

The Relation of Fragmentation Frequency to Fragment Number
in *Enchytraeus japonensis* NAKAMURA, 1993 (Oligochaeta, Enchytraeidae)
cultured Several Years under Laboratory Conditions

YOSHIO NAKAMURA*

Abstract

Over a period of six and a half years, *Enchytraeus japonensis* fragmented repeatedly with a wide range in the number of survival days under laboratory conditions. In the case of head fragments, in which only the head fragment with an original head was transferred to new media after each fragmentation, the maximum fragmentation frequency until death was 122 times while cultured with ground oats at 20°C. The fragmentation frequency, fragment number per fragmentation, and the interval between successive bouts of fragmentation averaged 35.3 times, 6.3 fragments and 20.4 days, respectively. In the case of tail fragments, in which only the tail fragment with an original tail was transferred to new media after each fragmentation, the highest fragmentation frequency was 85. The fragmentation frequency, fragment number, and the interval averaged 11.4, 6.1 and 24.0, respectively. In both cases, there was no tendency for the fragment number or interval days to increase or decrease with the increase of fragmentation frequency.

Key words: fragmentation frequency, fragment number, interval, enchytraeids, *Enchytraeus japonensis*, Japan

Introduction

In 1959, the enchytraeid species, *Enchytraeus fragmentosus*, which reproduces asexually by fragmentation was firstly recorded (Bell, 1959). Since then, seven species have been reported to use this reproductive mode (Christensen, 1959; Nielsen and Christensen, 1963; Bouguenec and Giani, 1987; Nakamura, 1993; Dózsa-Farkas, 1995a). Christensen (1961) states that the normal method of reproduction in enchytraeids is by mutual transference of spermatozoa between hermaphrodites. But various reproductive modes are reported in addition to fragmentation (parthenogenesis by Christensen, 1961; self-fertilization by Dózsa-Farkas, 1995b).

Enchytraeids are small terrestrial and aquatic worms of the phylum Annelida, class Oligochaeta. They belong to microdrilids and are often called 'potworms' or 'enchy'. Most species are 3-15 mm long with a body diameter of 0.03-0.5 mm, and are usually white in color. Up to the present, about six hundred species have been described (Nielsen and Christensen, 1959; Nakamura, 2000). In terrestrial ecosystems, enchytraeids are actively involved in decomposition of organic matter, in nutrient cycling, and in soil formation (Kasprzak, 1982).

The first evidence in Japan of the fragmenting species,

Enchytraeus japonensis, was collected from an eco-farmed crop field under conditions of no-tillage agriculture, an organic mulch of chopped crop residues and mowed weed, and no chemicals (Nakamura, 1993). The specimen was obtained in the second year after the change from conventional farming to eco-farming. Finding this species was serendipitous. During a routine survey of soil animals at the above mentioned crop field, several enchytraeid larvae were extracted and cultured, because identification of the enchytraeid is based on live and full grown specimens. One of the cultured specimens is broken into several fragments, and continued to fragment. Thereafter *Enchytraeus* larvae collected from routine sampling over nine years were also cultured, but no additional specimens which carried out fragmentation were found (Nakamura *et al.*, 2003). It is unknown why it disappeared.

This species resembles another fragmenting species obtained from culture, *Enchytraeus bigeminus* NIELSEN et CHRISTENSEN, 1963, in morphological characteristics, but the analysis of general protein and isozyme patterns shows that the two species are different (Schmelz *et al.*, 2000). *E. japonensis* is a widely used new experimental material for regeneration studies (Myohara *et al.*, 1999). Its fragmentation can be induced by decapitation, and its egg production by re-feeding starved worms in wet leaf mold or in aged agar medium (Inomata *et al.*, 2000).

In Oct. 1991, one specimen of this species, *E. japonensis*, was collected, and now, in Dec. 2003, it is cultured at various laboratories. One question about these cultured worms is whether they will continue to fragment eternally to support their population. When a worm is cut into

Received 8 March 2004.

Accepted 31 May 2004.

*Laboratory of Crop Sciences, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama city, Ehime, 790-8566, Japan

several fragments, the most anterior fragment with the head or the most posterior one with the tail regenerates new segments except the head or tail, respectively. Another question is whether the original head or tail fragment is endowed with a fragmentation limit. The present study investigates the fragmentation potential in the original head and original tail fragments under laboratory conditions.

