研究資料

DNA barcoding identified the exotic terrestrial isopod *Porcellio scaber* (Crustacea, Isopoda, Oniscidea) on the Kyushu mainland, western Japan

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DNA バーコーディングを用いた九州からの外来性ワラジムシ類 *Porcellio scaber* の発見

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Porcellio scaber Latreille, 1804 was found on the Kyushu mainland for the first time. The specimens were identified based on DNA barcoding and the following five morphological characteristics: 1) posterior margin of pereonite 1 is almost straight, 2) epimerons 1–4 have obvious sinuous posterior margins, 3) exopodites of pleopods 1 and 2 bear monospiracular lungs, 4) the posterior tip of the telson tapers, and 5) the flagellum of the second antenna is composed of two segments. Moreover, the specimens collected from a park in Fukuoka City showed carapace color variation.

Keywords：Color variation, Fukuoka, Invasive species, Porcellionidae

九州本土において初めてワラジムシ *Porcellio scaber* を確認した。本研究で採集した標本は，１）第１胸節背板の後縁中央部がほぼ直線であること，２）第１–４胸節背板の側部後縁がＳ字になること，３）第１，２腹肢外肢が１個の呼吸穴を持つこと，４）腹尾節の先端が尖ること，５）第２触角の鞭部が２節で構成されること，および，DNA バーコードによる類似度検索により *P. scaber* であると判断した。また，福岡市内で採集された標本には体色に変異が認められた。

キーワード：体色変異，福岡，外来種，ワラジムシ科

Introduction

Exotic species are species that were transferred from their native distribution to other areas by human activities (Ontario, 2013). The number of exotic species is rising with the global increase of human travel, and these species have the potential to cause extinction of native species (Gurevitch and Padilla, 2004). The most effective management of exotic species is to prevent invasion of species. However, if they have invaded a new site, it is important to detect them early so they can be eradicated or controlled (Simberloff *et al*., 2013).

Precise identification of species often requires professional skill and specimens with sufficient morphological characters to identify species. For example, adult male morphological characteristics are usually important for identifying terrestrial isopods (e. g., Kashani, 2015; Taiti and Wynne, 2015), it implies that female specimens are difficult

2016. 7 . 7 受付；2016. 11. 15受理

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to precisely identity. DNA barcoding is a powerful tool that can resolve this issue. This approach is a way to identify species using a short genetic sequence and enables species identification of specimens without sufficient morphological characters (Hebert *et al*., 2003). There is some possibility of not collecting specimens with full taxonomic characteristics at early stages of invasion because of a small number of individuals. Thus, the DNA barcoding is expected to be able to identify exotic species in early stage of invasion.

The aim of this study was to report the first collection of exotic terrestrial isopods, *Porcellio scaber* Latreille, 1804, from the Kyushu mainland, western Japan. All of the specimens were female, and it is difficult to identify *P. scaber* individuals based on female morphological characters, therefore we used DNA barcoding to identify species. Moreover, we describe morphological characters, including color variation, and determine DNA sequences of additional genetic regions for future DNA-barcode studies.

Materials and Methods

Examined specimens

Two females were collected from under leaf litter at the Minato-100-Nen-Park in Fukuoka City, Fukuoka Prefecture (N33.6494, E130.4148) on 14 October 2015 by Tomoki Iwasaki. One female was collected from under leaf litter at the Akama Kitaguchi Mini Park in Munakata City, Fukuoka (N33.80879, E130.56780) on 1 December 2015 by Shigenori Karasawa. The specimens were preserved in 99.5% ethanol until morphological observation and molecular analysis.

Morphological observation

Photographs were obtained based on multifocused montage images using a digital microscope VHX-2000 (KEYENCE Corporation, Japan). The specimens for scanning electron microscopy were dried at room temperature, coated with gold, and photographed using a JCM-5100 scanning electron microscope (JEOL, Japan).

