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Statistical Distinction between Normal and Amputated Specimens in a Field-Collected Sample of the Earthworm, *Amynthas lautus* (Ude)

Chu-Fa Tsai[1,*] and Su-Chen Tsai[1]

[1]*Habitat and Ecosystem Division, Taiwan Endemic Species Research Institute, Chichi, Nantou, Taiwan 552, R.O.C.*
Tel: 886-49-2761331 ext. 215. Fax: 886-49-2764600 or 2761582.

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Chu-Fa Tsai and Su-Chen Tsai (2001) Statistical distinction between normal and amputated specimens in a field-collected sample of the earthworm, *Amynthas lautus* (Ude). *Zoological Studies* **40**(1): 21-28. The interactive outlier rejection procedure, which repeatedly performs a linear regression analysis between total length and segment length with the outlier rejection at the 95% confidence limits, is apparently a useful statistical method in distinguishing tail-amputated specimens (outliers) and normal specimens (within the confidence interval) in field-collected samples of the pheretimoid earthworm, *Amynthas lautus*. After removing amputated specimens, there was an asymptotic relationship between segment number and total length of normal specimens; segment number increased with total length and reached a plateau in aclitellate worms and remained at the plateau in clitellate worms. Left skewness of the segment-frequency distribution of the field-collected sample indicated the presence of tail-amputated specimens in clitellate worms, but the presence of both tail amputation and growth effect in aclitellate worms.

Key words: Oligochaeta, Length, Segment number.

The number of segments is one of the important systematic characters in specific descriptions of oligochaete earthworms. Gates (1972) indicated that in the taxonomic study of oligochaetes, the number of segments should be determined from normal individuals; but this is not always possible, as sometimes every specimen, even with a large sample, lacks a portion of the tail. Murchie (1960) showed that many field-collected earthworms tend to have fewer segments than reared ones. It is a common phenomenon that a field-collected sample of earthworms consists of both normal specimens with the original number of segments and amputated specimens with some missing tail segments.

Autotomy is one of the common responses of terrestrial megascolecid earthworms to tactile stimuli such as touching or grasping (Edwards and Bohlen 1996). It occurs only in the posterior portion (Murchie 1960, Gates 1972, Vail 1972). As the earthworms are an important component of the diet of many species of vertebrates (MacDonald 1983), predation is perhaps a primary cause of autotomy, though there might be other unknown causes. Regeneration of lost tail segments is common, but regeneration capacity varies by species and by site of amputation, so that the full number of segments is not always regenerated (Gates 1972). Distinguishing between normal specimens and amputated specimens is sometimes simple, but it is not always possible by examining tail structure alone.

The above situation raised the question of how to distinguish between normal specimens and tail-amputated specimens in a field-collected sample of earthworms.

Amynthas lautus (Ude) is a common terrestrial pheretimoid earthworm in Taiwan (Tsai 1964), southern China (Chen 1933), and the Ryukyu Islands, Japan (Ohfuchi 1956). In Taiwan it is found in the foothills. According to the definition of ecological groups of earthworms (Bouche 1977), we may categorize *A. lautus* as an endogenic earthworm, of medium size and with a short life, which burrows in the upper 10-15 cm of soil, and is subjected to low predation.

*To whom correspondence and reprint requests should be addressed.

We used *A. lautus* as a study species to 1) develop a statistical method to distinguish normal specimens and amputated specimens in a field-collected sample, 2) estimate amputation frequency, 3) estimate total length and segment number of normal and amputated specimens, 4) determine the relationship between segment number and total length in normal specimens, and 5) determine the effects of amputation on the frequency distribution of segment number and total length.

MATERIALS AND METHODS

Specimen collection and preparation

Earthworms were collected by digging and hand sorting at a field near the parking lot in the Forest Recreation Park of National Taiwan University, Hsitou, Nantou County, in central Taiwan, between 10 and 18 Dec. 1998. The field was composed primarily of clay and some gravel, and was covered with short grass. In the collection, we kept unharmed earthworms, and discarded those damaged by shoveling. In total, 99 specimens of *A. lautus*, including 51 aclitellates (without a clitellum) and 48 clitellates (with a clitellum formed by segments XIV-XVI) were collected. They were kept alive and brought to the laboratory.

