C. The relationship of developmental steps with time was illustrated in Figures 18, 19, and 20 using data from representative individuals. Time for development from step 0 to step 20 varied from 10 to 21 days among individuals in the AKN population (Fig. 18). It varied from 9 to 15 days in males and females of the MIZ and males of MIZ. Duration for molting and development from steps 1 to 20 had a sigmoid curve relationship.

A dose-mortality curve (Kouno, 1951), applicable to sigmoid curve relationships, was applied to

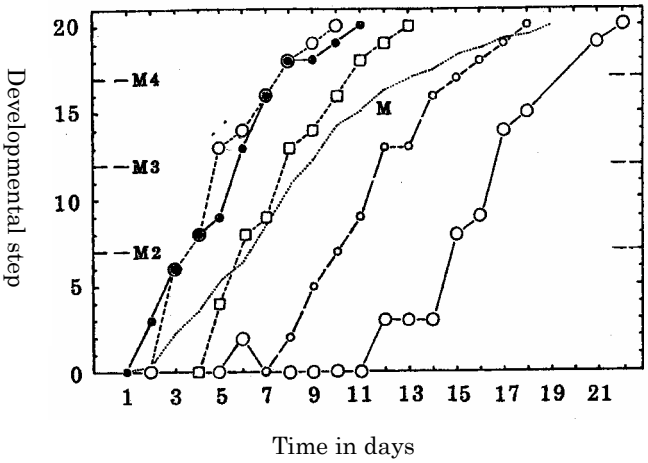

FIG. 18. Relationship between developmental steps of juveniles and time in the AKN females. Dotted line (M): average of 10 juveniles; Other lines: Both extremes and moderate individuals; M2, M3, and M4 stand for completion of the second, third, and fourth molt, respectively.

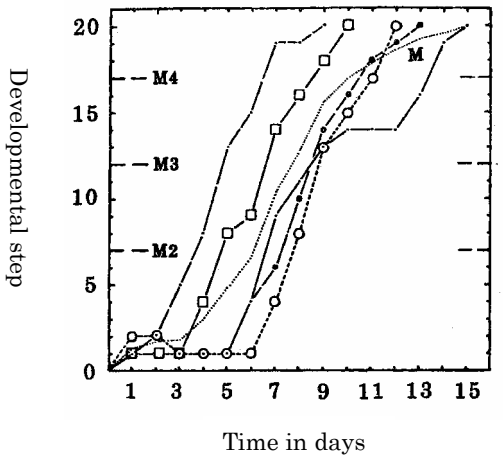

FIG. 20. Relationship between developmental steps of juveniles and time in MIZ males. Dotted line (M): Average of 5 individuals; Other lines: Both extremes and moderate individuals. M2, M3, and M4 : The same as in Fig. 18 .

TABLE 35. Regression line between the attainment value (probit) and time (logarithm) obtained from developmental steps of AKN and MIZ populations.

Population	Sex	Regression	Correlation	Predicted time for development			
			r[a]	7[b]	10[b]	12[b]	17[b]
AKN	♀	$\dot{Y}$= 2.3679 + 3.3320T[c]	0.9916**	4.72	6.17	7.35	12.63
MIZ	♀	$\dot{Y}$=1.5807 + 5.2947T	0.9970**	3.74	4.42	4.94	6.94
MIZ	♂	$\dot{Y}$= 1.0777 + 4.9286T	0.9871*	5.22	6.25	7.03	10.14

[a]* $P = 0.05$; ** $P = 0.01$.
[b] Developmental step 7 : J2 cuticle shed; 10: middle of whole developmental process; 12: J3 cuticle shed; 17: J4 cuticle shed.
[c] T = Time in days (logarithm); $\dot{Y}$= Attainment value (probit).

analyze the relationship between rates of molting and development in different populations as follows.

$$Y = (N/20) \times 100$$

The variable Y is an attainment value expressed as a percentage of the completion of development (20 steps). The variable N is the number of steps attained by each individual juvenile in a given time. The average attainment values (Y) of each group were converted to "probit" values and plotted against a logarithmic time scale as shown in Figure 21. The theoretical time needed for development through three molts was predicted by using the regression formulae in Table 35. The results indicated, for example, that the time to attain step 17 in which shedding of the J4 cuticle is completed was greater in AKN females than that for MIZ females.

As shown above, rate of molting and juvenile development could be evaluated using the developmental step method, and statistical analysis of differences between populations could be determined by probit conversion of the attainment values.

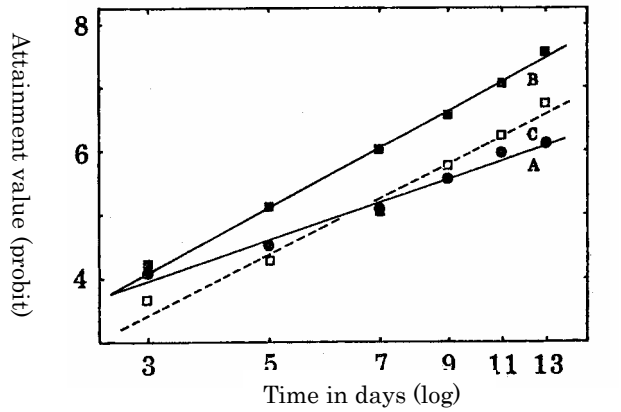

FIG. 21. Relationship between attainment value (probit) in molting and development and time in days (logarithm). A: AKN female; B: MIZ female; C: MIZ male.

3. pH of incubating medium and juvenile development

Rates of the molting and development of juveniles were tested with varying pH levels of the incubating medium.

Materials and methods

Nematode populations used: The MIZ, HAW, and TEX populations of MNT, the ASH-a population of MRT, and the SIB and AKN populations of MAT were used in this study. Numbers of J2 of each population were prepared in the same way as in the previous experiments. Varying pH levels of the incubation medium were prepared by adding two kinds of buffer solutions (Na2HP04 plus KH2P04 and

TABLE 36. Recipe for preparation of pH levels of incubating water medium and pH treatments.

	Aconitic acid plus sodium hydroxide buffer (A)					Sodium phosphate plus potassium phosphate buffer (B)				Distilled water (C)
Treatment code		1	2	3	4		5	6	7	8
Original solution	M/2 A-acid*	20**	20	20	20	M/15 Na2HPO4	1	7	9.5	0
	M/2 NaOH	15	76	103	126	M/15 KH2PO4	9	3	0.5	0
	Distilled water	165	104	77	54		0	0	0	0
	pH	2.4	4.1	5.0	5.8		5.85	7.2	8.04	6.7
Test solution	Orig. solution	1	1	1	1		1	1	1	-
	Distilled water	9	9	9	9		9	9	9	10
	pH	2.9	4.3	5.3	6.1		6.0	7.3	8.2	6.7

*A-acid: 1,2,3-Propenetricarboxylic acid (C6H6O6); ** ml each.

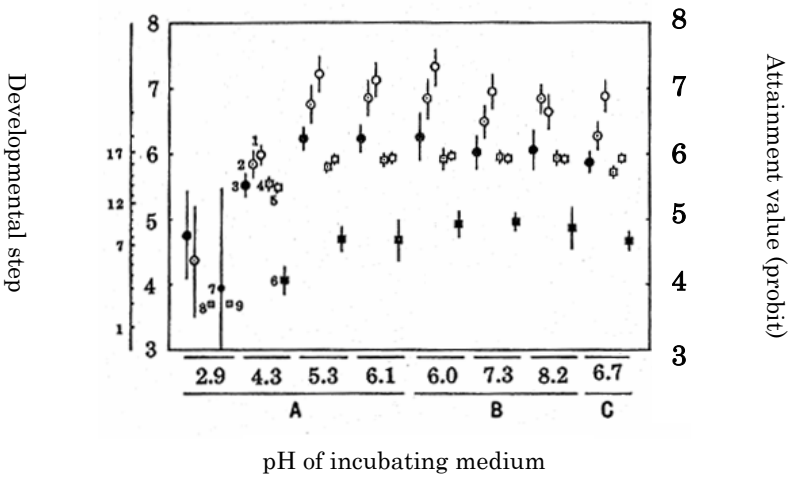

FIG. 22. Effects of pH of incubating water medium on juvenile development. 1: TEX; 2: HAW; 3: MIZ; 4: AKN; 5: SIB; 6: ASH-a population; 7, 8, and 9: Dead juveniles of MIZ, TEX, and AKN, respectively; A and B: Kind of buffer (see Table 36); C: Distilled water as control. Average and 95% confidence limit.

aconitic acid (C6H606) plus NaOH), as shown in Table 36. Test solution for incubation of juveniles was prepared by mixing 1 ml original buffer solution and 9 ml distilled water. Twelve fresh second-stage juveniles of each population were placed in a Seinhorst dish for fixing nematodes with 1.5 ml test solution. The dishes with test solution and juveniles were placed in large petri dishes (mentioned before) with a small amount of tap water and kept in an incubator regulated at 27 to 28 ˚C in the dark. The pH levels of incubation medium (test solution) ranged from 2.9 to 8.2. Distilled water was used as the control (pH 6.7). After 1 week of incubation in the incubator nematodes were examined under a compound microscope at 400 × magnification to observe the developmental steps. Developmental differences were compared by probit conversion of the attainment values. Juvenile incubation tests included five replications (five dishes).