Materials and Methods

Animals and cultures

The enchytraeid worm, *E. japonensis*, which had been cultured for several years in the laboratory of Tohoku National Agricultural Experiment Station, was used (Fig. 1A). One fully grown worm from a stock culture was placed in a small glass dish (30 mm diam., 15 mm high) with a 1.5% agar solution. The dish was covered with

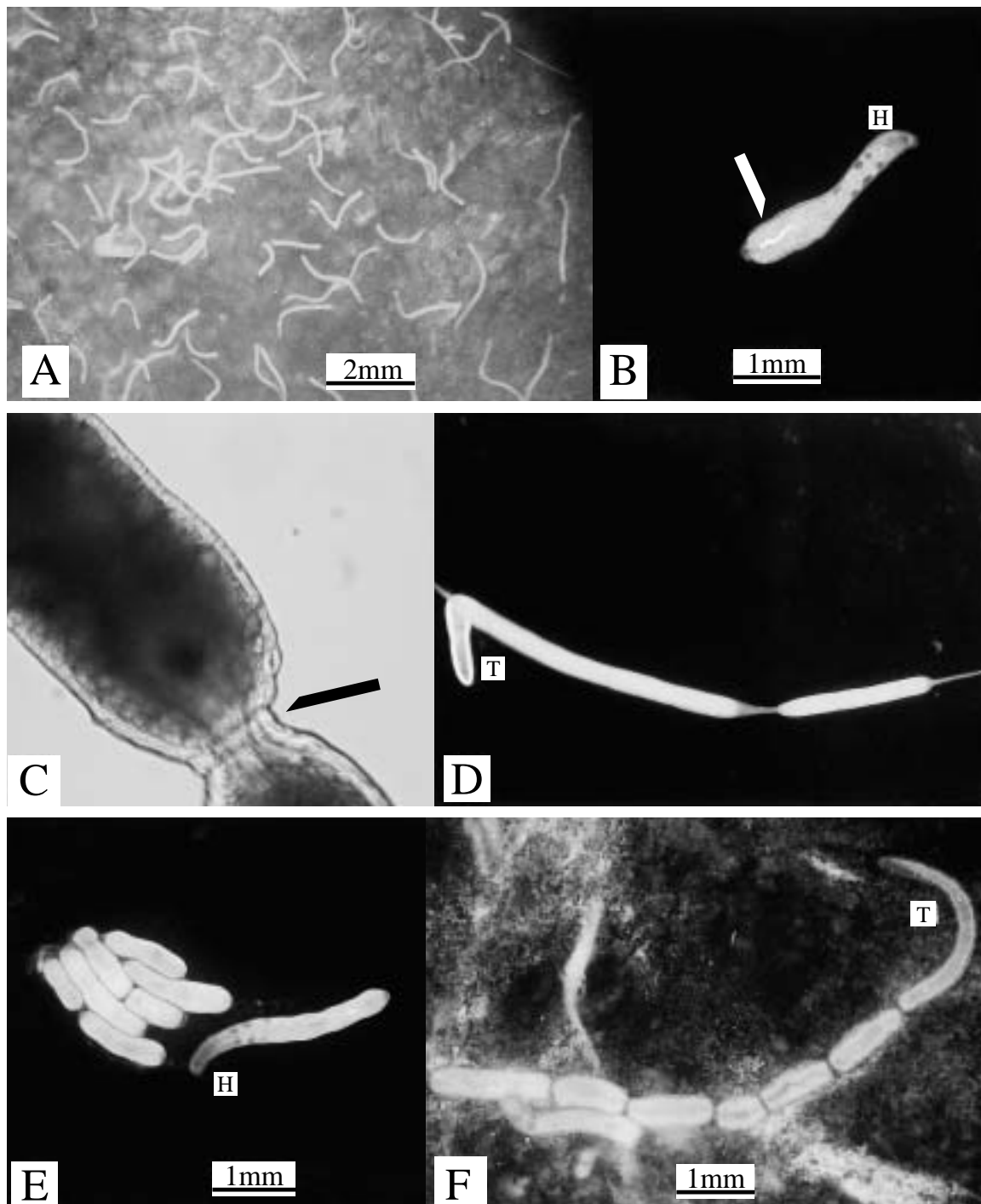


Fig. 1 (A-F). A: Stock culture of *Enchytraeus japonensis*; B: Head fragment (arrow shows nerve cord); C: Body circular muscle constriction before fragmentation; D: Tail fragment with an expanded epidermis which connects with the other fragment; E: Fragmentation finished (tail fragment is transported to a new dish); F: Worms staying in jellied ground oats (white bubbles). H: head, T: tail