The length of an earthworm is strongly affected by its contraction and relaxation. The length of a relaxed worm may be two, three, or even more times longer than that of a strongly contracted one (Gates 1972). In this study we kept the anesthetization, fixation, and preservation processes consistent for all specimens, using the following procedures. 1) Specimens were anesthetized in a 30% ethyl alcohol-water solution until there was no response to touch with a needle (about 3 to 5 min). 2) They were removed from the solution, held vertically with their tails up, and laid horizontal and straight on a piece of paper. Extending and straightening the specimens were by gravity alone. 3) The specimens and the paper were placed horizontally in a container filled with ethyl alcohol vapor for about 20 min. This ensured the death of the specimens to avoid possible coiling and contraction in the subsequent fixation process. 4) The specimens together with the paper were transferred horizontally into a 10% formalin-water solution for fixation. Pouring small amounts of the solution with a pipette onto the specimens facilitated submersion. The fixation period was about a week. 5) The paper was removed, and the fixed specimens were transferred to flowing water overnight and then preserved in a 75% ethyl alcohol-water solution. Putting the specimens on paper in the procedure enabled us to maintain their shapes and sizes unchanged during the processes of anesthetization and fixation.

Data collection

For each of the preserved specimens, we measured total length from the prostomium to the anus at a precision level of 1 mm, and counted total segment number under a dissection microscope. The clitellum was counted as 3 segments. We calculated average segment length by dividing total length by total segment number.

Interactive outlier rejection procedure

The interactive outlier rejection procedure (Neter et al. 1990) was used to distinguish between amputated specimens and normal specimens in the field-collected sample (normal and amputated specimens combined). The use of this procedure was based on three assumptions. 1) In the growth of earthworms, total length increases proportionally (linearly) with segment length. 2) Tail amputation decreases total length but not segment length. Therefore, amputated specimens may fall as outliers outside the negative 95% confidence limit of the total length-segment length relationship line. 3) Tail amputation does not increase total length, and thus, there should be no outliers outside the positive 95% confidence limit of the relationship line.

Based on the above assumptions, in the interactive outlier rejection procedure, the first linear regression analysis between total length (dependent variable) and segment length (independent variable) was conducted for the field-collected sample, and outlier points (data) were identified at the negative 95% confidence limit (Neter et al. 1990). After removing the outlier points, the second linear regression analysis was conducted by using the data remaining within the 95% confidence interval, and then new outlier points at the negative 95% confidence limit were identified and removed. The above regression analysis and outlier rejection process were conducted repeatedly until the final analysis prior to the appearance of an outlier at the positive 95% confidence limit. Outliers at the negative 95% confidence limits of all regression analyses in the procedure were considered amputated specimens, and those remaining within the 95% confidence interval of the final analysis were considered normal specimens.

Asymptotic function relationship analysis

Segment number was plotted against total length for the field-collected sample, in which normal and amputated specimens were identified by the interactive outlier rejection procedure. After removal of amputated specimens, the asymptotic relationship analysis was conducted between segment number (N_s) and total length (L_t) of normal specimens, using the following equation:

$$N_s = a(1 - e^{bLt})$$

where *a* is constant, and *b* is the regression coefficient.

Frequency distribution and skewness determination

The segment-frequency distribution and the length-frequency distribution were established for each of aclitellate, clitellate, and total worms (aclitellates and clitellates combined) of the normal specimens and the field-collected sample. Evans (1946) indicated that the absence of left skewness in the segment frequency distribution of *Allolobophora chlorotica* (Savigny) was attributed to mitigation by caudal regeneration of the effect of predation. However, Gates (1972) considered it to occur because of the absence of predation. In this study we assumed that the segment frequency distribution of a normal earthworm population of an age group (cohort) is symmetrical, and thus, the occurrence of left skewness in a field-collected sample may indicate the presence of specimens with some tail segments amputated. Skewness (g_1) of the distribution was calculated by the following equation (Snedecor 1959):