Results

At low pH (2.9 and 4.3) development of juveniles was delayed and most juveniles died (Fig. 22). At pH 2.9, mortality rates for juveniles of TEX, ASH-a, and SIB were 100%; those of MIZ, HAW, and AKN were 80%, 92%, and 90%, respectively. At pH 4.3, 15.4% of juveniles of MIZ, 20.5% of ASH-a, and 11.5% of SIB died, but juveniles of the other populations tested did not die at the same pH. At pH 5.3 and higher pH, mortality rates of juveniles ranged from 0 to 6% in all populations tested. Developmental steps of dead individuals ranged from 1 to 4 in most cases, and were rarely steps 5 to 12. Juveniles of

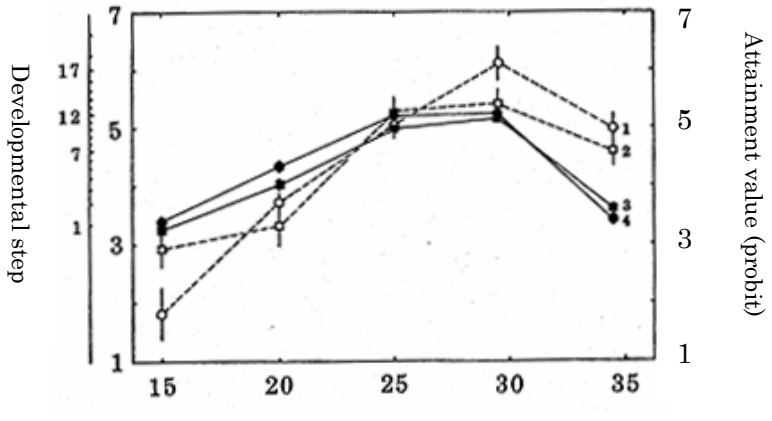

FIG. 23. Effects of incubation temperature on juvenile development. 1: HAW; 2: MIZ; 3: ASH-a; 4: AKN. Average of 60 individuals with 95% confidence limit.

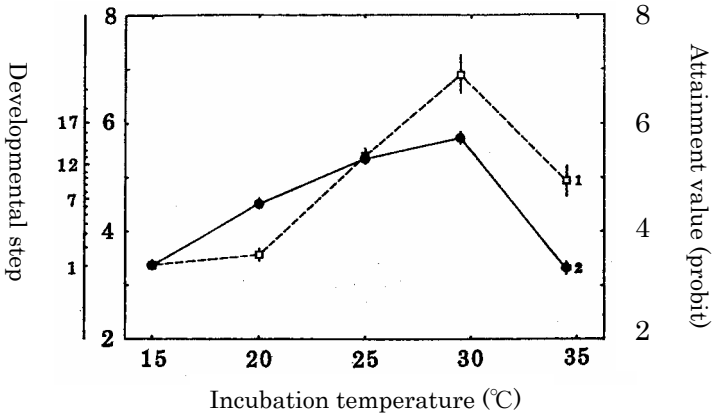

FIG. 24. Effects of incubation temperature on juvenile development. 1: TEX; 2: SIB. Average of 60 individuals with 95% confidence limit.

TEX, HAW, MIZ, AKN, and SIB had developed to step 16 or more during 1 week of incubation at pH 5.3 and higher pH treatments, while ASH-a juveniles remained at step 7. Treatments of pH 5.3, 6.1, 7.3, 8.04, and 6.7 (control) did not affect juveniles of any population or cause significant differences in development.

In conclusion, in this set of experiments, pH of the incubation medium had only a negligible effect on juvenile development except for extremely low pH, such as pH 2.9 or 4.3.

4. Temperatures and juvenile development

Effects of temperatures on juvenile development were tested to compare different populations.

Materials and methods

Nematode populations tested and preparation of juveniles: The same as in the previous experiment 20 freshly hatched juveniles of each nematode population were placed in a Seinhorst dish (mentioned above)

TABLE 37. Soil temperatures tested and experimental methods.*

Expt code	Treat. code	Av. soil temp. & range (˚C)	Expt. length (day)	Interv. for pot harv. (day)	Popul. Tested	Nematode number inoculated per pot				
						Male	Female	J2	Total	♂/♀
1	1)	30.5±1.7	36	1	MIZ,	287	389	37	713	0.7
	2)	15.0±1.0	70	4	HAW	178	339	0	517	0.5
2	1)	33.4±1.0	40	1	MIZ,	482	632	133	1,247	0.8
	2)	23.0±1.3	65	2**	HAW	402	644	342	1,388	0.6
3	1)	26.4±1.5	54	1	MIZ	577	843	9	1,431	0.9
	2)	16.9±1.5	153	4	HAW	388	767	10	1,165	0.5
4	1)	33.0±1.0	35	1	MIZ	773	1,043	888	2,704	0.7
		33.0±1.0	35	1	HAW	741	1,037	2,146	3,924	0.7
		33.0±1.0	35	1	TEX	702	880	2,009	3,591	0.9
		33.0±1.0	35	1	ASH-a	0	1,001	27	1,029	0
		33.0±1.0	35	1	SIB	0	1,346	243	1,589	0
		33.0±1.0	35	1	AKN	0	1,101	882	1,983	0
5	1)	29.8±1.2	40	1	TEX	105	343	1,044	1,492	0.3
	2)	25.2±1.8	40	1	M×H	227	489	729	1,445	0.5
	3)	19.9±1.4	50	2**	AKN	0	864	1,053	1,917	0
	4)	15.9±1.3	103	4						
6	1)	29.2±1.7	40	1	ASH-a	0	659	167	826	0
	2)	25.8±1.5	40	1	ASH-b	0	698	109	807	0
	3)	19.9±1.3	65	2**	SIB	0	1,006	188	1,194	0
	4)	16.6±1.5	177	4						

* Treat. code = Treatment code; Expt. length (day) = Experimental length in days; Av. soil temp & range (˚C) = Average soil temperature with fluctuation range (˚C); Interv. for pot harv. (day) = Interval in days for pot harvest; Popul. tested = Population tested. ** After 21 days, pot harvest was carried out every day (1-day interval).

TABLE 38. Relationship between soil temperatures and time for development of MIZ and HAW populations of MNT.

Soil temp.*	Popul. code*	Secr. of gel. matrix**	Male attr.**	Ovipos.**	Term of egg*	Juv. stage* ♂	Juv. stage* ♀	Gener. time* ♂	Gener. time* ♀
33.4	MIZ	7	7	8	4	5	6	17	18
	HAW	6	7	7	3	8	8	18	18
30.5	MIZ	4	4	9	7	7	8	23	24
	HAW	4	7	10	4	8	9	22	23
26.4	MIZ	5	6	9	7	9	8	25	24
	HAW	4	6	9	4	11	11	24	24
23.0	MIZ	8	11	14	8	14	14	36	36
	HAW	7	9	11	7	14	14	32	32
16.9	MIZ	13	13	29	28	32	40	89	97
	HAW	13	21	29	20	44	43	93	92

* Soil temp. = Soil temperature (˚C); Popul. code = Population code; Term of egg = Term of egg stage (day); Juv. stage = Juvenile stage length in days; Gener. time = Generation time in days (least length in days from penetration of females to emergence of next young females or males). ** Secr. of gel. matrix = First secretion of gelatinous matrix in days after 24-hour inoculation period; Male attr. = First male attraction (males staying around female body) in days after 24-hour inoculation period; Ovipos. = First oviposition in days after 24-hour inoculation period.

with 1.5 ml distilled water and kept in incubators regulated at 15, 20, 25, 29, or 34 ˚C in the same way as in the previous experiment for 1 week in the dark. After incubating for 1 week, juveniles were examined for development under a compound microscope (400× magnification) and the probit conversion and attainment averages were compared with a 95% confidence limit.

Results

As shown in Figures 23 and 24, the development of juveniles increased with increasing incubation temperatures from 15 to 25 ˚C in all populations tested, but populations of MNT, especially HAW and TEX, developed more rapidly at 30 ˚C than at 25 ˚C, contrasting with populations of MRT and MAT, whose developmental rate at 30 ˚C equaled that at 25 ˚C. Additionally it was noteworthy that juvenile development in the latter populations was depressed more severely by an incubation temperature of 34 ˚C than in the former ones, as indicated by developmental only to step 1 for the latter, and to about step 10 for the former. Virtually no development occurred in juveniles of the latter at 34 ˚C, while juveniles of the former had developed to early stages of molting of the J3 cuticle at the same temperature. Among the three populations of MNT, MIZ juveniles developed more slowly than those of the other two (TEX and HAW) at 30 ˚C. As presented above, incubation temperatures affected the juvenile development of six populations, and development at 34 ˚C was noticeably different between juveniles of MNT and those of the other two (MRT and MAT).