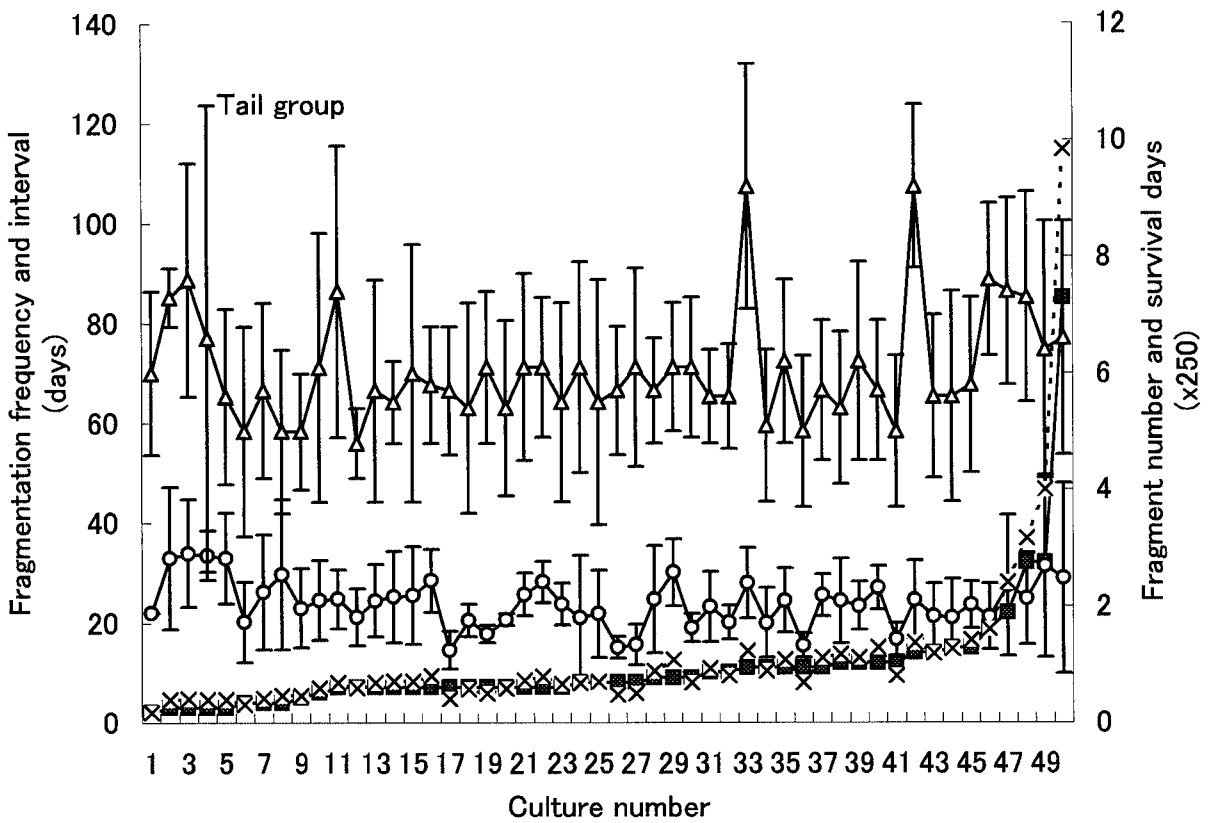
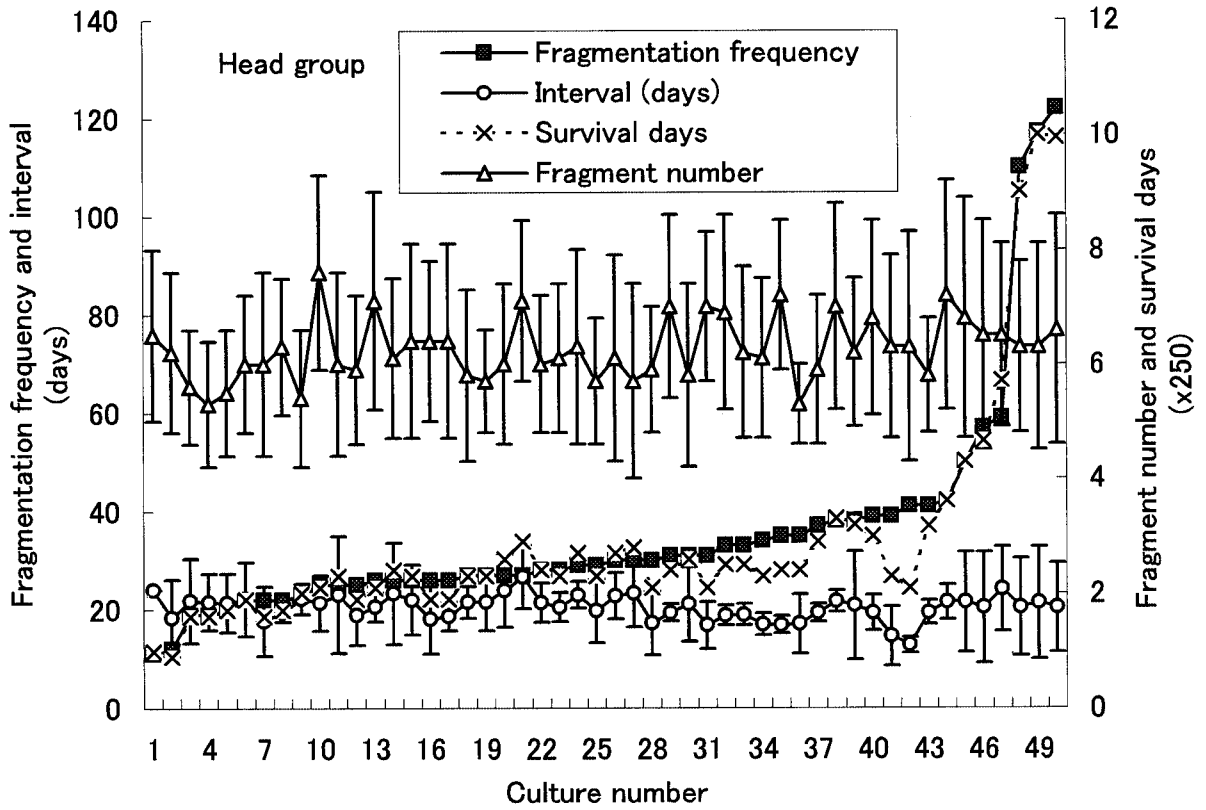