$$g_1 = k_3/(k_2)(k_2)^{1/2}$$
$$k_2 = S_2/(n - 1)$$
$$k_3 = nS_3/(n - 1)(n - 2)$$

where k_2 and k_3 are, respectively, mean squares corresponding to the average of the second and third powers of deviations from the mean; S_2 and S_3 are the sums of squares and cubes of deviations from the mean; and *n* is number of samples. A g_1 value equal to 0 indicates a symmetrical distribution. A value larger or smaller than 0 indicates right or left skewness. The test of significance of the difference of the g_1 value from 0 was made by *t*-test with an infinite number of degrees of freedom at the 1% level ($p < 0.01$, *t*-value = 2.576)(Snedecor 1959).

RESULTS

Total length-segment length relationship

In the interactive outlier rejection procedure, three performances of the linear regression analysis between total length and average segment length with outlier rejection at the 95% confidence limits were found to be sufficient to reach the analytical stage prior to the appearance of the first outlier at the positive 95% confidence limit (Fig. 1). The appearance of the first outlier at the positive 95% confidence limit was a sign to terminate the procedure at the prior analysis. The result showed that in the field-collected sample, 5 out of 51 aclitellates (9.8%) and 15 out of 48 clitellates (31.3%) were found to be outliers (Table 1). When the outliers were removed, there were 79 specimens within the 95% confidence limits, and they showed that total length increased linearly and proportionally with segment length.

Based on the three assumptions on which the interactive outlier rejection procedure was used, it is reasonable to consider that the outliers were amputated specimens, while the specimens within the

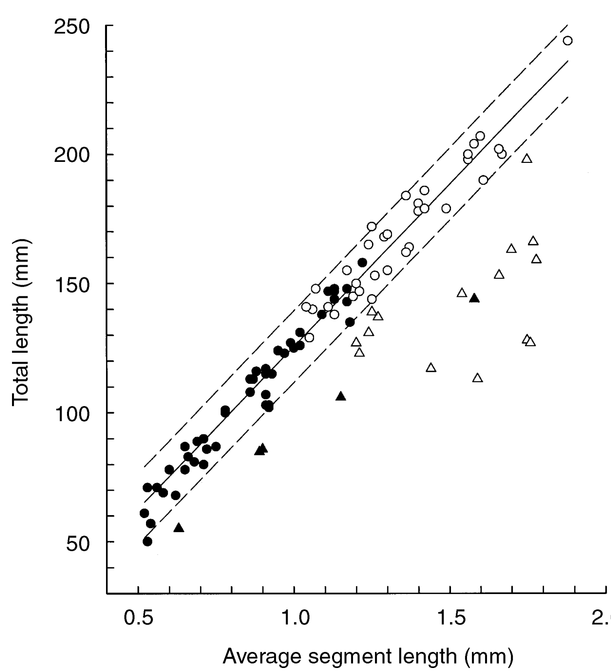

Fig. 1. Final linear regression relationship between total length and average segment length in the interactive outlier rejection procedure for *Amynthas lautus* collected at the Hsitou Forest Recreation Park, Nantou Co., between 10 and 18 December 1998 (●: normal aclitellates; ○: normal clitellates; ▲: amputated aclitellates; Δ: amputated clitellates; —: the regression line of normal specimens; ---: 95% confidence limits of the regression line).

95% confidence limits were normal specimens.

After removal of amputated specimens from the field-collected sample, total length for normal specimens increased with segment length (L_s)(Fig. 1) as expressed by the following linear regression relationship equation:

$$L_t = -4.026 + 129.09L_s + e$$
df = 78, $r = 0.986$ ($p < 0.01$)

Aclitellate and clitellate worms shared the same relationship equation line (Fig. 1), suggesting that growth of total length by increasing segment length occurred at both life stages at a similar rate.