5. Soil temperatures of culturing pots and developmental time for one generation

Effects of soil temperature on the life cycle of six laboratory populations were studied to determine developmental zero (basal temperature) and effective accumulative temperature of those nematode populations.

TABLE 39. Relationship between soil temperatures and time for development of ASH-a and ASH-b populations of MRT, and SIB population of MAT.

Soil temp.*	Popul. code*	Secr. of gel. matrix**	Ovipos.**	Term of egg*	Juv. stage* ♂	Juv. stage* ♀	Gener. time* ♂	Gener. time* ♀
29.2	ASH-a	5	7	5	17	10	29	22
	ASH-b	-	-	-	7	10	19	22
	SIB	-	-	-	-	8	-	21
25.8	ASH-a	3	7	9	-	10	-	26
	ASH-b	-	-	-	-	10	-	27
	SIB	-	-	-	-	9	-	25
19.9	ASH-a	7	21	8	25	16	54	45
	ASH-b	-	-	-	-	22	-	49
	SIB	-	-	-	-	21	-	52
16.6	ASH-a	31	47	24	-	57	-	128
	ASH-b	-	-	-	-	70	-	144
	SIB	-	-	-	-	62	-	136

* The same as in Table 38; ** the same as in Table 38.

TABLE 40. Relationship between soil temperatures and time for development of TEX, M×H of MNT, and AKN populations of MAT.

Soil temp.*	Popul code*	Secr. of gel. matrix**	Male attr.**	Ovipos.**	Term of egg*	Juv. stage*		Gener. time*	
						♂	♀	♂	♀
29.8	TEX	-	-	-	-	6	6	18	18
	M×H	-	-	-	-	7	7	19	19
	AKN	6	9	9	6	-	8	-	23
25.2	TEX	-	-	-	-	9	9	27	27
	M×H	-	-	-	-	11	11	28	28
	AKN	5	13	13	9	-	12	-	34
19.9	TEX	-	-	-	-	10	11	31	32
	M×H	-	-	-	-	11	12	30	31
	AKN	9	17	17	10	-	19	-	46
15.9	TEX	-	-	-	-	22	22	73	73
	M×H	-	-	-	-	16	18	75	77
	AKN	17	33	33	34	-	-***	-	-***

* The same as in Table 38; ** the same as in Table 38; *** no females emerged within the experiment (103 days).

Materials and methods

Nematode populations used: In addition to the populations that were used in the previous test, population M×H (crossed population between MIZ and HAW; see section I for details) and population ASH-b were used, for a total of eight populations. Tests were conducted with 13 temperatures of soil from a minimum of 15 ˚C to a maximum of 33.4 ˚C, as shown in Table 37. Since all temperature treatments could not be applied equally to all nematode populations, six experiments were selectively conducted with different combinations of temperatures and nematode populations. Number of nematodes inoculated differed from 517 to 3,924 per pot (small clay pots as mentioned later) among experiments. Inoculated nematodes were prepared as described in section I. Extraction of nematodes was by SVB. Tomato seedlings (cv. Fukuju No. 2, four-leaf stage) were separately transplanted to small clay pots with "steam-sterilized mixed soil" and grown in a greenhouse for 1 week. After that, 2 to 4 ml of nematode suspension containing the given nematode numbers as shown in Table 37 was inoculated into two holes (7-mm diam. and l cm deep) around the plant in each pot. After inoculation, pots of the same nematode population were embedded in steam-sterilized sand retained by plastic containers (see section I for details) at about 3-cm spacing to avoid quick desiccation and placed in a greenhouse at 20 to 30 ˚C for the first 48 hours to allow nematodes to penetrate tomato roots. After the 48-hour penetration, inoculated pots within plastic containers were transferred to a greenhouse or growth chamber with 8,000-lux illumination (fluorescent lamps for 12 hours and dark for 12 hours) and regulated at the necessary temperatures. Each container was moved within the greenhouse or the growth chamber once a week to diminish the effect of uneven temperature by position. Tomato plants were fertilized with 1 ml of diluted (1/2 in strength) Hoagland solution once a week and water, which was adjusted to the same temperature of the greenhouse or growth chamber, was added when necessary. A set of 40 to 50 pots was prepared for one temperature treatment per population.

One pot was randomly selected and processed to extract nematodes by SCF at varying intervals (1, 2, or 4 days according to the temperature treatments as shown in Table 37), and nematodes were examined for beginning of eclosion of eggs, duration of juvenile stages (J2 after hatched to the end of juvenile development including three molts of the J2, J3, and J4), generation time (a life cycle), and nematode numbers (fresh J2, developing juveniles, males, and females) under a compound microscope with 60 to 400× magnification. Host plant roots were carefully removed and washed with tap water. Then, they were also examined for commencement of production of a gelatinous matrix and oviposition by females under a stereo microscope with 60× magnification.

Results

Times in days of gelatinous matrix production, oviposition, juvenile stage, and generation (one life cycle) were determined (Tables 38, 39, and 40).

Female parasitism and beginning of oviposition: Penetration of roots by young females was observed commonly on all nematode populations 24 hours after inoculation. Usually, the anterior part (head and neck) of females penetrated roots at an approximately right angle and the lip region reached the endodermis of roots. The posterior part (more than half) of the female body remained outside the root tissues. The gelatinous matrix produced by females on roots in MIZ and HAW populations of MNT was first observed at 26 to 30 ˚C, 4 to 5 days after the 24-hour inoculation period (hereafter, time in days is expressed in days after the 24-hour inoculation period). Appearance of the matrix was delayed more at lower

TABLE 41. Differences in development at 33 ˚C between three populations of MNT and three of MRT or MAT (days).

Biol. type	Population	Beginning of hatch*	Term of juvenile stage		Generation time	
			Male	Female	Male	Female
MNT	MIZ	10	7	7	17	17
	HAW	11	6	6	17	17
	TEX	10	5	5	15	15
MRT	ASH-a	28	-**	-**	-	-
MAT	SIB	22	-**	-**	-	-
	AKN	27	-**	-**	-	-

* Days after 24-hour inoculation time; ** no males or females emerged during experimental period (35 days).

or higher temperature treatments in MRT (ASH-a) and MAT (AKN) populations. It was first observed at 25 to 29.8 ˚C, 3 to 6 days after 24-hour inoculation (no examination for other populations including TEX, $M^{x}H$, SIB for gelatinous matrix secretion was made). Attraction of males, which gathered around females enclosed in the gelatinous matrix and eggs, was observed at about the same time as the secretion of the gelatinous matrix in most cases, and occasionally there was a lapse of 1 or 2 days between secretion of the gelatinous matrix and male attraction in MNT populations. Initiation of oviposition by females occurred earlier with increasing temperature. It began on days 7 to 8 at 33.4 ˚C in the MNT population, and on days 7 to 9 in MRT and MAT populations at 29 to 30 ˚C (no data on oviposition for MRT and MAT at temperatures greater than 30 ˚C).

Term of egg stage: The period of the egg stage was estimated to be the time from the beginning of oviposition until the first occurrence of J2 in soil. It lasted for 3 to 4 days at 33.4 ˚C in MNT populations and increased in time with a decrease in soil temperature. The egg stage lasted 20 to 28 days at 16.9 ˚C (Table 38). It was interesting that the length of the egg stage was shorter in HAW than in MIZ at all soil temperatures tested. Similarly, the egg stage in MRT and MAT was 5 to 6 days at 29 and 30 ˚C and 24 to 34 days at around 16 ˚C (Tables 39 and 40). Some differences also occurred between ASH-a and AKN populations. Hatch of juveniles at 33 ˚C was greatly delayed in the ASH-a population of MRT and the SIB and AKN populations of MAT as compared to nematodes of MNT. The former needed 22 to 28 days until emergence of juveniles, while the latter hatched 10 to 11 days after inoculation (Table 41).

Length of the juvenile stage: The juvenile stage here includes J2, J3, and J4 with three molts to the young female or male stage. At 33 ˚C, juveniles of each population of MNT needed 5 to 7 days in both sexes for completing development, and at lower temperatures, such as 16.9 ˚C, MIZ and HAW populations required 40 days for their development. The TEX population required 22 days at 15.9 ˚C for development (Tables 38 and 40). On the other hand, juveniles of MRT and MAT developed in the shortest time at about 30 ˚C, but there was no difference in development time for ASH-a and ASH-b between 30 ˚C and 25 ˚C. The most striking difference between MNT and MRT or MAT populations was observed at 33 ˚C. Here, juveniles of the MNT all finished their development within a week, while those of the MRT and MAT did not develop to young females or males at all during the experimental period of 35 days (Table 41).

Generation time: The shortest time for one generation in the MNT was observed to be 15 to 17 days at 33 ˚C, and it increased with declining soil temperatures. Differences between sexes were not significant, but the TEX population had some unexpected generation periods. It was 15 days at 33 ˚C, being only 2 to 3 days shorter than the other two, MIZ and HAW. However, it was 73 days at 15.9 ˚C, in contrast to more than 90 days for MIZ and HAW at 16.9 ˚C (Tables 38 and 40). Nematodes of MRT and MAT did not complete their generation at 33 ˚C (Table 41), but a generation was completed in 21 to 23 days at 29.2 to 30.5 ˚C. This generation time was similar to that of MIZ and HAW. At low temperatures, around 16 ˚C, it took 128 to 144 days for the MRT and MAT populations to complete a generation.