Fig. 2. Fragmentation frequency, fragment number, interval, and number of survival days in *Enchytraeus japonensis* under laboratory conditions. Standard errors of the mean appear as vertical bars. Upper: head fragment group; lower: tail fragment group

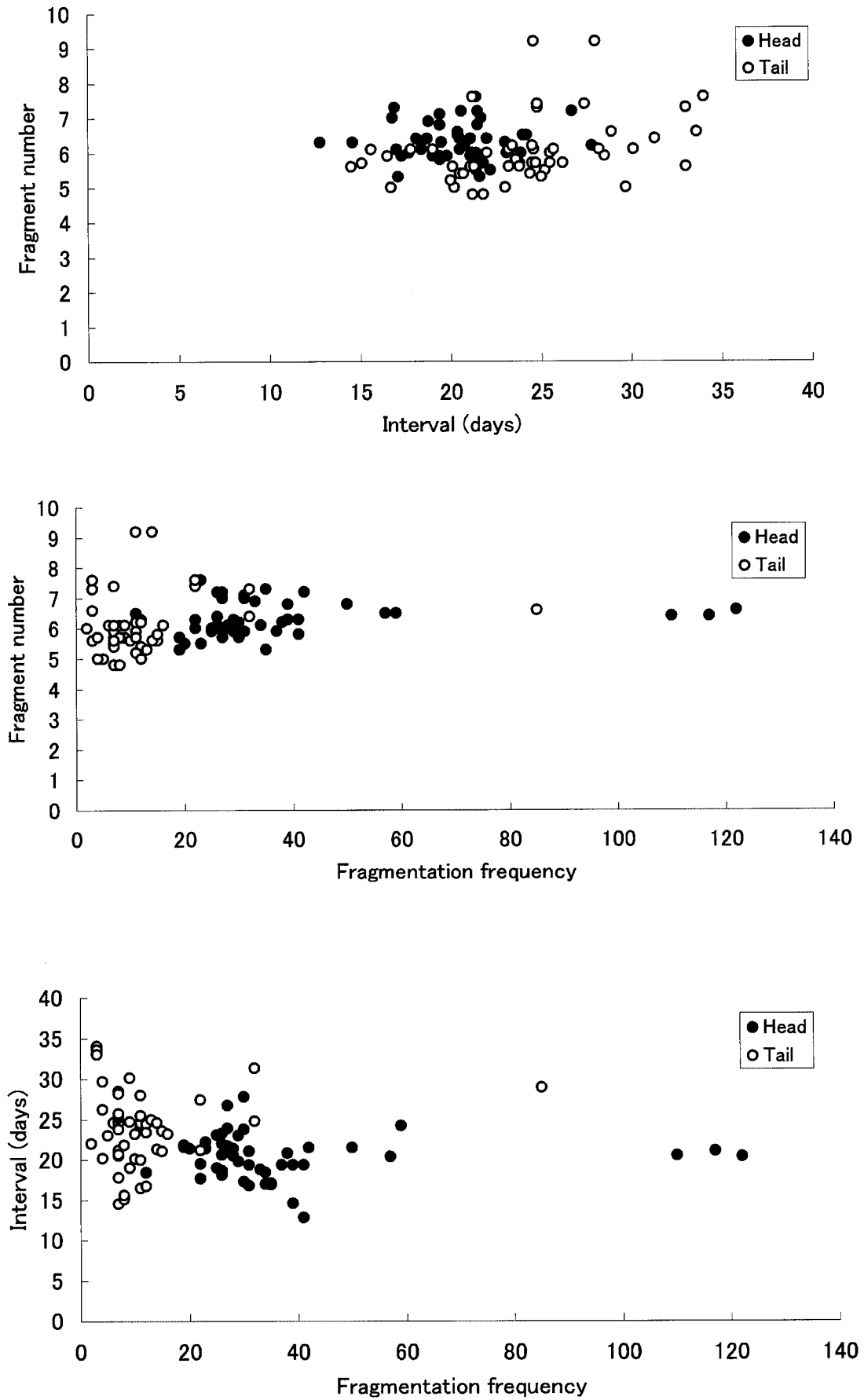


Fig. 3. Relationship among fragmentation frequency, fragment number, interval, and number of survival days in *Enchytraeus japonensis* under laboratory conditions.

