

**Morphological and molecular analyses of *Bandonia boninensis* (Arachnida:
Opiliones: Assamiidae) in the Bonin (Ogasawara) Islands**

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Abstract

To clarify the morphological and phylogenetic differentiation of *Bandonia boninensis* Suzuki 1974 in Chichi-jima Island of the Bonin (Ogasawara) group of Islands, we studied the external morphological characters and performed sequencing of the cytochrome *c* oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA) and 28S rRNA of nuclear DNA (nrDNA). The sequences of COI and 28S rRNA were identical among the individuals of *B. boninensis*. These results suggest that *B. boninensis* experienced a rapid expansion of its distribution in Chichi-jima Island without undergoing any morphological and molecular differentiation.

Key words: Harvestmen, morphology, phylogeny

Introduction

Oceanic island archipelagos are profoundly interesting ecosystems to explore the evolutionary patterns and processes. The Bonin (Ogasawara) Islands (26°30' to 27°40' N, and 142°00' to 142°15' E), which consist of about 30 small islands, are located in the western Pacific Ocean, approximately 1000 km south of the Japanese mainland, and have never been historically connected to any continent or archipelago. Therefore, the fauna and flora in these islands are very disharmonic and a high proportion of endemism has been recognized (Ono et al. 1991; Ohbayashi et al. 2003).

Four species of the harvestmen were recorded in the Bonin Islands, namely, *Bandona boninensis* Suzuki 1974, *Hologagrella minatoi* Suzuki 1974, *Verpulus boninensis* Suzuki 1978, and *V. similis* Suzuki 1978 (Suzuki 1974; Suzuki 1978). Among them, *B. boninensis* was first described based on the single female individual collected from Chichi-jima Island



Fig. 1 *Bandona boninensis* from Chichi-jima Island of the Bonin Islands. A) BL: body length); B) CW: carapace width; C) CL: carapace length; D) Fe1L: first femur length; E) Fe2L: second femur length; F) Fe3L: third femur length; G) Fe4L: fourth femur length; H) PTL: palpus-tarsus length and I) Chel. L: chelicerae length. Bar=1mm.

(Suzuki 1974) (Fig. 1). Since all of the collected samples were identified as females, Suzuki (1978) suggested that *B. boninensis* is a parthenogenetic species, which reproduces asexually without fertilization. In addition, Tsurusaki (1991) supported the suggestion of Suzuki (1978) based on a broad survey of the harvestmen in the Bonin Islands, wherein male individuals of this species were not identified. Recently, Zhang et al. (2010) discovered *B. boninensis* in the Yunnan Province of south China, which is a new distribution area of this species; in contrast to previous studies, they not only found females but also one male individual, implying that *B. boninensis* in this area were not parthenogenic, although the frequency of occurrence of the male individual was very low. This report suggested a possible existence of male *B. boninensis* in Chichi-jima Island; however, males are not easily discovered because of their low frequency of occurrence.

In general, genetic variation within a species is a fundamental component of biological diversity, and the distribution of this variation within a species is shaped by processes that are extrinsic to the species, such as ecological events or selective regimes, as well as intrinsic factors, such as the type of mating system (Avice 2000). Considering these factors in a geographical context, the structuring of this variation is a product of the current genetic exchange within a species as well as the historical relationships between the populations. However, parthenogenesis inexorably accumulates deleterious mutations with no mechanisms to get eliminate them, and is sufficient to significantly reduce genetic variation (Lushai et al. 1998; Simon et al. 2003). Therefore, it is possible that there are very low genetic variations in *B. boninensis* in Chichi-jima Island considering that this species is parthenogenic. Thus, information regarding the nucleotide variations is indispensable for the conservation of *B. boninensis* in this area.

