

Update on northernmost distribution of the luminous earthworm *Microscolex phosphoreus* (Dugès, 1837) in Japan, and their DNA and size variations

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Abstract: The luminous earthworm *Microscolex phosphoreus* was collected from Akita City in Akita Prefecture (39°43'N). This is the new record of northernmost distribution of this species in Japan. We also collected the specimens again from Sendai City, where the previous northernmost record in 2016, in Miyagi Prefecture. All specimens collected in Akita City were in adult stage, while collected in Sendai City and Kasugai City (Aichi Prefecture) at almost the same time contained both in juveniles and adults.

Key words: Bioluminescence, Northernmost distribution, Akita Prefecture, *Microscolex phosphoreus*.

INTRODUCTION

The luminous earthworm *Microscolex phosphoreus* (Dugès, 1837) is cosmopolitan (Rota *et al.*, 2018), and the distribution in Japan has been recorded (Yamaguchi, 1935; Oba, 2012; Oba *et al.*, 2011a, 2011b, 2016), especially in recent years, from the central region of Japan (Honshu, Shikoku, Kyushu). On the other hand, this species was not recorded from northern most area, Hokkaido, and southern most region, Ryukyu. Previously, we reported the distribution of this species from the city parks of Yamagata City in Yamagata Prefecture (38°13'N) and Sendai City in Miyagi Prefecture (38°16'N) of Tohoku Region, where the far north of Honshu (Oba *et al.*, 2016). In this study, we reported a new record of the northernmost distribution of *M. phosphoreus* from Akita Prefecture, and the comparison of their DNA and size variations to those of other localities.

MATERIALS AND METHODS

The earthworms *Microscolex phosphoreus* were surveyed from Aomori Prefecture, Akita Prefecture, Miyagi Prefecture and Aichi Prefecture in November 2018. The specimens were collected by carefully digging up the topsoil near casts and by hand sorting. The worms were killed in 30% (v/v) ethanol, photographed and transferred to 99.5% (v/v) ethanol for long term preservation, molecular analysis and subsequent morphological studies. The materials were deposited in Department of Environmental Biology, Chubu University. The number of segments, body length and diameter (at segment XX) of the collected specimens were counted and measured under a stereomicroscope (WRAYMER LW-800 series). Only the adult *M. phosphoreus* were measured and used to analyzed, with juvenile from all sampling sites being exclude.

Total DNA was extracted from the posterior part of samples using Lysis Buffer for PCR (Takara). The fragment of

mitochondrial cytochrome *c* oxidase I (*COI*) was amplified with the Tks Gflex™ DNA Polymerase (Takara) using universal primers (Folmer *et al.*, 1994), as follows: 1 μ l of Tks Gflex DNA Polymerase (1.25 unit/ μ l), 25 μ l of 2x Gflex PCR buffer (Mg²⁺, dNTP plus), 1 μ l each of LCO1490 (forward) and HCO2198 (reverse) universal primer, 19.5 μ l of sterilized distilled water and 2.5 μ l of crude lysate with Lysis buffer. The PCR reaction mixtures were heated to 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min. The concentration and quality of the amplicons were tested with 1% agarose gel against a DNA standard marker in 1x TAE buffer and detected under UV transillumination. For sequencing, the PCR products were directly sent to Macrogen Inc. (Japan) without purification. All *COI* sequences were aligned using the ClustalW algorithm in MEGA7 v. 7.0.18 (Thompson *et al.*, 1994; Kumar *et al.*, 2016) and manually checked by eyes. The sequences were checked and compared with other sequences available in the GenBank databases at the National Center for Biotechnology Information (NCBI) using BLAST similarity search tool (<http://www.ncbi.nlm.nih.gov>).

RESULTS

In late November 2018, luminous earthworm *Microscolex phosphoreus* surveys were conducted in 4 locations in Japan (Aomori prefecture, Akita Prefecture, Miyagi Prefecture and Aichi Prefecture). In our field study, we did not get specimens in Hachinohe, Aomori. The detailed distribution of the habitats and the collection information of collected earthworms *M. phosphoreus* in 3 locations are given below.