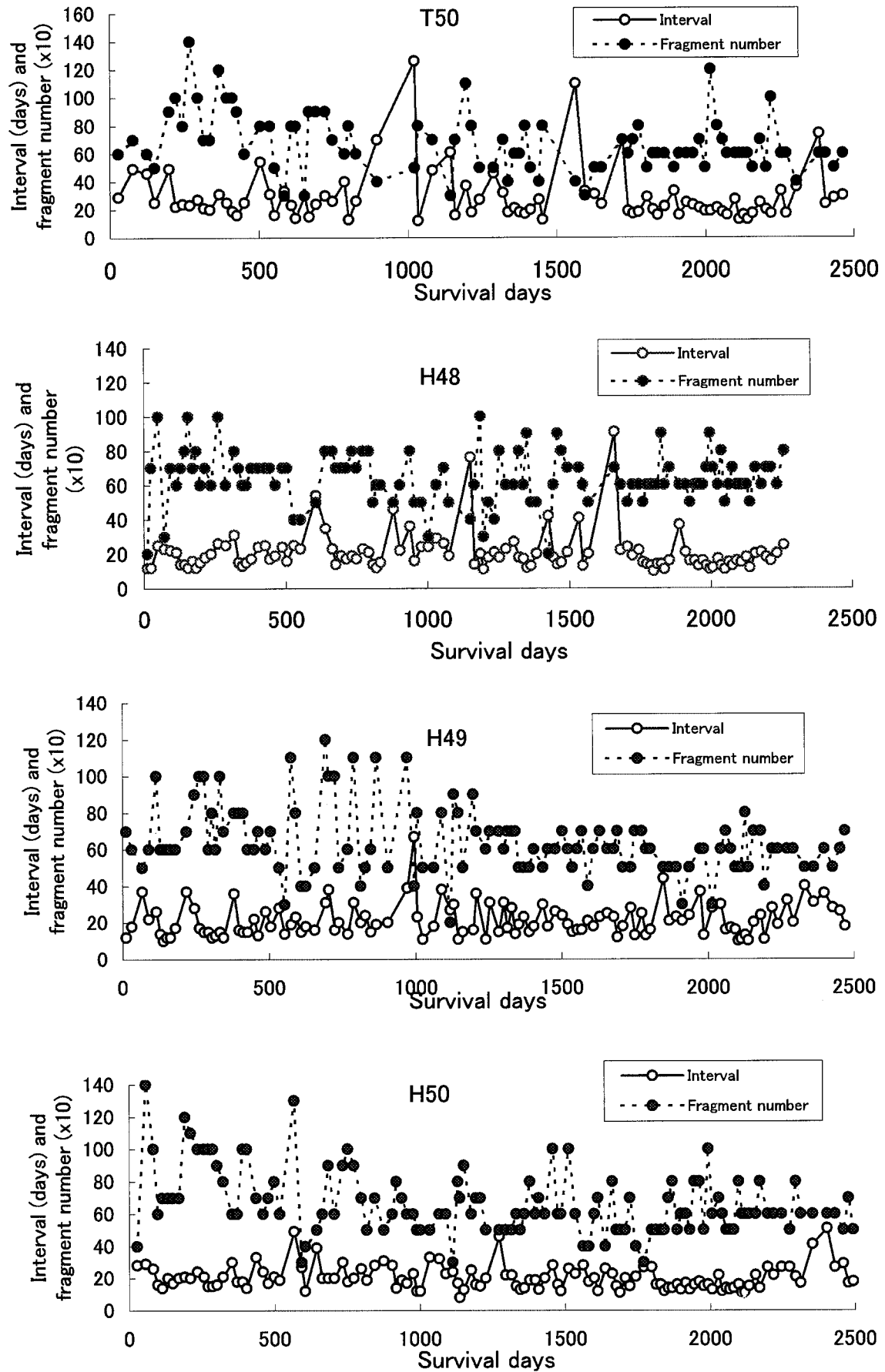


Fig. 4. Fragment number and interval in *Enchytraeus japonensis* with a large number of survival days under laboratory conditions. H: head fragment; T: tail fragment; The Arabic numerals show the culture number in Fig. 1

another of the same size and sealed with vinyl tape to prevent desiccation. Ground oats, in a quantity in excess of the worm's needs, were placed on the agar substrate to provide food, and the dishes were stored in the dark at 20 °C. One hundred dishes were prepared, and half of them were used for a head group, the rest for a tail group. When the experiment commenced, a bamboo stick was used to gently place the head (Fig. 1B) or tail fragment on the agar substrate in a new small dish. Thereafter, the head fragment with an original head in the head group or the tail one with an original tail in the tail group was repeatedly transported into a new glass dish with an agar substrate until death. At each daily inspection under a light microscope it was ascertained whether fragmentation had taken place. When fragmentation had taken place, the number of fragments and the number of days between successive bouts of fragmentation (interval) were recorded.