The number of phylogenetic studies based on molecular data has increased enormously in recent years, and most of the recent studies focus on closely related species or variation within the species. In particular, the use of molecular markers has considerably improved our knowledge about how the past events shape the genetic diversity within a species (Avice 2000). Moreover, in conjunction with advances in molecular techniques and analytical methods on the haplotype data, some gene regions that provide sufficient intraspecific variation have been identified and successfully used in addressing various population and evolutionary questions in a number of species (Olsen and Schaal 1999; Caicedo and Schaal 2004; Londo et al. 2006; Muller et al. 2006). Recent phylogenetic studies on the harvestmen were based on analysis of the genes with a high substitution rate such as the cytochrome *c* oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA) and 28S rRNA of nuclear DNA (nrDNA) (Kumekawa et al. 2014; Pinto-da-Rocha et al. 2014; Kumekawa et al. 2015). Therefore, a phylogenetic tree was reconstructed using these genes to clarify the genetic variation in *B. boninensis* in Chichi-jima Island of the Bonin Islands and to assess the parthenogenetic ability of this species in this area.

Materials and Methods

Morphological analyses

The collection sites of the seventeen *B. boninensis* individuals selected in this study are presented in Fig. 2 and Table 1. Morphological analysis was performed by the measurement of body length (BL), palpus-tarsus length (PTL), carapace length (CL), chelicerae length (Chel. L) and width (CW), and femur lengths of the first to the fourth leg (Fe1L-Fe4L), for each individual of *B. boninensis* (Fig. 1). Measurements were made using the Imaging software cellSens standard (Olympus Co., Tokyo).

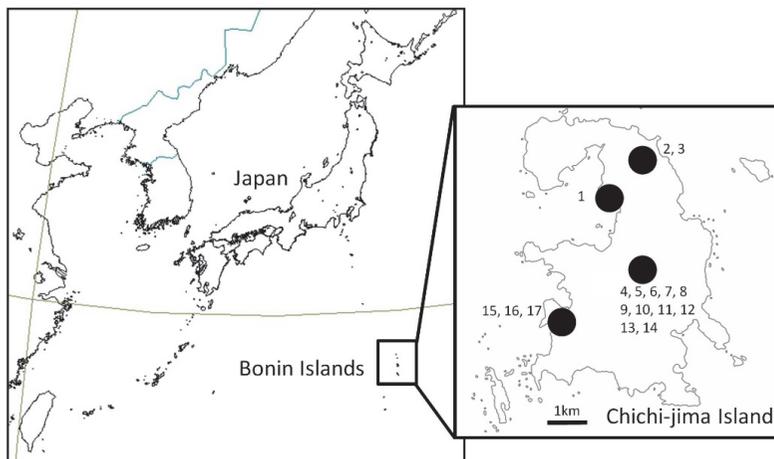


Fig. 2 Sampling location of *Bandona boninensis* in Chichi-jima Island of the Bonin Islands.

Table 1. Summary of *Bandona boninensis* analyzed in this study. OTU name correspond to our phylogenetic results (Figs. 3 and 4).