Locality: North side of Research Center of Advanced Materials for Breakthrough Technology, Akita University Campus, 1-1-1 Hondo, Akita, Akita Prefecture, Japan (39°43'N, 140°08'E).

Date: 21 and 22 November, 2018



Figure 1. Photographs showing (A) Microhabitat of *M. phosphoreus* at Akita University Campus, Akita Prefecture; (B) *M. phosphoreus* in the sampling site in Akita Prefecture; (C) Bioluminescence of *M. phosphoreus* collected from Akita Prefecture.

Collectors: Yuichi Oba and Kenshu Fujiwara

Number of samples: 21 adults

Locality: Yanagimachi Park, 1-14-23 Ichiban-cho, Sendai, Miyagi Prefecture, Japan (38°15'N, 140°52'E).

Date: 30 November, 2018

Collectors: Seesamut Teerapong, Ikuhiko Kin and Gaku Mizuno

Number of samples: 82 adults and 19 juveniles

Locality: Southern side of Miura Memorial Library, Chubu University Campus, 1200 Matsumoto-cho, Kasugai, Aichi Prefecture, Japan (35°16'N, 137°00'E).

Date: 26 November, 2018

Collectors: Seesamut Teerapong and Ikuhiko Kin

Number of samples: 26 adults and 17 juveniles

The northernmost recorded of *M. phosphoreus* in this study was in Akita Prefecture. The microhabitat in Akita University campus is shown in Figures 1A-1B. The bright green bioluminescence when the earthworm was forcibly handled is shown in Figure 1C.

A total of 72 individuals of *M. phosphoreus* from the 3 sampling sites (Akita Prefecture, Miyagi Prefecture and Aichi Prefecture) were counted in segments and measured in diameter and length (mm). Some specimens damaged to be examined were excluded. The length of 30 mm to 35 mm (N = 26) was the most abundant, follow by length of 25 mm to 30 mm (N = 23) and only one sample was recorded for length less than 15 mm. The lengths of *M. phosphoreus* from all sampling sites were normally distributed (Fig. 2). The relationships between number of segments, length and diameter of *M. phosphoreus* were plotted in Figure 3A-3C, showing that the Akita specimens are large in diameter (1.49 ± 0.31 mm) compared with Miyagi (0.94 ± 0.12 mm) and Aichi populations (1.12 ± 0.16 mm). The relationship of the number of segments, length and diameter showed low correlation between parameters. Adult *M. phosphoreus* (N = 72) had an average number of segment of $72.39 \pm$

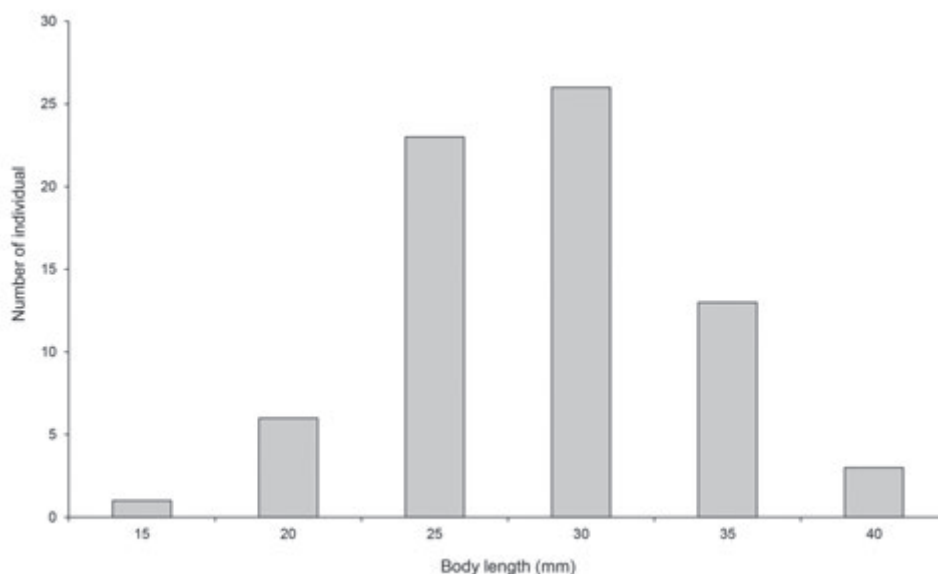


Figure 2. The frequency distribution of length of *M. phosphoreus* in this study (N = 72).