Statistical evaluation

Data were subjected to an analysis of variance, and Fisher's least significant difference for means and coefficient of correlation were calculated.

Results

In the initial phase of fragmentation under laboratory conditions, body circular muscle constriction occurred in several parts of the body (Fig. 1C). Thereafter, the fragmented body moved backward and forward (Fig. 1D), or again constricted in the middle of the body and twisted in order to promote the succeeding separation. Each fragment moved here and there, and one could not distinguish where the fragment was located (Fig. 1E). In a new dish, the transported head or tail fragment also moved here and there, and sometimes went under a part of the sealed vinyl tape. After regeneration of the tail or head had finished, worms gathered to the food. The ground oats food changed gradually into jelly, which resulted from preoral digestion (Reichert *et al.*, 1966). Several worms did not depart from the jelly oats until the next fragmentation (Fig. 1F). Such behaviour was often observed in both groups throughout the experimental period.

Head fragment (Head group)

Of the 50 specimens examined, three broke into fragments more than 110 times, and survived beyond 2200 days (Fig. 2). The fragmentation frequency increased with an increase in survival days, and the maximum fragmentation frequency was 122, associated with a survival period of 2459 days. The fragmentation frequency of the remaining worms was less than 59 times, and the survival period was less than 1433 days. The interval between successive bouts of fragmentation ranged from 165 to 7 days, with a mean of 20.4 days at 20 °C. The fragment number in one fragmentation ranged from 14 to 2, with a mean of 6.3. There was no tendency for the fragment number or the interval to increase or decrease

with an increase in the fragmentation frequency or survival days (Fig. 3).

Three heads which survived beyond 2200 days showed a wider variation in fragment number in the first half of their lives than in the latter half, and converged on an average fragment number as time went by (Fig. 4). Each of the other specimens showed no tendency for the fragment number and interval to increase or decrease as time went on.

Tail fragment (Tail group)

Of the 50 specimens examined, only one fragmented more than 80 times and survived beyond 2400 days (Fig. 2). The fragmentation frequency increased with the expansion of survival days, and the maximum fragmentation frequency was 85, while the maximum number of days survived was 2463. The fragmentation frequency of the remaining worms was less than 32 times, and the number of days survived less than 1003. The range was from 126 to 10 days, and the mean was 24.0 days. The fragment number ranged from 14 to 2, and the mean was 6.1. There was no tendency for the fragment number or interval to increase or decrease with the addition of fragmentation frequency or survival days (Fig. 3).

The tail with the longest survival period showed a wider variation of fragment number in the first half of its life than in the latter one, and converged on an average fragment number with the lapse of time (Fig. 4). Each of the remaining specimens showed no tendency for the fragment number or interval to increase or decrease as time went on.

Discussion

Since the first description of fragmenting enchytraeid species (Bell, 1959), seven fragmenting species have been reported. Studies on them have established that they are able to reproduce sexually in addition to fragmenting (Christensen, 1959 and 1973; Vena *et al.*, 1969; Hamilton and Hess, 1971; Dózsa-Farkas, 1996; Myohara *et al.*, 1999). In the case of the present species, *E. japonensis*, fragmentation was also induced by re-feeding starved worms in wet leaf mold or in aged agar medium; also, the starvation was not essential for, but did promote the induction of sexual maturation (Myohara *et al.*, 1999; Inomata *et al.*, 2000). Sexual maturation was density dependent, as in *E. bigemius* (Christensen, 1973) and *E. dudichi* DÓZSA-FARKAS, 1995 (Dózsa-Farkas, 1996). The fragmentation of another species, *Cognettia sphagnetorum* (VEJDOVSKY, 1877), the predominating species in a pine forest soil in Sweden, would be advantageous in recovering after the dry periods that occur with fairly high regularity at the site (Lundkvist, 1982). During the present experimental period of seven and a half years, however, specimens of both groups, head and tail, reproduced asexually. And they certainly regenerated tails from head fragments or heads from tail fragments. This is thought to indicate that the conditions did not change abruptly or

Table 1. Fragmentation frequency, fragment number, number of survival days and interval between fragmentation bouts in *Enchytraeus japonensis* under laboratory conditions. (Each group consists 50 specimens)

	Head group				Tail group			
	Max	Min	Mean	(sd)	Max	Min	Mean	(sd)
Fragmentation frequency until death	122	11	35.3	(22.6)	85	2	11.4	(12.3)
Fragment number (per one fragmentation)	14	2	6.2	(0.5)	14	2	6.0	(0.9)
Survival days	2459	265	726	(476)	2463	44	275	(358)
Interval (days between fragmentations)	165	7	20.4	(2.7)	126	10	24.0	(4.7)

drastically during the experimental period. Because in the present study only one worm was cultured in each dish, density dependence of the type in sexual reproduction is unknown, and further investigation is needed.