Segment number-total length relationship

In figure 2, segment number was plotted against total length for the field-collected sample, in which normal and amputated specimens were already identified by the interactive outlier rejection procedure. Amputated specimens are sparsely and randomly scattered in the area of low segment number of the figure, whereas normal specimens are crowded in the area of high segment number, and show an asymptotic function relationship expressed by the following equation:

$$N_s = 127.28(1 - e^{-0.037Lt})$$
$R^2 = 0.27$, df = 78

According to the above equation, the segment number of normal specimens increased with total length for aclitellate worms until an average length of 100 mm, when it reached a plateau of 127 segments. Change (metamorphosis) from aclitellate to clitellate worms occurred at lengths between 125 and 170 mm. The specimens larger than 170 mm were all clitellates (Fig. 2). The maximum length in the sample was 244 mm. Apparently growth by increasing segment number occurred only in aclitellates, and not in clitellates. Based on the above asymptotic function equation, the first segment (N_s = 1) was estimated to occur at a total length of 0.21 mm, the size of beginning segmentation.

Segment-frequency distribution

For the field-collected sample, the g_1 values of the segment-frequency distributions of aclitellate, clitellate, and total worms were negative and significantly different from 0 (Table 1), suggesting that their distributions were skewed toward the left (Fig. 2). After amputated specimens were removed from the field-collected sample, the segment-frequency distribution of normal specimens of clitellate worms had a g_1 value which did not significantly differ from 0 (Table 1), suggesting a symmetrical distribution (Fig. 3). For clitellate worms, the removal of amputated specimens from the field-collected sample shifted the distribution from being skewed to being sym-

Table 1. Total length, segment number, and skewness (g_1 value) of the frequency distribution of aclitellate, clitellate, and total worms (aclitellates and clitellates combined) of normal specimens, amputated specimens, and a field-collected sample (normal and amputated specimens combined) of *Amynthas lautus* collected between 10 and 18 December 1998 at the Hsitou Forest Recreation Park, Taiwan

	No.	Total length (mm)		Segment number		g_1 Value	
		x ± S.D.	Range	x ± S.D.	Range	Length	Segment
Normal specimens							
Aclitellate	46	106 ± 28.0[a]	50-158	124 ± 8.1[b]	96-133	–0.063	–1.303[c]
Clitellate	33	171 ± 26.0	119-244	127 ± 5.5[b]	118-138	0.623	0.1494
Total	79	133 ± 42.0	50-244	124 ± 7.3[b]	96-138	0.139	–1.227[c]
Amputated specimens							
Aclitellate	5	95 ± 32.3[a]	55-144	93 ± 3.8	87-96	–	–
Clitellate	15	142 ± 21.3	113-198	95 ± 14.0	71-115	–	–
Total	20	130 ± 31.5	55-198	95 ± 12.9	71-115	–	–
Field sample							
Aclitellate	51	105 ± 20.3[a]	50-158	121 ± 12.1	87-133	–0.025	–1.343[c]
Clitellate	48	161 ± 28.1	113-244	116 ± 28.1	71-138	0.568	–1.223[c]
Total	99	132 ± 39.9	50-244	118 ± 15.1	71-138	0.1032	–1.414[c]

[a]Significant difference from the corresponding values of clitellates at the 1% level ($p < 0.01$, *t*-test).
[b]Significant difference from the corresponding values of amputated specimens at the 1% level ($p < 0.01$, *t*-test).
[c]Significant difference from 0 at the 1% level ($p < 0.01$, *t*-test).

metrical.

Unlike clitellates, the segment-frequency distribution of aclitellates of normal specimens retained its left skewness as did that of the field-collected sample. The removal of amputated specimens did not affect the skewness of the distribution. The same situation was found for the total sample (Table 1; Fig. 3).

Length-frequency distribution

The length-frequency distributions of aclitellate, clitellate, and total worms were all symmetrical with g_1 values which did not significantly differ from 0 for normal specimens as well as for the field-collected sample. The removal of amputated specimens did not affect the symmetry of the length-frequency distribution of the field-collected sample (Table 1; Fig. 4).