Developmental rate, effective cumulative temperature, and developmental zero (basal temperature): The developmental rate for one generation had a linear relationship to soil temperature for each population when analyzed by linear regression (Table 42). Correlation coefficients between the two variables on generation time were positive and highly significant for each population except for AKN. Slopes of the regressions were largest in SIB followed by ASH-a and TEX, and then MIZ, ASH-b, HAW, and M×H in descending order. The AKN had the lowest regression slope, although it was not significant. Effective cumulative temperatures (Ÿ) were estimated from an equation derived from linear regression. The largest value was 458.1 (degree-days: DD) for AKN (not significant), and the smallest value was 307.7DD for SIB.

Developmental zero ranged from 9.3 ˚C (M×H male) to 14.1 ˚C (SIB female). In general, nematodes

TABLE 42. Relationship between developmental velocity of eight laboratory populations and soil temperatures on generation time.

Biol. type	Population	Sex	Regression	Cor. coef.[a]	Devel. zero	Ef. cum. temp.
MNT	MIZ	♂	Ÿ= 0.002822T－0.036731	0.98615**	13.0	354.4
		♀	Ÿ= 0.002735T－0.035086	0.97539**	12.8	365.6
	HAW	♂	Ÿ= 0.002700T－0.032828	0.98566**	12.2	370.4
		♀	Ÿ= 0.002663T－0.032143	0.97920**	12.1	375.5
	TEX	♂	Ÿ= 0.002902T－0.030804	0.98149**	10.6	344.6
		♀	Ÿ= 0.002927T－0.031620	0.98463**	10.8	341.7
	M×H	♂	Ÿ= 0.002520T－0.023472	0.95091*	9.3	396.8
		♀	Ÿ= 0.002563T－0.024789	0.95895*	9.7	390.2
MRT	ASH-a	♀	Ÿ= 0.002949T－0.038955	0.99089**	13.2	339.1
	ASH-b	♀	Ÿ= 0.002715T－0.037155	0.96069*	13.7	368.3
MAT	SIB	♀	Ÿ= 0.003250T－0.045783	0.99673**	14.1	307.7
	AKN[b]	♀	Ÿ= 0.002183T－0.022973	0.97830-	10.5	458.1

[a] Cor. coef. = Correlation coefficient; Devel. zero = Developmental zero; Ef. cum. temp. = Effective cumulative temperature (degree-days: DD).
[b] Calculated with only three points of variable; * = Cor. coef. significant at 0.05 level; ** = significant at 0.01 level. Ÿ = Predicted rate of development; T = Soil temperature.

of the MNT had a relatively low developmental zero ranging from 9.3 to 13 ˚C (MIZ male), while those of the MRT and MAT had higher values ranging from 13.2 ˚C (ASH-a) to 14.1 ˚C (SIB female) except for AKN. Developmental rate for the juvenile stage was also correlated with soil temperature as shown in Table 43. The relationship between juvenile stage interval (days) and soil temperature was similar to that between generation time and soil temperature, mentioned above, with the exception of those for the M×H and AKN populations, which were not significant based on the correlation coefficient. Developmental zero in juveniles was about 10 ˚C in both sexes of the TEX population, but most other populations showed more than 13 ˚C with significant correlation coefficients except for M×H and AKN

The slope of the linear regression for egg development was larger in MIZ and HAW populations than in ASH-a and AKN, and therefore the effective cumulative temperature was smaller in the former than the latter . Developmental zero was lower in the AKN and ASH-a populations than in the MIZ and HAW.

Effective cumulative temperatures of eight populations were plotted against developmental zeros as shown in Figure 25. There were negative correlations between effective cumulative temperature and developmental zero, with the exception of TEX and AKN. The M×H population had the largest effective cumulative temperature but its developmental zero was lowest, while the SIB's effective cumulative temperature was smallest and its developmental zero was highest among populations tested. Other populations were distributed along a curved line between M×H and SIB populations.

TABLE 43. Relationship between developmental velocity of eight laboratory populations and soil temperatures on juvenile stage interval (days).

Biol. type	Population	Sex	Regression	Cor. coef.[a]	Devel. zero	Ef. cum. temp.
MNT	MIZ	♂	Ÿ= 0.008822T－0.123370	0.95518**	14.0	113.4
		♀	Ÿ= 0.007801T－0.102857	0.96824**	13.2	128.2
	HAW	♂	Ÿ= 0.007466T－0.102785	0.95867**	13.8	133.9
		♀	Ÿ= 0.007215T－0.098174	0.95001**	13.6	138.6
	TEX	♂	Ÿ= 0.008424T－0.083929	0.97843**	10.0	118.7
		♀	Ÿ= 0.008651T－0.091347	0.98731**	10.6	115.6
	M×H	♂	Ÿ= 0.005078T－0.018460	0.92030-	3.6	196.9
		♀	Ÿ= 0.005694T－0.03607	0.94889-	6.3	176.6
MRT	ASH-a	♀	Ÿ= 0.006501T－0.078719	0.94177-	12.1	153.8
	ASH-b	♀	Ÿ= 0.007231T－0.100467	0.96806*	13.9	138.3
MAT	SIB	♀	Ÿ= 0.009019T－0.131366	0.99063**	14.6	110.9
	AKN[b]	♀	Ÿ= 0.007275T－0.094661	0.99178-	13.0	137.5

[a], [b] See Table 42 for details.

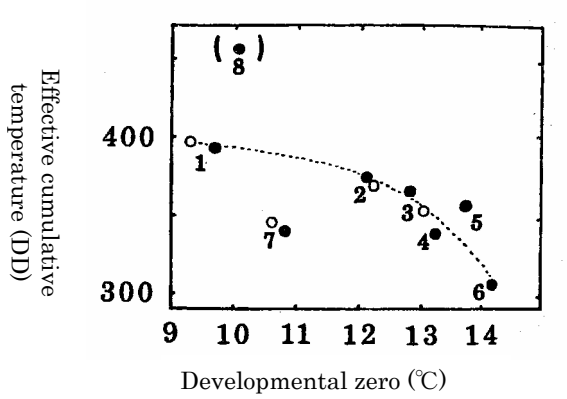

FIG. 25. Relationship between effective cumulative temperatures and developmental zeros in eight laboratory populations. Black circle: female; empty circle: male; 1: M×H; 2: HAW; 3: MIZ; 4: ASH-a; 5: ASH-b; 6: SIB; 7: TEX; 8: AKN (no significance in correlation coefficient between the two variables).

As shown above, there were clear differences in response to soil temperature between the MNT population and MRT or MAT, especially at high temperatures, such as 33 ˚C or 33.4 ˚C. The temperature response was distinctive because juveniles of MRT and MAT did not develop to young females, while those of MNT developed normally to young females or males at the same temperatures.

6. Discussion

The juveniles of *R. reniformis* were observed for development to males or young females after molting 3 times in the absence of a host. The results agreed generally with the report by Linford and Oliveira (1940). They suggested there were four molts of the juveniles after hatching but in the current study only three molts of the J2, J3, and J4 cuticles were observed. The first molting in this nematode was reported to occur within the eggshell before hatch. It is the same as in other nematode species (Nakasono, 1966; Sivakumar and Seshadri, 1971a). Examples of juveniles that can develop to adults without feeding are known in males of *Tylenchulus semipentrans* (Van Gundy, 1958) and *Meloidodera floridensis* (Hirschmann and Triantaphyllou, 1973). The J2 of *Paratylenchus aciculus* also develop to adult females without feeding after hatch (Kashio et al., 1975). The J2 of *P. projectus* take some food at the second stage but lack a stylet needed to feed at the third and fourth stage of development (Rhoad and Linford, 1961). Nematode stages that do not appear to feed during development also occur in *Meloidogyne* nematodes (Triantaphyllou, 1960; Triantaphyllou and Hirschmann, 1960).

Twisting, shortening, and elongating movements of the juvenile body coincided with the morphological changes that occurred on a regular basis during molting and development of the second, third, and fourth stages of *R. reniformis*. This agrees with reports by Linford & Oliveira (1940) and others (Brazcski et al., 1971; Hechler, 1967; Hirschmann and Triantaphyllou, 1973; Roman and Hirschmann, 1969). Twisting and shortening of the body appeared to assist the juvenile in shedding the old cuticle. Measurements of body length and width during molting and development of juveniles were made in this study. The rates for shortened body length and width were different between juveniles of MNT and MRT. It probably suggests some physiological differences between the two. Male individuals elongated more than J2 after completing a molt and developed without feeding. A similar phenomenon is also known for males of *T. semipenetrans* (Van Gundy, 1958).