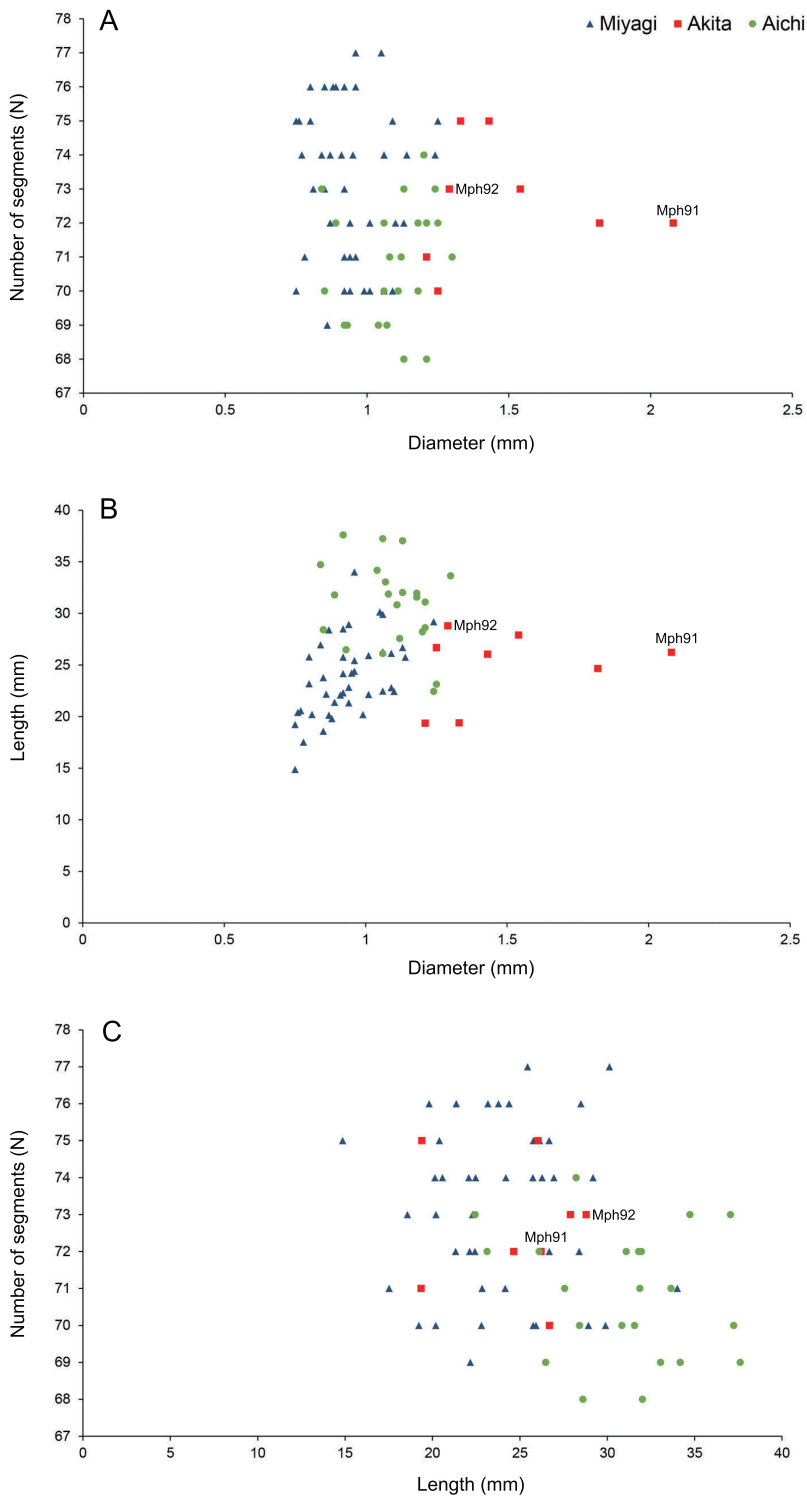


Figure 3. (A) Number of Segments plotted against the diameter (mm) of *M. phosphoreus* collected in 3 prefectures. (B) Length (mm) vs Diameter. (C) Number of segments vs Length. The plots of the specimen Mph91 and Mph92 (Akita population) were specified in figures.