Inomata *et al.* (2000) cultured *E. japonensis*, which was acquired from the same original stock as the present study, and reported that the fragmentation interval was from 13 to 15 days at 24°C. This is a fair amount shorter than what was found in the present study: 20 days in the head group, and 24 days in the tail group at 20°C. This may be due to the difference in temperature, as demonstrated in *E. dudichi* by Dózsa-Farkas (1996), or the fragments without head and tail segments may regenerate more quickly. Comparing the data concerning the fragment number of other fragmenting *Enchytraeus* species and the present species, *E. japonensis*, the latter had a high capability for fragmentation, since it was 4-7 in *E. bigeminus* (Christensen, 1964 and 1973), 3-11 in *E. fragmentosus* (Bell, 1959), 3-6 in *E. variatus* (Bouguenec and Giani, 1989) and 7-18 in *E. dudichi* (Dózsa-Farkas, 1996).

The fragmentation frequency increased with the increase in survival days. Such a trend was clear in the tail group. The fragmentation frequency, survival days, and fragment number were more numerous or longer in the head group than in the tail group. On the other hand, the interval between successive bouts of fragmentation was longer in the head group than in the tail group. No significant difference was observed between the two groups in any of the four factors mentioned above. But there was a slight tendency for the fragment number to decrease in the head group and increase in the tail group with the increasing number of days. The specimens of the head and the tail groups were each divided into two categories, long and short life spans. In the case of a worm with an exceptionally large number of survival days, as in three of the head and one of the tail group, the fragment numbers showed a wider variation in the first half of their lives than in the latter half, and converged on an average fragment number with the increasing number of survival days. Such a tendency was clearly observed in the worm with the largest number of survival days in the tail group. These four worms lived for between 2200 and 2500 days, although there was a large difference in the fragmentation frequency between the two groups, head and tail.

It was believed that the maximum number of generations would be 167 to 196 from the first fragmentation in a stock culture under laboratory conditions; this was based

on the interval calculated in the present study, although an accurate interval was not known except for the head and tail fragments. The cause of death could not be proved in the present study. But the maximum fragmentation frequency, 122 in the head group and 85 in the tail group, indicates that the fragmentation capacity is higher in the head fragment than in the tail one. And its capacity may be charged with a limit, like the fission limit of *Paramecium aurelia*, Protozoa (Sonneborn, 1954) and the phenomenon of Hayflick (1965). Since fragmentation of the present species can be induced by decapitation (Inomata *et al.*, 2000), the question is whether the present fragmentation capacity is higher or lower than that in fragmentation stimulated by decapitation. The fragmentation frequency and interval were many and short in the head group, and few and long in the tail group, respectively. This suggests the existence in both groups of some substance like telomerase, and further studies, especially on biogenic activity, are needed.

Acknowledgements: My thanks are due to Dr. T. FUJIKAWA of Shigenobu-cho, Ehime pref. and Mr. K. NAKAMURA of Kyusyu Univ. for the stock management and observation of specimens, and Dr. Y-N. NAKAMURA of Kinki Univ. and Dr. S. TOCHINAI of Hokkaido Univ. for their kind advice.

References

- Bell, A.W. (1959) *Enchytraeus fragmentosus*, a new species of naturally fragmenting Oligochaete worm. *Science*, 129: 1278.
- Bouguenec, V. and N. Giani (1987) Deux nouvelles espèces d'*Enchytraeus* (Oligochaeta, Enchytraeidae) et redescription d'*E. bigeminus* Niel. & Chr. Remarques sur le genre *Enchytraeus*. *Annls Limnol.*, 23: 9–22.
- Bouguenec, V. and N. Giani (1989) Biological studies upon *Enchytraeus variatus* Bouguenec & Giani 1987 in breeding cultures. *Hydrobiol.*, 180: 151–165.
- Christensen, B. (1959) Asexual reproduction in the Enchytraeidae (Oligo.). *Nature*, 184: 1159–1160.
- Christensen, B. (1961) Studies on cyto-taxonomy and reproduction in the Enchytraeidae with notes on parthenogenesis and polyploidy in the animal kingdom. *Hereditas*, 47: 387–450.
- Christensen, B. (1964) Regeneration of a new anterior end in *Enchytraeus bigeminus* (Enchytraeidae, Oligochaeta). *Vidensk. Medd. Dansk aturh. Foren.*, 127: 259–273.
- Christensen, B. (1973) Density dependence of sexual reproduction in *Enchytraeus bigeminus* (Enchytraeidae). *Oikos*,