In order to differentiate developmental stages of nematodes, lengths of body, stylet, and gonads are commonly used as criteria in many species that repeat feeding and growing at each developmental stage (Golden, 1956; Hirschmann, 1962; Mamiya, 1975; Yagita, 1975; Yuen, 1966). This method, however, is not so applicable to juveniles of *R. reniformis* because such growth or increase in body or stylet length does not occur at each stage, although gonad development does occur (Nakasono, 1980). An attempt was made to use the 21 developmental steps for evaluating the degree of development in juveniles of this nematode. Attainment values evaluated as the percentage of the completed developmental steps to the total steps required for development were converted to probit values for statistical analysis. A similar method was used to compare molting in animal-parasitic nematodes (Sommerville, 1957). This method seems to be reasonable to analyze differences in physiological characters, such as juvenile development.

Extremely low pH, such as 2.9 and 4.3, of the incubation medium severely affected the development of juveniles of *R. reniformis*. There was some doubt that chemicals in the buffer solution (aconitic acid and NaOH) influenced juveniles and may have been responsible for some of the dead juveniles. It was not possible to separate chemical toxicity and pH effects in this study. Similar effects were pointed out for soybean cyst nematode hatching solution (Okada, 1977). Development of juveniles of all populations tested was not affected at a pH of 5.3 to 8.3. Molting in J4 of *Paratylenchus nanus* is not affected by pH 4 to 7.0 (Fisher, 1966b). Reproduction of *T. semipenetrans* is affected in soils by pH lower than 4.3 but is not affected at pH 4.0 to 7.3 (Van Gundy and Martin, 1961). In animal-parasitic nematodes there is an optimum pH for molting (Rogers and Sommerville, 1960). Plant-parasitic nematodes do not appear to have an optimum pH for molting and development. However, extremely low or high pH values are deleterious and can be lethal.

Temperatures affected molting and development of *R. reniformis* in both incubation medium and pot soils; in particular, juvenile development of MRT and MAT was severely inhibited at around 33 ˚C, while development of MNT juveniles was only slightly retarded at the same temperature. At low temperatures, such as 16 to 17 ˚C, the former needed a longer period (days) for completion of a generation than did the latter. The developmental zero (basal temperature) was relatively higher in the former than in the latter. These facts suggest that MNT populations and MRT or MAT are different from each other in adaptability to temperature. Tolerance of high temperatures in the former provides an explanation for their wide distribution and ability to reproduce in tropical areas, while intolerance for high temperatures in the latter suggests their distribution is restricted to subtropical or temperate zones. On the other hand, lower values for the developmental zero observed with the former suggests they can survive even in cool climate areas found in temperate zones. The effective cumulative temperature (DD) for one generation was greater in populations with a lower developmental zero (MNT) than those of populations having a higher developmental zero (MRT and MAT). A similar phenomenon occurs in some insects (Utida, 1957), but the developmental zero is usually higher in southern or tropical insects than in northern or temperate ones. This differs from *R. reniformis*. MRT and MAT populations that have a higher developmental zero than that of MNT are widely distributed in more northern areas than the latter in Japan (Figs. 1 and 3). The smaller DD of MRT and MAT populations compared with that of MNT might explain this; namely, a smaller DD is more advantageous for the former to complete a generation in northern climactic conditions. It is interesting that the DD of HAW is close to that of MIZ but not to that of TEX. The TEX population was peculiar in having a lower developmental zero and smaller DD compared with other populations tested (Fig. 25). The TEX population appears to be similar to that reported by Rebois (1973). This MNT population had an optimal temperature for development of 29.5 ˚C with 19 days or less for one generation. The temperature requirements for different populations of *R. reniformis* appear to be involved in the geographical distribution in the original localities.

The developmental zero in other nematodes is known to be variable between species and within species. The developmental zero appears to be related to geographical distributions as follows: 4.4 ˚C for *Heterodera schachtii* (Jones, 1975), 10 ˚C for *H. glycines* (Ichinohe, 1955), 9.0 ˚C for *Meloidogyne* sp. in North America (Tyler, 1933), l0.08 ˚C for *M. incognita*, 8.8 ˚C for *M. hapla* (Vrain et al., 1978), and 12 ˚C for *M. incognita* in Kumamoto prefecture, Japan (Gotoh et al., 1973). Similar variation in optimal temperature for reproduction occurs in populations of *Globodera pallida* and *G. rostochiensis* distributed in New Zealand, Peru, and England (Foot, 1978; Franco, 1979).

It is considered that such variations between species and within species of plant-parasitic nematodes involve genetic variations and natural selection to climate, cultivation methods, crop species or cultivars, and other environmental factors (Ellenby and Smith, 1975; Hominick, 1979). Laboratory populations of *R. reniformis* used in this study were all cultured in the same way in the same greenhouse and each population repeated at least 10 generations before being used in this study. Therefore, all of them appear to have received similar selection pressure in the greenhouse during the period of population propagation. However, the results of experiments here showed that each population still maintained its own characteristics in developmental response to environment, especially to varying temperatures. The populations present in the original geographic localities should have similar characteristics to the nematodes cultured continuously in the greenhouse. However, some distinctive characters may have been lost during extended greenhouse culture.

VIII. Differences in Host Preference of Laboratory Populations

1. Comparison of host preference among six laboratory populations

Rotylenchulus nicotiana (Yokoo & Tanaka, 1954), synonym for *R. reniformis*, was first reported from a field in Aukune city, Kagoshima prefecture, in which tobacco (*Nicotiana tabacum* L.) was cultivated, but this plant was not a very good host for that nematode when tested under greenhouse conditions (Nakasono and Ichinohe, 1967). Another population of *R. reniformis* belonging to MAT from a great burdock field of Tokorozawa city, Saitama prefecture, increased well on African marigold (*Tagetes erecta* L., cv. Gold Smith), but French marigold (*T. patula* L., cv. Fantango) suppressed its population density when tested in the field (Nakasono, 1980). This result appeared to be somewhat different from that observed by Linford and Yap (1940) for the MNT Hawaiian population of *R. reniformis*. As the final study here, a pot experiment was done to test host preference for six laboratory populations of *R. reniformis* in the greenhouse.

Materials and methods

Nematode populations used: MIZ and HAW populations of MNT, ASH-a and ASH-b ones of MRT,

TABLE 45. Inoculated nematode numbers per pot of six laboratory populations of *R. reniformis.*

Biological type	Population	Male (%)	Young female (%)	J2 (%)	Total	♂/♀
MNT	MIZ	4,102 (33)	4,770 (38)	3,609 (29)	12,481	0.8
	HAW	2,625 (36)	3,962 (54)	745 (10)	7,332	0.7
MRT	ASH-a	0	2,804 (88)	361 (11)	3,165	0
	ASH-b	0	5,380 (86)	868 (13)	6,248	0
MAT	SIB	0	5,233 (83)	1,026 (16)	6,259	0
	AKN	0	2,745 (42)	3,738 (58)	6,483	0

SIB and AKN ones of MAT. Nematodes for inoculum were propagated as described in section I and extracted from soils by SVB.

Plants tested: Nine plants were tested as hosts as follows: nira in Japanese (*Allium tuberosum* Rottl., cv. unknown); cabbage (*Brassica oleracea* L. var. *capitata* L., cv. Takara Kanran); watermelon (*Citrullus vulgaris* Schrad, cv. Wase Shimaou); carrot (*Daucus carota* L. var. *sativa* Dc., cv. Senkou 3 Zun); sweet potato (*Ipomoea batatas* Lam., cv. Norin No. 2); tobacco (*Nicotiana tabacum* L., cv. NC 95); African marigold (*Tagetes erecta* L., cv. Mommothnum); wheat (*Triticum vulgare* L., cv. Norin No. 61); cowpea (*Vigna sinensis* Endl., cv. unknown).

Growing plants: Three to six seeds of test plants mentioned above were buried in big clay pots with "steam-sterilized mixed soil" (see section I) after seeds were surface-sterilized with Ruberon (organic mercury, "1000 time solution"). For sweet potato a vine cutting was planted in each pot. Four pots per plant and 36 pots in total for nine kinds of test plants were prepared for one nematode population, the pots of which were divided randomly into six sets regardless of plant species and countersunk into steam-sterilized river sand within six plastic containers (mentioned in section I). Containers of test plants were arranged randomly on beds in the greenhouse; and after seeds of each test plant germinated, containers were randomly moved from place to place on beds every 2 weeks. At the same time 9 ml of diluted Hoagland solution (1/2 strength) was supplied to each pot. As the experiment was done during the late autumn and winter season, soil temperatures, which were monitored with an automatic thermorecorder, were comparatively low with an average of 19.8 ˚C and fluctuated between 15 and 21 ˚C.

Nematode inoculation: More than 2,700 young females of each population were inoculated to each pot as shown in Table 45. Populations of MIZ, ASH-b, SIB, and AKN were each inoculated 65 days after test plants were seeded and the remaining HAW and ASH-a populations were placed in pots 75 days after seeding for convenience; 5 ml of nematode suspension containing individuals as shown in Table 45 was poured into five holes (0.7-mm diam. and 1-cm deep) made around the plant in pot soil.