Differences between amputated and normal specimens

Amputated specimens had an average segment number of 93 for aclitellates and 95 for clitellates, which were significantly fewer than the 124 and 127 of normal specimens, respectively (Table 1). The average number of segments lost in amputation was 28.3 for aclitellates, which did not significantly differ from the 31.2 for clitellates (t-test, $p > 0.05$). For total worms, the average number of segments lost in amputation was 30. The segment numbers of amputated specimens being dispersed in figure 2 indicates that amputation might occur at any tail portion without a particular site of breaking off in autotomy.

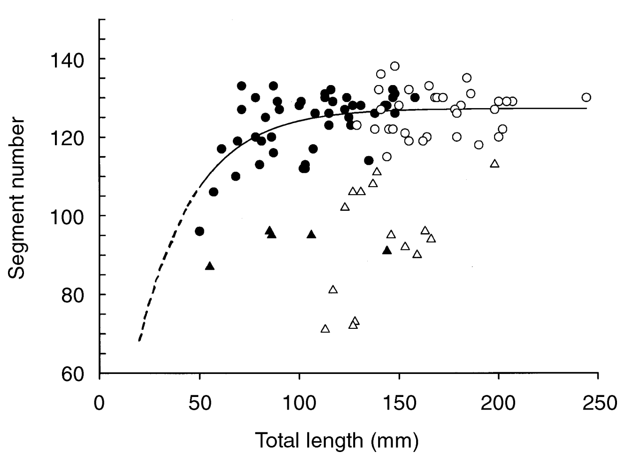

Fig. 2. Relationship between segment number and total length of *Amynthas lautus* collected at the Hsitou Forest Recreation Park, Nantou Co., between 10 and 18 December 1998 (●: normal aclitellates; ○: normal clitellates; ▲: amputated aclitellates; Δ: amputated clitellates; —: asymptotic relationship line of normal specimens; ---: predicted relationship line).

In contrast to segment number, total length of amputated specimens had an average of 95 mm for aclitellates and 142 mm for clitellates. These did not significantly differ, respectively, from the 106 and 171 mm of normal specimens (Table 1).

DISCUSSION

In this study we found that the interactive outlier

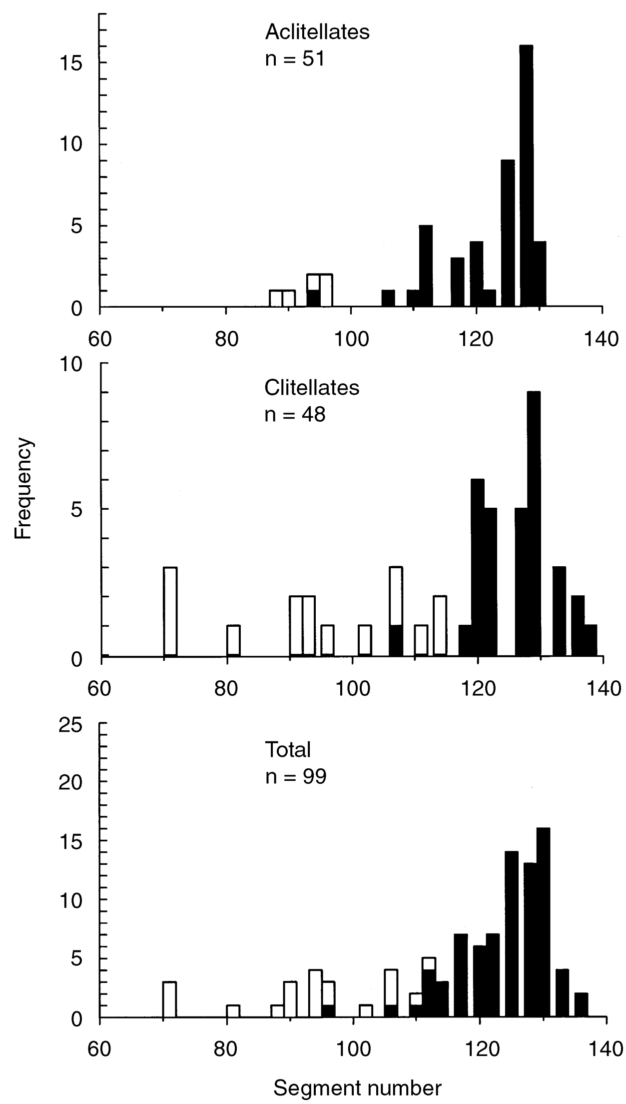

Fig. 3. Segment-frequency distribution of aclitellate, clitellate, and total worms (aclitellates and clitellates combined) of *Amynthas lautus* collected at the Hsitou Forest Recreation Park, Nantou Co., between 10 and 18 December 1998 (■: normal specimens; □: amputated specimens).