Molecular analysis

One *P. scaber* collected from the Minato-100-Nen-Park in Fukuoka City was used for molecular analysis. One *P. scaber* found in Tokyo (N35.728269, E139.715181) was also analyzed in order to determine the genetic difference between specimens in Tokyo (Kanto region) and Kyushu. A partial region of the mitochondrial cytochrome c oxidase subunit I (COI) gene was sequenced for

Table 1. Species, localities, and DDBJ accession numbers.

Species	Locality	Accession no.			
		COI	12S	18S	28S
Porcellio scaber	Fukuoka, Japan	LC126629	LC126631	LC126633	LC126635
Porcellio scaber	Tokyo, Japan	LC126628	LC126630	LC126632	LC126634
Porcellio scaber	British Columbia, Canada	KM611741	-	-	-
Porcellio scaber	British Columbia, Canada	KM611704	-	-	-
Porcellio scaber	British Columbia, Canada	KM611660	-	-	-
Porcellio scaber	British Columbia, Canada	KM611577	-	-	-
Porcellio scaber	British Columbia, Canada	KM611545	-	-	-
Porcellio scaber	Massachusetts, USA	HQ978726	-	-	-
Porcellio scaber	Poitiers, France	LK052885	-	-	-
Porcellio scaber	Gdansk, Poland	DQ305142	-	-	-
Burmoniscus ocellatus	Iriomotejima Is., Japan	AB626177	-	-	-
Ligidium ryukyuense	Amami-Oshima Is., Japan	AB626262	-	-	-
Mongoloniscus koreanus	Fukuoka, Japan	LC017826	-	-	-
Mongoloniscus vannamei	Fukuoka, Japan	LC017827	-	-	-
Porcellio laevis	Siracusa, Italy	FN824122	-	-	-
Porcellio laevis	Siracusa, Italy	FN824123	-	-	-
Porcellionides pruinosus	Siracusa, Italy	FN824140	-	-	-
Porcellionides pruinosus	Australia	KR424607	-	-	-

DNA barcoding analysis. In addition, we sequenced three genetic regions for future study; nuclear 18S and 28S ribosomal RNA (rRNA) genes and the mitochondrial 12S rRNA gene. DNA extraction protocol, PCR conditions, and primers used were described in Karasawa *et al.* (2014). The sequence data have been deposited in DDBJ/EMBL/GenBank (COI: LC126629, 12S: LC126631, 18S: LC126633, 28S: LC126635).

Identification of species based on DNA barcoding was carried out using the BOLD Identification System for COI (http://www.barcodinglife.org/index.php/IDS_OpenIdEngine) on 2 May 2016. Sequence data were input into the system, which in turn showed candidate species that genetically matched the input data. We used the All Barcode Records on BOLD search database.

To visualize the validity of DNA barcoding, we constructed a phylogenetic tree with two exotic species (*Porcellio laevis* Latreille, 1804 and *Porcellionides pruinosus* (Brandt, 1833)), three morphologically resembling species found in Japan (*Burmoniscus ocellatus* (Verhoeff, 1928), *Mongoloniscus koreanus* Verhoeff, 1930 and *M. vannamei* (Arcangeli, 1927)), and six *P. scaber* specimens in other countries based on the COI sequence data. The sequence data of these specimens were obtained from the GenBank database (Table 1). Multiple sequence alignment was carried out by MUSCLE in SeaView 4. 5. 4 (Gouy *et al.*, 2010), and all gaps were excluded from subsequent analysis. Maximum Likelihood (ML) analysis was performed using MEGA 7 (Kumar *et al.*, 2016). The best-fit models of sequence evolution determined by the Bayes information criterion (BIC) in the program MEGA 7 was HKY+G model. Bootstrap support was assessed using 100 replicates.