Examination for nematode infection and reproduction: Sixty days after nematode inoculation, pot soils and plant roots were carefully separated and soils were processed to extract nematodes by SCF. Nematodes recovered were identified as either newly emerged individuals or survivors of the original inoculum and counted separately under a stereo microscope. Plant roots were washed with tap water and stored in small vials with 5% formalin solution to determine numbers of females infecting roots and eggs produced.

Results

Adult females on roots, numbers of eggs produced, and numbers of newly emerged nematodes are presented for each test plant in Tables 46 to 51.

Sweet potato and marigold were parasitized by all populations tested, but female numbers on roots and rates (%) of parasitizing females (% of females on

TABLE 46. Host preference of six laboratory populations of *R. reniformis* for sweet potato as evaluated by parasitizing females and newly emerged nematodes.

Biol. type	Popul.	Females on root*	Infection rate***	J2 in soil*	♂+♀ in soil*	Eggs/♀ on root	Newly emerged nematodes/ ♀on root***	Rate****
MNT	MIZ	108	2.26	3,861	282	73	111	2.52
	HAW	119	3.00	4,956	123	51	94	2.82
MRT	ASH-a	20	1.28	17	0	32	33	0.24
	ASH-b	12	0.22	13	0	47	48	0.11
MAT	SIB	22	0.42	8	0	31	31	0.13
	AKN	57	2.07	33	0	55	56	1.16

* Average numbers per pot,;note J2 and ♂+♀were newly emerged (second state); ** Parasitizing female % to inoculated young females; *** (J2 + ♂+♀+eggs)/♀; **** Rate of newly emerged nematodes per inoculated young females.

TABLE 47. Host preference of six laboratory populations of *R. reniformis* for marigold as evaluated by parasitizing females and newly emerged nematodes.

Biol. type	Popul.	Females on root*	Infection rate**	J2 in soil*	♂+♀ in soil*	Eggs/♀ on root	Newly emerged nematodes/ ♀on root***	Rate****
MNT	MIZ	168	3.52	3,501	33	82	103	3.63
	HAW	7	0.18	405	10	78	137	0.24
MRT	ASH-a	0.3	0.00	0	0	5	5	0.00
	ASH-b	0.3	0.01	13	3	38	91	0.01
MAT	SIB	8	0.15	0	0	27	27	0.04
	AKN	8	0.03	0	0	46	46	0.13

*, **, ***, ****: Same as that in Table 46 for details.

TABLE 48. Host preference of six laboratory populations of *R. reniformis* for tobacco as evaluated by parasitizing females and newly emerged nematodes.

Biol. type	Popul.	Females on root*	Infection rate**	J2 in soil*	♂+♀ in soil*	Eggs/♀ on root	Newly emerged nematodes /♀on root***	Rate****
MNT	MIZ	52	1.09	1,928	21	87	124	1.35
	HAW	72	1.82	5,108	80	78	150	2.72
MRT	ASH-a	0	0	0	0	0	0	0
	ASH-b	4	0.08	0	0	58	58	0.04
MAT	SIB	4	0.07	0	0	42	42	0.03
	AKN	10	0.37	0	0	63	63	0.23

*, **, ***. ****: See Table 46 for details.

TABLE 49. Host preference of six laboratory populations of *R. reniformis* for cowpea as evaluated by parasitizing females and newly emerged nematodes.

Biol. type	Popul.	Females on root*	Infection rate**	J2 in soil*	♂+♀ in soil*	Eggs / ♀ on root	Newly emerged nematodes /♀on root***	Rate****
MNT	MIZ	2	0.04	7	0	16	20	0.01
	HAW	9	0.23	136	0	71	86	0.02
MRT	ASH-a	0	0	0	0	0	0	0
	ASH-b	7	0.26	0	0	49	49	0.06
MAT	SIB	0	0	0	0	0	0	0
	AKN	0.5	0.02	0	0	63	63	0.01

*, **, ***, ****: See Table 46 for details

TABLE 50. Host preference of six laboratory populations of *R. reniformis* for "nira" as evaluated by parasitizing females and newly emerged nematodes.

Biol. type	Popul.	Females on root*	Infection rate**	J2 in soil*	♂+♀ in soil*	Eggs / ♀ on root	Newly emerged nematodes / ♀on root***	Rate****
MNT	MIZ	01)	—	193	—	—	—	—
	HAW	28	0.71	171	13	45	52	0.37
MRT	ASH-a	0	0	0	0	0	0	0
	ASH-b	0.3	0.01	0	0	20	20	0.00
MAT	SIB	0	0	0	0	0	0	0
	AKN	2	0.07	23	0	16	28	0.02

*, **, ***, ****: See Table 46 for details. 1) Probably failure in detection of females on roots because newly emerged J2 were extracted from pot soils.

TABLE 51. Host preference of HAW population for watermelon, carrot, cabbage, and wheat as evaluated by parasitizing females and newly emerged nematodes.

Plant1)	Females on root*	Infection rate**	J2 in soil*	♂+♀ in soil*	Eggs / ♀ on root	Newly emerged nematodes / ♀on root***	Rate****
W M	5	0.12	7	0	41	42	0.05
CR	6	0.15	165	0	31	59	0.09
CB	0	0	0	0	0	0	0
WH	0		0	0	0	0	0

*, **, ***, ****: See Table 46 for details. 1) WM = Water melon; CR = Carrot; CB = Cabbage; WH = Wheat.

roots per female numbers inoculated per pot) were greater in sweet potato than in marigold. ASH-a and –b showed especially lower numbers in merigold than other populations (Tables 46 and 47). All populations, except ASH-a parasitized tobacco and populations of MNT, tended to have more females and higher infection rates than other populations (MRT and MAT, Table 48). On cowpea, parasitism was observed by only a few females of all populations; in fact, ASH-a and SIB populations had no females on roots and no reproduction (Table 49). On "nira," the HAW population had 28 females on roots, which was more than other populations had. ASH-a and SIB did not have females parasitizing this plant (Table 50).

It was interesting that watermelon and carrot were infected with the HAW population (Table 51) but not with other ones (MIZ, ASH-a, ASH-b, SIB, and AKN). This was a difference between HAW and MIZ, both of which belong to the same biological type (MNT). Cabbage and wheat were not host plants for all populations tested here (Table 51). Among Japanese populations, the MIZ population had a higher rate of parasitizing females than the others (ASH-a, -b, SIB, and AKN) on all test plants.

Numbers of newly emerged juveniles (J2), young females, and males (second generation) were all greater in both the HAW and the MIZ populations of MNT than in the MRT and MAT populations; namely, the HAW and MIZ had 400 to 5,000 J2 and 10 to 280 young females and males combined per pot on sweet potato, marigold, and tobacco, whereas populations of MRT and MAT had 8 to 33 J2 only on sweet potato, except for the ASH-b, which had 13 J2 and 3 young females on marigold. Similar differences were also observed on cowpea and "nira" between MNT and MRT or MAT populations. The HAW population had second-generation J2 on watermelon and carrot, while others did not have any second-generation J2 on the same plants.

In sum, the host preference as evaluated by parasitizing female numbers and rates obviously differed between the MNT population and the MRT or MAT-population; this was especially apparent on sweet potato, marigold, and tobacco. Furthermore, there were some differences even between the two populations (HAW and MIZ) of MNT and between populations of MRT and MAR.

2. Discussion

Quantitative analysis of host preference in nematodes evaluated by measuring numerically such characters as infection rate, egg production, and (or) reproduction rate on test plants can provide useful data for separating nematode species or physiological races. The identification method for the most common root-knot nematodes (*Meloidogyne* spp.), described by Sasser (1952), involves host reactions. The International *Meloidogyne* Project by Taylor and Sasser presented an identification method for *Meloidogyne* species utilizing six test plants, in which gall indices of 0, 1, 2, 3, 4, and 5 were assigned based on root galling and indices 0 to 2 were then designated with a minus sign (−) and 3 to 5 with a plus (+) (Taylor and Sasser, 1978). Since *R. reniformis* does not cause galls on host roots, identification criteria for *Meloidogyne* cannot be applied to *R. reniformis,* but the idea of using numerical comparisons provides a useful suggestion for this study. On the other hand, physiological races of soybean nematode, *Heterodera glycines*

TABLE 52. Comparison of infection rates of six laboratory populations *Rotylenchulus reniformis* on 9 test plants as percentage to that of HAW.

Biol. type	Population	SP*	MG	TB	CP	NR	WM	CR	CB	WH
MNT	HAW	100	100	100	100	100	100	100	0	0
	MIZ	90.8	2,400	72.3	22.2	(−)1)	0	0	0	0
MRT	ASH-a	16.8	4.3	0	0	0	0	0	0	0
	ASH-b	10.1	4.3	5.6	77.8	1.1	0	0	0	0
MAT	SIB	18.5	114.3	5.6	0	0	0	0	0	0
	AKN	47.9	114.3	13.9	5.6	7.1	0	0	0	0

* SP = Sweet potato; MG = Marigold; TB = Tobacco; CP = Cowpea; NR = Nira; WM = Watermelon; CR = Carrot; CB = Cabbage; WH = Wheat. 1) Newly emerged J2s were extracted from pot soils, so infection seems to be positive.