- 24: 287–294.
- Dózsa-Farkas, K. (1995a) *Enchytraeus dudichi* sp. n., a new fragmenting *Enchytraeus* species from Iran (Oligochaeta, Enchytraeidae). *Opusc. Zool. Budapest*, 27/28: 41–44.
- Dózsa-Farkas, K. (1995b) Self-fertilization: an adaptive strategy in widespread enchytraeids. *Eur. J. Soil Biol.*, 31: 207–215.
- Dózsa-Farkas, K. (1996) An interesting reproduction type in enchytraeids (Oligochaeta). *Acta Zool. Acad. Sci. Hungaricae*, 42: 3–10.
- Hamilton, M. & R. T. Hess (1971) Axenic production of ova by *Enchytraeus fragmentosus* (BELL). *Canadian J. Zool.*, 49: 1199–1120.
- Hayflick, L. (1965) The limited in vitro lifetime of human diploid cell strains. *Experim. Cell Res.*, 37: 614–636.
- Inomata, K., F. Kobari, C. Yoshida-Noro, M. Myohara and S. Tochinai (2000) Possible neutral control of asexually reproductive fragmentation in *Enchytraeus japonensis* (Oligochaeta, Enchytraeidae). *Invertebrate Repro. Develop.*, 37: 35–42.
- Kasprzak, K. (1982) Review of enchytraeids (Oligochaeta, Enchytraeidae) community structure and function in agricultural ecosystems. *Pedobiologia*, 23: 217–232.
- Lundkvist, H. (1982) Population dynamics of *Cognettia sphagnetorum* (Enchytraeidae) in a Scots pine forest in Central Sweden. *Pedobiologia*, 23: 21–41.
- Myohara, M., C. Yoshida-Noro, F. Kobari & S. Tochinai (1999) Fragmenting oligochaete *Enchytraeus japonensis*: a new material for regeneration study. *Develop. Growth Differ.*, 41: 549–556.
- Nakamura, Y. (1993) A new fragmenting enchytraeid species, *Enchytraeus japonensis* from a cropped kuroboku soil in Fukushima, northern Japan (Enchytraeids in Japan 5). *Edaphologia*, 50: 37–39.
- Nakamura, Y. (2000) Checklist of enchytraeids (Oligochaeta: Enchytraeidae) of the world. *Misc. Publ. Tohoku Natl. Agr. Exp. Stn.*, 24: 29–104. (In Japanese with English abstract.)
- Nakamura, Y., H. Shiraishi & M. Nakai (2003) Earthworm and enchytraeid numbers in soybean-barley fields under till and no-till cropping systems in Japan during nine years. *Mem. Fac. Agr., Ehime Univ.*, 48: 19–29.
- Nielsen, C. O. and B. Christensen (1959) The Enchytraeidae, critical revision and taxonomy of European species. *Natura Jutlandica*, 8/9: 1–160.
- Nielsen, C. O. and B. Christensen (1963) The Enchytraeidae, critical revision and taxonomy of European species. *Natura Jutlandica*, 10 (suppl. 2): 1–19.
- Reichert, A., U. Mothes-Wagner & K. -A. Seitz (1996) Eco-histological investigation of the feeding behaviour of the enchytraeid *Enchytraeus coronatus* (Annelida, Oligochaeta). *Pedobiologia*, 40: 118–133.
- Schmelz, R. M., R. Collado & M. Myohara (2000) A taxonomic study of *Enchytraeus japonensis* (Enchytraeidae, Oligochaeta): morphological and biochemical comparisons with *E. bigeminus*. *Zool. Sci.*, 17: 505–516.
- Sonneborn, T. M. (1954) The relation of autogamy to senescence and rejuvenescence in *Paramecium aurelia*. *J. Protozool.*, 1: 38–53.
- Vena, J. A., R. T. Hess and M. L. Gotthold (1969) Attainment of sexuality in *Enchytraeus fragmentosus* Bell under laboratory conditions. *Experimentia* (Basel), 25: 761.

摘 要

中村好男：長期飼育条件下におけるヤマトヒメミミズの死亡までの碎片分離回数，碎片数および間隔

わが国ではじめて記録されたヒメミミズ科の碎片分離種，ヤマトヒメミミズ *Enchytraeus japonensis* NAKAMURA, 1993は，1991年に畑土壌から採集された。碎片分離で増殖する個体群から成長した100個体を選び出し，個体ごとに寒天培地上に添加した粉碎オートミールを餌に飼育した。碎片分離直後の50個体の碎片から頭部を含む頭部碎片を，新しい容器で飼育し，その後毎回の碎片分離時に頭部碎片のみを，新しい容器に移し継代飼育した。また同じように50個体の碎片から尾部を含む尾部碎片のみを継代飼育した。その結果，死亡までの碎片分離回数，1回の碎片数，碎片間隔日数および生存日数の平均は，頭部碎片が35.3回，6.2片，20.4日および726日；尾部碎片が11.4回，6.0片，24.0日および275日であった。最大の碎片分離回数と生存日数は，頭部碎片が122回と2459日，尾部碎片が85回と2463日であった。頭部碎片と尾部碎片とも，1回の碎片数と碎片間隔日数が，碎片回数の増加，すなわち生存日数の増加にともなって，それぞれ増加あるいは減少するという傾向は認められなかった。