Results and Discussion

Precise species identification of *P. scaber* requires male morphological characteristics (Vandel, 1962; Gruner, 1966), but unfortunately we cannot collect male specimens. Both male and female *P. scaber* are known to have the following morphological characteristics (Vandel, 1962; Gruner, 1966; Hopkin, 1991; Schmidt, 2003): there were three lobes on the anterior margin of the cephalon (Fig. 1A), the posterior margin of pereonite 1 is almost straight (Fig. 1A), epimerons 1–4 have obvious sinuous posterior margins (Fig. 1A), exopodites of pleopods 1 and 2 bear monospiracular lungs (Fig. 1B and C), the posterior tip of the telson tapers (Fig. 1A), and the flagellum of the second antenna is composed of two segments (Fig. 1D). The specimens examined had all of these morphological characters. However, some of these characters were shared with congeneric species (e.g., Schmidt, 2003).

To ensure proper species identification, we used the DNA barcoding approach which can identify

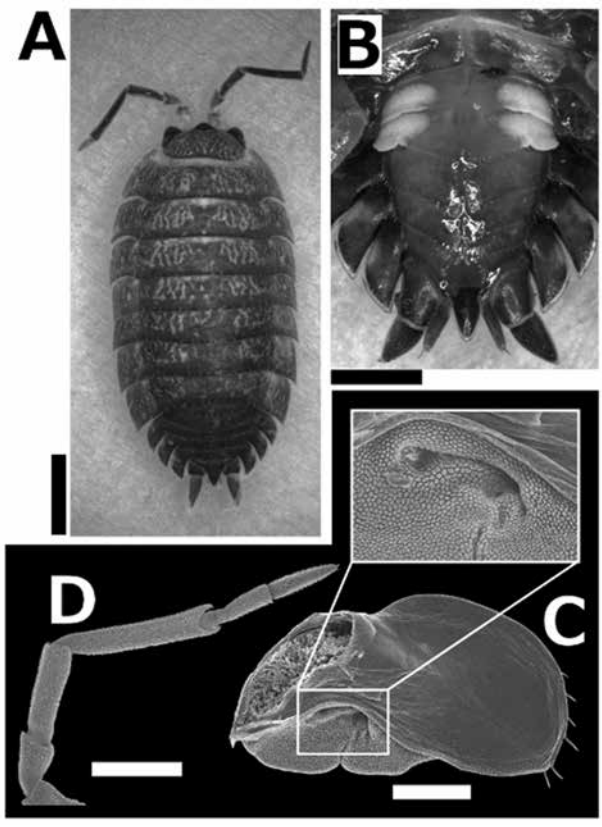

Fig. 1. Photographs and scanning electron micrographs of *Porcellio scaber collected* from Fukuoka, Kyushu. A: Dorsal surface, female collected from Minato-100-Nen-Park; scale bar, 2 mm. B: Ventral side of the abdomen, female collected from Akama Kitaguchi Mini Park; scale bar, 1 mm. C: Ventral side of exopodite 1, the upper white square indicates monospiracular photographed from a different angle, female collected from Minato-100-Nen-Park; scale bar, 0.3 mm. D: Second antenna, female collected from Minato-100-Nen-Park; scale bar, 1 mm.

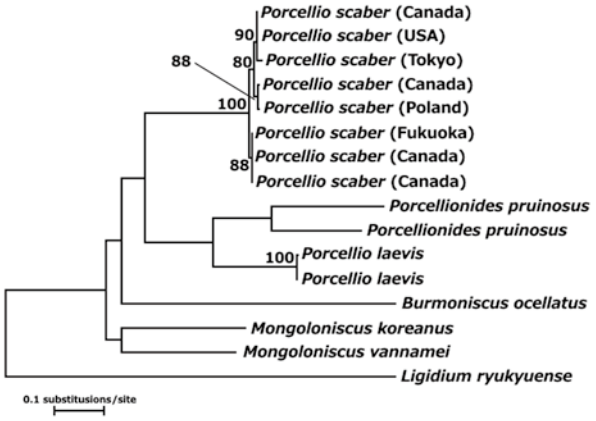

Fig. 2. ML phylogenetic tree based on combined COI sequence data. Bootstrap values exceeding 70% are shown at each relevant node.