TABLE 53. Host preference of 6 laboratory populations of *Rotylenchulus reniformis* for 9 test plants expressed by the symbol + for positive infection or − for negative.

Biol. type	Population	SP*	MG	TB	CP	NR	WM	CR	CB	WH
MNT	HAW	+	+	+	+	+	+	+	−	−
	MIZ	+	++1)	+	+		−	−	−	−
						(+)2)				
MRT	ASH-a	+	−	−	−	−	−	−	−	−
	ASH-b	+	−	−	+	−	−	−	−	−
MAT	SIB	+	++1)	−	−	−	−	−	−	−
	AKN	+	++1)	+	−	−	−	−	−	−

* See Table 52 for plant names. 1) Percentage was more than 100% (see Table 52). 2) New J2 emerged in soils though parasitizing females on roots were not successfully detected. The relative infection rate more than 10 was referred to as the positive infection (+) and equal or less than 10as the negative (−).

Ichinohe, are identified by differences in numbers of parasitizing females or cysts on genetically different soybean cultivars or strains as test plants, in which the highest susceptible cultivar, "Lee," is used as a standard and infection rates and cyst numbers on four other test cultivars or strains of soybean are compared to numbers on the standard. A percentage value more than 10 is designated as a positive infection (+), but a value equal to or less than 10 is designated as no infection (−) (Golden et al., 1970).

In the present study, young female numbers for inoculum varied among populations, ranging from 2,700 to 5,300, but infection rates of all populations (parasitizing females) approximately paralleled the number of females in inoculum. Thus, the HAW population, which had infection rates commonly higher on all test plants except for marigold, was used as a standard for assigning relative values (%) of infection rates (Table 52). When the relative value was more than 10, infection on the plant was referred to as positive (+), whereas it was considered negative (−) when the value was equal to or less than 10 (Table 53). This method appears to provide a way to judge relative differences in the ability for a nematode population to infect plants. However, there are some problems in relation to genetic changes in a nematode population. For example, populations that were judged as negative (−) on tobacco in Table 53 varying relative infection rates, ranging from 0 to 5.6 (Table 52). This variation has meaning. According to Triantaphyllou (1975), physiological races in *H. glycines*, mentioned above, involve the gene frequency in nematode populations at the time of the experiment and are not constant but changeable with generations. In fact, some populations of *H. glycines*, which showed low relative infection rates (less than 10) at first and were initially identified as the same race, increased their relative infection rate up to 85% after successive culture on the same resistant cultivars of soybean (Riggs et al., 1977). It is evident that their race changed in response to selection pressure by culturing on the same cultivars. Indian populations of *R. reniformis* belonging to the MNT were reported to undergo similar race changes (Dasguptaand Seshadri, 1971a; b). In addition to these examples for nematodes with amphimictic reproduction, a similar phenomenon has been known in parthenogenetic nematodes, such as *Meloidogyne* spp. (Nishizawa, 1974; Okamoto and Mitsui, 1974; Riggs and Winstead, 1959). Likewise, infection rates for the parthenogenetic populations of *R. reniformis*, such as MRT and MAT, would be altered gradually through genetic changes by planting repeatedly the same resistant cultivars or resistant crops in infested fields.

According to Table 53, six laboratory populations tested seem to be identified as distinct races but when tobacco and cowpea are removed as test plants the SIB and AKN populations, both of which were originally from geographically close localities, Kagoshima prefecture, become the same race. The AHS-a and -b populations, which originated from the same field in Chiba prefecture, form another race. Finally, the MIZ and HAW populations appear to be independent from each other. These results suggest that the physiological race in plant-parasitic nematodes, which is separated by the host preference or infection rate, can easily be differentiated to varying extents by numbers and qualities of test plants or cultivars used as criteria. In the International *Meloidogyne* Project by Taylor and Sasser, 150 populations of *M. incognita* collected from the worldwide localities were separated into four races, *M. arenaria* was placed into two races, and *M. javanica* and *M. hapla* were not divided into races by the six test plants, namely tobacco, cotton, bell pepper, watermelon, groundnut, and tomato (Taylor and Sasser, 1978). They emphasized that the physiological races in *Meloidogyne* spp. in the world were not as numerous as expected. Since there are many more important local or domestic crops than those six test plants in respective areas or countries in the world, using such locally important crops or resistant cultivars in combination with those six test plants would help to reveal not only commonly recognized races in the world but also locally characteristic ones,

which are essential for biologically controlling nematode populations in diverse agricultures of the world.

Some difference in tobacco reaction to the AKN population between the present study and that report by Nakasono and Ichinohe (1967) must be related to difference in cultivars used and(or) a change in gene frequency in the laboratory population maintained under greenhouse conditions for a prolonged time.

Finally, it was evident that variations in host preference by populations of both MNT and MRA or MAT were quite wide and populations of the former had a much wider host preference than the latter two.

IX. Concluding Discussion

Rotylenchulus reniformis, distributed in either cultivated or native lands through the geographically wide ranges from the temperate to tropical zones in the world, is known to attack diverse kinds of crops and plants, and its wide variation in morphological characters led Dasgupta et al. (1968) to characterize it as a "polymorphic species." They also identified Japanese populations as a variation type in the same species. The purpose in the present study was to elucidate and discuss the biological significance of polymorphism or polymorphic characteristics in this species by examining field and laboratory populations for variations in morphological and physio-ecological characters.

There appear to be two generalizations that Dasgupta et al. adopted for characterizing and describing *R. reniformis* as "polymorphic species" as follows: The first generalization was that numbers of specimens collected from geographically different broad areas in the world including cultivated and wild lands could be divided into two groups, which were different in frequency of male occurrence, so-called "male-numerous group" and "male-lacking one." In most cases, specimens in the former group were small in body length and other morphological characters, while the latter group had larger values for those characters with much variation, ranging from intermediate to extremely large. Overall, that nematode collection consisted of a complex group of nematodes, which showed continuous variations in morphological characters, ranging from small to large. The second generalization was that the presence or absence of males seemed not to be important for the taxonomy in this nematode because male occurrence in some populations was highly variable and affected by environmental factors. For example, collection of this nematode was attempted two times at the same locality and habitat, in Ratunapura, Sri Lanka. The first collection included only young females without any males and specimens were the large type, but the second one had both young females and males, in about equal numbers and morphological characters, showing intermediate or bridged features between the first collection and the specimens collected in the type locality of *R. reniformis*.

Japanese populations of *R. reniformis* appear to agree with the definition of the polymorphic species by Dasgupta et al. (1968), in having a wide variation in morphological characters. The morphological variation and (or) overlapping measurements between different populations in this nematode species, however, have been considered until now only through a quantitative consideration of characters. If we can add a qualitative aspect to the examination of those characters, the meaning of the morphological variation becomes more clear. The principal components analysis (PCA) adopted here to study variation in measurements of morphological characters in Japanese populations of *R. reniformis* is "a multivariate technique and looks for a few linear combinations of the original variables that can be used to summarize a data set, losing in the process as little information as possible"* [13] (Okuno, 1967a,b). Barraclough and Blackith (1962) state that application of PCA to morphological data on nematodes is a possible method to obtain quantitative and qualitative information for effective comparison. Coomans (1971) also emphasizes that statistical analysis of multivariate data on nematodes would give good results when information is sufficient and precise. As shown in Figures 6 and 7, distribution of scores on the first and second principal components seemed to define differences in the size and shape of each population more clearly than the simple and morphometric comparison of measurements (Table 1), which has been used commonly in conventional nematology. It was interesting that scores on the maps were distributed in a pattern showing three groups, and populations of these groups corresponded to the members of three morphological groups characterized by lengths of body and hyaline portion in tail (Figs. 4 and 5).

The three morphological groups in *R. reniformis* populations appear obviously to be different in their physiological properties, and they correspond to the biological types characterized by male frequency of occurrence, namely the first group, in which nematodes are relatively slender and longer in body size, corresponds to the "male-rare type" (MRT), producing mainly females with rare males in each generation, the second one, in which individuals are slender and short in body size, corresponds to the "male-numerous type" (MNT), reproducing with both males and females, both occurring at about the same frequency in each generation, and the third one, in which nematodes are relatively large in body width and interme-

[13] Cited partly from Brian S. Everitt & Graham Dunn (2001) : Applied Multivariate Data Analysis, 342pp. London, for correct statement by the translator.

diate in body length, corresponds to the "male-absent type", usually producing only females without males. Since frequency in male occurrence was not affected by environmental changes, such as soil temperature, host plant taxa, or host nutrition, sexes in this nematode appear to be determined genetically but not environmentally. A difference in male occurrence between two sampling times at the same locality and habitat can be considered not to show change of male frequency in the same population caused by environmental difference or alterations between the first sampling and the second. Rather the difference may be due to existence of biologically different (two) types of populations at neighboring (micro-) habitats in the same locality or field. It seems to be likely that mixing of those populations has resulted from some artificial factors, such as cultivation work, in the field.