2.31. In the field surveys, potential predators were not observed during collection.

The *COI* sequences of 2 samples from Akita population had 100% nucleotide identity with *M. phosphoreus* according to the BLAST analysis. The nucleotide sequences were deposited at the GenBank under accession number LC455933 (sample ID, Mph91) and LC455934 (sample ID, Mph92).

DISCUSSION

Current record in Akita prefecture is the northernmost distribution of *Microscolex phosphoreus* in Honshu (Akita Sakigake Shimpō, 2018), whereas no reported in Hokkaido and in this study we did not get *M. phosphoreus* in Hachinohe, Aomori Prefecture. The distribution of adult and juvenile earthworms across the 3 prefectures in Japan were examined. In total, mature earthworms were more abundant (78.18%) than non-mature. Akita prefecture had only adult specimens (100%), followed by Miyagi prefecture (81.19%) and Aichi prefecture (60.47%). According to the geographical location, we suggest that the earthworm likely grows more rapid in northernmost part than southern parts in a cool temperate climate.

In this study, morphometric variations in *M. phosphoreus* were investigated. Total lengths in 3 localities varied from 14.86 mm to 37.61 mm, and number of segments from 68 to 77. The specimens of *M. phosphoreus* shows variation in number of segments and body dimensions, Dyne & Jamieson (2004) reviewed on the taxonomy of *M. phosphoreus* and described 10–55 mm in length with 73–91 segments, Taravera & Pérez (2009) reported 20–55 mm in length of *M. phosphoreus* in the western Canary Islands. In the earliest description in morphological variation of this species in Japan, Yamaguchi (1935) examined the Kanagawa specimens, showing 25–53 mm long (condition unknown) and 74–76 in segment number. In the most recently reported by Rota *et al.* (2018) examined the Siberian specimens, showing 35–55 mm long (living condition) and up to 47 mm after fixation with 73–79 segments. The lengths and segment numbers of *M. phosphoreus* investigated in this study are in the range of variations of the previous reports. A 658 base pair of the mitochondrial *COI* sequences from two Akita Prefecture's specimens showed 100% nucleotide identity to the *M. phosphoreus* of accession numbers LC108792 (Yamagata Prefecture, Japan; previous northernmost record), LC339499 (Fukushima Prefecture, Japan), AB750641 (Hyogo Prefecture, Japan), LC198324 (Shimane Prefecture, Japan), and others. The molecular studies on the genetic diversity of the mitochondrial *COI* gene have been reported in Japan by Oba *et al.* (2011a, 2011b, 2012, 2015, 2016) and worldwide scale by Rota *et al.* (2018), and our present results showed the inclusion of the sequences from Akita Prefecture in the clade of the specimen from Japan and Australia, revealing no pattern in distribution. We found that Akita Prefecture's specimens were larger in diameter but the length and range of segment numbers were not different from other two populations. We chose the largest and one of the smallest specimens in diameter from Akita Prefecture for DNA sequencing, but as shown above, there was not sequence differences between them. It is still uncertain which factor is causative of the large in diameter of Akita specimens compared to those of other two localities. In conclusion, this study reported the new record of the northernmost distribution of *M. phosphoreus* in Japan in Akita Prefecture. It showed not only the highest percentage of mature earthworms but also the largest in diameter compared with other 2 sampling sites in southern regions. These results highlight for land use and management for the maintenance of this soil organism, which play dominant engineering roles in soil ecosystem.

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ホタルミミズの北限記録更新とその遺伝子および体サイズの解析

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要約：発光性貧毛類ホタルミミズ *Microscolex phosphoreus* を秋田県秋田市（北緯 39°43'）から採集した。日本北限記録となるので報告する。同じ時期に、先の北限記録のひとつであった宮城県仙台市でも採集した。採集した個体は、秋田市産のものはすべて成体であったが、仙台市と春日井市（愛知県）のものは成体と幼体の両方が含まれていた。

キーワード：生物発光，北限記録，秋田県，ホタルミミズ