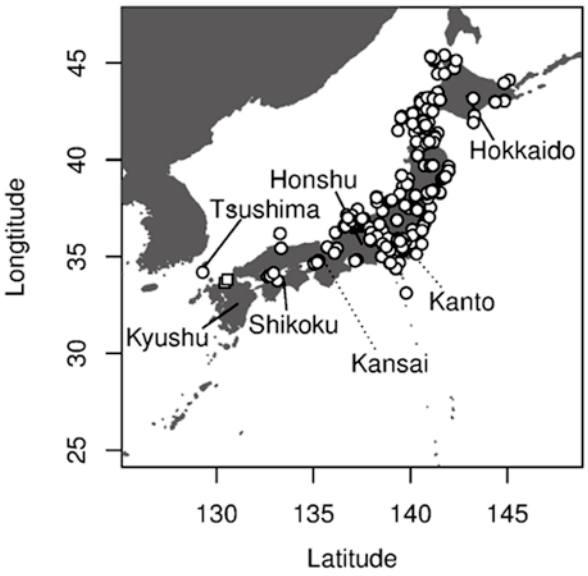

Fig. 3. Map of *Porcellio scaber* collection sites in Japan. Circles indicate sites that have been reported in the Japanese terrestrial isopod distribution database (http://isopoda.sakura.ne.jp/map/map.php), and squares indicate new collection sites.

female specimens because genetic data are used (Hebert *et al*., 2003). The BOLD Identification System selected the top 99 candidate specimens with more than 98% similarity to specimens collected from Kyushu, and all except for eight unidentified specimens in the database were *P. scaper*. In addition, the phylogenetic tree revealed that *P. scaber* in Fukuoka Prefecture was included in a clade composed of eight specimens collected from four countries (Fig. 2). Therefore, we identified these specimens as *P. scaber*. *Porcellio scaber* is also known to show distinctive variation in carapace coloration (Bhella *et al*., 2006), and both orange and gray types were collected from the park in Fukuoka City.

Porcellio scaber is distributed worldwide, but in most areas, outside of Europe, the species is considered exotic (Schmalfuss, 2003); while it has been suggested that this species represents six subspecies (WoRMS Editorial Board, 2016), and the taxonomic treatment remains unclear. Solving the taxonomic confusion is important for precise identification, but it requires further analysis beyond the subject of this present study. In Japan, the species is also thought to have been introduced by human activity (Nunomura, 2007). Japan comprises four main islands: Hokkaido, Honshu, Shikoku, and Kyushu (Fig. 3). Nunomura (1998) reported that *P. scaber* was not found in the Kansai region of western Honshu during 1967–1977, and its distribution might be limited to Eastern Honshu and Hokkaido. Later, *P. scaber* were collected from Tottori, Osaka, Nara, and Hyogo in western Honshu, and Tokushima in Shikoku between 1992–1998 (Nunomura, 1998). In 2012, Hayashi (2012) found this species in Shimane, which is located in western Tottori, therefore the western limit of their distribution in Honshu has been considered to be Shimane. Nunomura (2011) also collected this species from Tsushima Island, which is north of the Kyushu mainland. However, *P. scaber* has not been collected on the Kyushu mainland until now. The results of the present study indicate that this species recently spread to the southwest part of Japan. The phylogenetic tree indicated that *P. scaber* in Fukuoka Prefecture genetically differed from *P. scaber* in Tokyo (Fig. 2), which implied that *P. scaber* in Fukuoka Prefecture might be introduced from another country and not Honshu.

Taxonomic status of terrestrial isopods is usually delimited using male morphological characters (e.g., Kashani, 2015; Taiti and Wynne, 2015), and some of the taxonomical characters may change as individual growth (Tanaka and Karasawa, 2016). Thus, identification based on immature and female specimens is difficult and has the potential to

cause misidentification, and the present study showed that DNA barcoding is useful for identifing these specimens.

Acknowledgement

This research was partially supported by the Ministry of Education, Culture, Sports, Science and Technology, a Grant-in-Aid for Young Scientists (B) (no. 26830145).

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