From the viewpoint of the male's role in reproduction, the presence of males is essential in the morphological second group, belonging of the MNT, and their reproduction is amphimictic, while males are not necessary for reproduction in the first and third groups, of the MRT and MAT, respectively, and they can reproduce parthenogenetically. Thus, males are essential for the second group but rarely occurring males in the first group appear to have usually no role in reproduction.

The attraction of males to females present on sweet potato roots in soil prior to mating was elucidated in this study. In male attraction tests on agar plates, MNT males were more strongly attracted by MNT females than by MRT or MAT females. Additionally, male behavior on agar plates differed distinctly according to the biological type of females present. Specifically, males of MNT stayed close to the females of the same biological type (MNT) for a prolonged period and some males exhibited copulating behavior, whereas they did not do so with the females of MAT or MRT. Therefore, it appears that crossing between MNT and MRT or MAT is unlikely. This suggests that MNT is reproductively isolated from MRT and MAT. Crossing between MRT and MAT is also unlikely. The attraction of MRT males by MNT females is an interesting topic that deserves more research. However, this topic was not pursued in the current study.

As to environmental factors, temperature in particular influenced juvenile development differently among the eight laboratory populations. Specifically, populations fell into two groups that differed in sensitivity to high temperate, about 33 ˚C. The first group severely suffered from the high temperature (33 ˚C) so that its juveniles could not develop to young females or males in water medium and in soil, while the second group was tolerant or adaptable and juveniles developed normally to young females or males. The first group (designated as "the temperature group" for convenience, here) included both the morphological first and the third groups, and the second one included the morphological second group as mentioned above. The temperature groups thus also corresponded to the biological types as described above; the first corresponded to the MRT and MAT and the second to the MNT. As further details, parthenogenetic populations (ASH-a, ASH-b, SIB, and AKN) are included in the first group and amphimictic populations (MIZ, HAW, TEX, and M×H) in the second one. Likewise, at low temperatures, 16 to 17 ˚C, juvenile development was slower in the first group than the second and the basal temperature also differed, with the first group having higher basal temperature than the second. So, it is conclusive that populations of MNT have a wider thermal range for development and reproduction than MRT and MAT populations. On the other hand, effective cumulative temperatures (DD) for development tended to be smaller in the parthenogenetic populations of MRT and MAT with higher basal temperatures than in the amphimictic MNT populations with lower basal temperatures. It is considered that the smaller effective cumulative temperature would be advantageous for the parthenogenetic populations to thrive in northern (cooler) marginal areas up to the isothermal line of 14 ˚C of annual average temperature around Tokorozawa city in Kanto district, Japan, because they are so sensitive to temperature changes and cannot develop before soil temperature rises above 13 ˚C in the spring and early summer. However, they can develop and complete the life cycle in a relatively small number of DD once the soil warms adequately for growth of plants and nematodes in the summer. In contrast, amphimictic populations of MNT in southern areas such as Awaji island, Nagasaki prefecture, and south western (sub-tropical) islands, including Amami, Okinawa island, and others, would appear less sensitive to temperature changes because they have adapted to warm or hot climate localities with little fluctuation year round over a long evolutionary period. Thus, their optimum range of temperature for development is wider than that of the parthenogenetic populations, but their relatively large DD may prevent establishment in such cool marginal areas even though they can commence to develop at temperatures as low as 10 to 13 ˚C. So, differences in geographical distribution between the parthenogenetic and amphimictic populations appear to be partly related to their thermal sensitivity characteristics.

As discussed above, the phenomenon of parthenogenetic populations of *R. reniformis* establishing in northern marginal and cool or mild areas is similar to "the geographical parthenogenesis" that is known in many animal species including some insects, such as weevils (Curculionidae) (Cuellar, 1977; Takenoushi,

1980; Tsuruzaki, 1983). This term, "geographical parthenogenesis," came from the observation that parthenogenetic species or populations tend to distribute and thrive in higher latitudes and altitudes, where the environment is usually severe and unstable, than do amphimictic species or populations.

From an ecological viewpoint, an explanation is as follows: Parthenogenetic species or populations can distribute and occupy a new habitat in relatively severe environments earlier than the amphimictic ones because (i) they have two times the intrinsic rate of population increase and (ii) they can start reproduction with a single individual so that they can establish a new colony more quickly (Cuellar, 1977). In *R. reniformis*, populations of MAT and MAT reproduce parthenogenetically so that they seem to be capable of establishing new colonies more quickly than those of MNT. In fact, their female numbers seem to be about two times that of MNT population, as estimated from some data, for example, in Tables 9 and 23, although the experiments were not done for this objective. To test this hypothesis, additional analytical experiments are needed under controlled conditions.

It is clear that parthenogenetic populations of *R. reniformis*, dealt with here, are adapted to cool or mild and warm climates as in temperate and sub-tropical zones. However, their phylogenetic origin is not known yet. Plant-parasitic nematodes are known to produce their offspring usually in one of two ways, parthenogenetic or amphimictic, and most populations or species with parthenogenetic reproduction show polyploidy and variation in their chromosome numbers among populations of the same species or species of the same genus (Triantaphyllou, 1971). They are assumed to have originated and evolved from ancestral amphimictic species through changes in karyotype by various ways, such as interspecific hybridization, under varying environments (Triantaphyllou, 1970). Recently, Takenouchi successfully produced artificially parthenogenetic polyploid weevils (*Callirhopalus, Scepticus,* and *Catapionus*) by low-temperature (3 ˚C) incubation of eggs laid by diploid amphimictic females of those genera after mating with their respective males (Takenoushi, 1980). This is considered to be important evidence supporting a phylogenetic origin for parthenogenetic weevils, distributed widely in cool habitats at high latitudes and altitudes. In the case of *R. reniformis*, parthenogenetic populations show a wide range in body size from being intermediate to large, suggesting polyploidy. By elucidating their karyotypes and mechanism of gametogenesis, phylogenetic relationships between parthenogenetic and amphimictic populations (n = 9 and 2n = 18) (Nakasono, 1966; Triantaphyllou, 1971) would be better understood. The example of weevils as cited above is very suggestive here.

The host range of amphimictic populations (MNT) was evidently wider than that of parthenogenetic ones (MRT and MAT). This suggests high genetic variability of the former, resulting from frequent gene exchanges and (or) recombination through copulation between males and females in each generation, which is prerequisite for reproduction. On the other hand, it was likely that some differentiation in host preference occurred among the parthenogenetic populations dealt with here. Even in parthenogenetic populations host preference would gradually be altered as the result of selection pressure with changes of cropping system, kinds of crops or cultivars, and (or) environmental factors (Triantaphyllou, 1975).

A biological species is defined as “groups of actually (or potentially) interbreeding natural populations which are reproductively isolated from other such groups” (Mayr, 1965; Mayr et al., 1953). The MNT populations of *R. reniformis* used in this study, such as Mizuho (MIZ), Hawaii (HAW), and Texas (TEX), are obviously groups with amphimictic reproduction and their biological identity as the same species is clear because of their successful interbreeding and sexual attraction to each other demonstrated in this study. On the other hand, populations of MRT (ASH-a and ASH-b) and MAT (SIB and AKN) form a group with parthenogenesis, but the possibility of interbreeding with females of the MNT appears to be negligible or very small because of the failure to achieve a successful copulation with males of MRT or MAT in this study in spite of some attraction of males on agar plates to MNT females. Further, the results of this study demonstrate that parthenogenesis in these populations is not a facultative characteristic as in some animals (Tsuruzaki, 1983) but an obligate characteristic. According to Dobzhansky, the definition of the biological species does not apply to organisms that produce offspring by asexual or parthenogenetic reproduction (Dobzhansky, 1972). The status of the *R. reniformis* populations used in this study as one distinct biological entity or distinct species cannot be concluded with the limited data presented. However, it can be concluded that at least some of these populations represent distinct biotypes that are adapted to distinct habitats. The amphimictic and parthenogenetic populations present in sweet potato fields of Mizuho, Nagasaki prefecture, represent at least two biologically different populations that appear to be reproductively isolated and independent of each other as biological entities. They also do not appear to share the same ecological niche.

Germani (1978) recognizes that the presence of males is also valuable for taxonomy of the genus *Rotylenchulus*. This is supported by the results of the present study.

Populations of *R. reniformis* used in this study

varied in morphological characteristics that identified in *R. reniformis* three distinct groups. Therefore a form of polymorphism was demonstrated in this study. These three groups also differed in a number of fundamental physiological and ecological characteristics. The polymorphism observed in these populations does not simply seem to be a case of a nematode with a highly varied phenotype, but rather the polymorphism seems to reflect basic physiological and ecological differences in populations of *R. reniformis*.

Further, it will be very interesting to proceed with more detailed morphological, cytogenetic, and (or) ecological studies on this nematode in the future, as they would contribute to understanding of the mechanism of physiological race and species differentiation, which is essentially important for nematode control in agriculture.

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