rejection procedure provided a reasonable and useful statistical method for distinguishing tail-amputated specimens and normal specimens in a field-collected sample of *Amynthas lautus*. However, it is likely that some amputated specimens lost only very short parts of their tails, or regenerated most of their lost tails, so that their total lengths might fall, as did those of normal specimens, within the 95% confidence interval of the total length-segment length relationship line. Therefore, the interactive outlier rejection procedure used in this study might cause a slight underestimation of the number of amputated specimens.

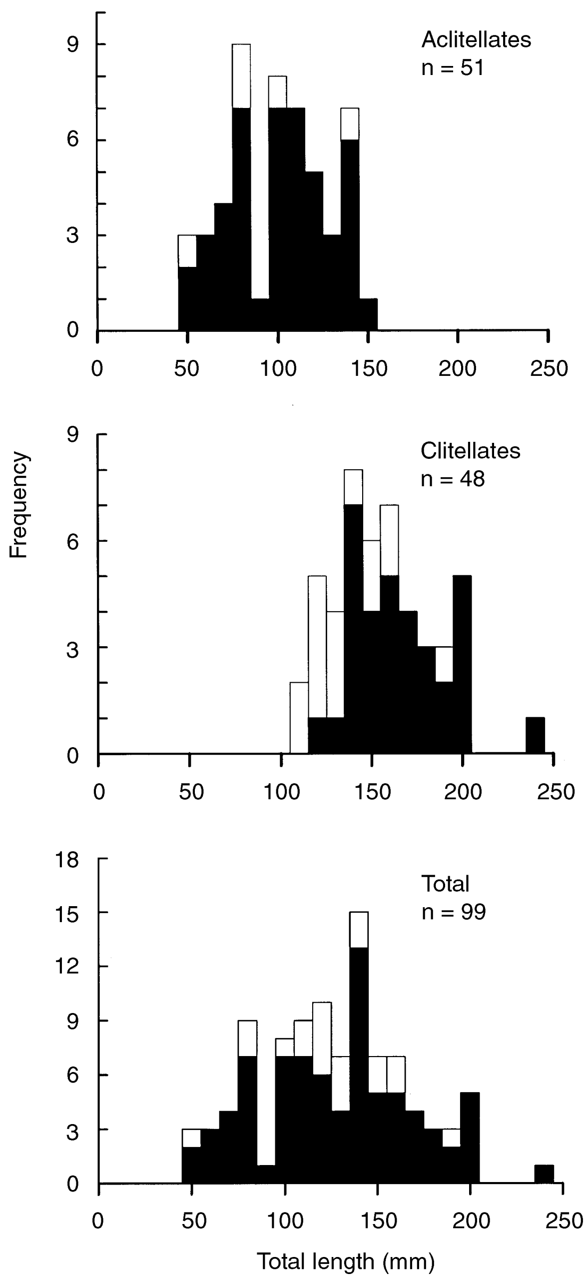

Fig. 4. Length-frequency distribution of aclitellate, clitellate, and total worms (aclitellates and clitellates combined) of *Amynthas lautus* collected at the Hsitou Forest Recreation Park, Nantou Co., between 10 and 18 December 1998 (■: normal specimens; □: amputated specimens).

Growth and amputation are two primary factors affecting total length and segment number of earthworms. In this study, amputation decreased the length and segment number for both aclitellate and clitellate worms; however growth increased both the length and segment number for aclitellates, but only the length for clitellates (Figs. 1, 2). In other words, total length grew continuously in both the aclitellate and clitellate stages, but segment number increased only in the aclitellate stage but not in the clitellate stage. Consequently, there was a comparatively large range in the length-frequency distribution but a small range in the segment-frequency distribution of normal specimens in both life stages (Table 1).

Because of the small range, segment numbers of most of the amputated specimens fell outside the minimum range of the segment frequency distribution of normal specimens, causing left skewness of the distribution of the field-collected sample (Fig. 3). When the amputated specimens were removed, the distribution became symmetrical for clitellate, but remained left skewed for aclitellate and total worms. Apparently, left skewness of the segment-frequency distribution of the field-collected sample was due to the presence of tail-amputated specimens in clitellate, but the presence of both amputated specimens and growth effect in aclitellate and total worms (Table 1; Fig. 3).

In contrast to segment number, the range of the length-frequency distribution of normal specimens was wide enough to accommodate the total lengths of almost all amputated specimens, so that the length-frequency distribution of the field-collected sample remained symmetrical as did that of normal specimens (Fig. 4). Therefore, the length-frequency distribution of the field-collected sample detected neither the presence of amputated specimens nor the effect of growth for aclitellate, clitellate, and total worms.

The percentage composition of tail-amputated specimens was 31.3% for clitellates, which was higher than the 9.8% for aclitellates. This difference was attributable to the size, maturation, and habit differences between the two life stages. As compared to aclitellates, clitellates are larger and more easily detected by predators. They are older and have lived longer, so that they have had greater exposure to predation. They are sexually mature and might move to the ground surface more frequently, particularly at night, for feeding and mating, and thus, they would encounter predators more often.

Gates (1972) indicated that some species of earthworms may have post-reproductive regeneration of the clitellum. Reynolds et al. (1974) recognized four categories in the age classification for lumbricid earthworms: 1) aclitellate juveniles with no easily recognizable genital markings, 2) aclitellate adults without a clitellum but obvious genital markings, 3) clitellate adults with a clitellum and genital markings, and 4) post-clitellate adults with clitellum degenerated but with obvious genital markings. As the clitellum is the organ that produces the cocoon essential for reproductive success, aclitellate adults (Reynolds et al. 1974) may be more reasonably considered as immature sub-adults. In this study, aclitellates may have included both juveniles and immature sub-adults. The post-clitellate adult (Reynolds et al. 1974) is apparently identical to the adult with post-reproductive regeneration of the clitellum (Gates 1972). In this study, whether *Amynthas lautus* has a post-reproductive degeneration of the clitellum (Gates 1972), or if it exists as a so-called post-clitellate adult (Reynolds et al. 1974) is unknown. However, the field-collected sample consisted of small aclitellates, large clitellates, but no large aclitellates (Fig. 2). These facts suggest that the month of the collection was in the breeding season, or that *A. lautus* has no post-reproductive regeneration of the clitellum, or the post-clitellate adult does not occur in this species. The interactive outlier rejection procedure is a useful method for distinguishing normal specimens from amputated specimens in a field-collected sample of earthworms, because it is based primarily on three reasonable assumptions; however, it remains to be verified by data of a population raised in the absence of predation.

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以統計分析判別野外採集之蚯蚓 *Amynthas lautus* (Ude) 樣本中的正常及斷尾個體

蔡住發[1] 蔡素蟾[1]

重複進行總體長與每一體節長間之線性回歸分析，並捨棄 95% 信賴區間 外的數值，即所謂的 interactive outlier rejection procedure。此種統計方法在野外採得之蚯蚓 *Amynthas lautus* 個體是否斷尾的判別上相當有用。在去除斷尾個體樣本後，正常個體的總體長與體節數之間呈現漸近關係；未達性成熟之蚯蚓，其體節數隨體長之增長而增加，終至到達高原期，於性成熟後則繼續維持在此高原期，其體節數並不隨體長之增長而增加。野外採得樣本之體節數頻度分布偏左，顯示成體中有斷尾個體之存在，而未成熟個體則同時受到斷尾及生長效應之影響。

關鍵詞：貧毛類，體長，體節數。

[1] 特有生物研究保育中心棲地生態組