Species	OTU name	Sex	Location			Sampling date	Accession No.			Reference
			Island	Village	Site		COI	28S		
<i>Bandona boninensis</i>	1	Adult ♀	Chichijima	Ogasawara	Sakairura	21 Feb, 2015	LC040968	LC040985		This study
	2	Adult ♀	Chichijima	Ogasawara	Mt. Asahi-yama	22 Feb, 2015	LC040969	LC040986		This study
	3	Adult ♀	Chichijima	Ogasawara	Mt. Asahi-yama	22 Feb, 2015	LC040970	LC040987		This study
	4	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040971	LC040988		This study
	5	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040972	LC040989		This study
	6	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040973	LC040990		This study
	7	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040974	LC040991		This study
	8	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040975	LC040992		This study
	9	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040976	LC040993		This study
	10	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040977	LC040994		This study
	11	Juv. -	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040978	LC040995		This study
	12	Juv. -	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040979	LC040996		This study
	13	Juv. -	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040980	LC040997		This study
	14	Adult ♀	Chichijima	Ogasawara	Mt. Chuou-san	25 Feb, 2015	LC040981	LC040998		This study
	15	Adult ♀	Chichijima	Ogasawara	Mt. taka-yama	26 Feb, 2015	LC040982	LC040999		This study
	16	Adult ♀	Chichijima	Ogasawara	Mt. taka-yama	26 Feb, 2015	LC040983	LC041000		This study
	17	Adult ♀	Chichijima	Ogasawara	Mt. taka-yama	26 Feb, 2015	LC040984	LC041001		This study
Out group										
<i>Pseudobiantes japonicus</i>	112	-	-	-	-	-	AB937947*	LC041002		This study
<i>Proscotolemon sauteri</i>	Prs1	-	-	-	-	-	AB937961*	LC041003		This study
<i>Kitungius insulanus</i>	474 Kii1	-	-	-	-	-	AB981641** AB937918*	LC041004 LC041005		This study This study
<i>Serracutisoma spelaicum</i>	Ses	-	-	-	-	-	KF726818	KF726706	Pinto-da-Rocha et al., 2014	
<i>Cynortellana pulchra</i>	Cyp	-	-	-	-	-	KF726835	KF726723	Pinto-da-Rocha et al., 2014	
<i>Gaibulus schubarti</i>	Gas	-	-	-	-	-	KF726836	KF726724	Pinto-da-Rocha et al., 2014	

*Kumekawa et al., 2014. **Kumekawa et al., 2015.

DNA Extraction, Amplification, and Sequencing

DNA extractions were performed using QIAGEN DNeasy kits (Qiagen, Valencia, CA), according to the manufacturer's protocol for animal tissue samples. The isolated DNA was resuspended in Tris-EDTA buffer and stored at -20°C until use.

The COI and 28S rRNA sequences from all the specimens were amplified using the previously published COI primers—LCO1490 (Folmer et al. 1994) and HCO output (Prendini et al. 2005), and 28S rRNA primers—ZX1 and ZR2 (Mallatt and Sullivan 1998). DNA amplification was followed according to the method described by Kumekawa et al. (2013): incubation at 94°C for 10 s, followed by 45 cycles of incubation at 94°C for 1.5 min, 48°C for 2 min, and 72°C for 3 min, with a final extension at 72°C for 15 min. DNA was amplified by polymerase chain reaction (PCR) in a 50- μ l reaction volume containing 50 ng total DNA, 10 mM Tris-HCl buffer (pH 8.3) with 50 mM KCl and 1.5 mM MgCl₂, 0.2 mM of each of the dNTPs, 1.25 U *Taq* DNA polymerase (TaKaRa, Tokyo, Japan), and 0.5 μ M of each primer. After amplification, the reaction mixtures were subjected to electrophoresis on 1% low-melting-temperature agarose gels for the purification of the amplified products. The purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (ABI PRISM DNA Sequencing kit, Perkin-Elmer Applied Biosystems, Tokyo, Japan) and ABI PRISM 3100-*Avant* Genetic Analyzer according to the manufacturers' instructions.

Data analysis

The ClustalW program was used to align the sequences for the construction of phylogenetic trees for *B. boninensis* and its allied species (Thompson et al. 1994). Moreover, we confirmed the insertions and deletions (indels) using MEGA6 (Tamura et al. 2013). In the neighbor-joining (NJ) method, the maximum composite likelihood model was employed with a bootstrap resampling of 1,000 replications. The maximum-likelihood (ML) method was reconstructed by MEGA 6 (Tamura et al. 2013) based on the model-test function for the best ML model analysis and the reliability of the branching patterns in ML trees was tested by bootstrapping (1,000 samples). The best models for COI and 28S rRNA were GTR + G + I and TN92 + G, respectively.

Results and Discussion

We determined the COI and 28S rRNA sequences to construct the molecular phylogenetic tree of *B. boninensis* and its allied species. The lengths of COI and 28S rRNA were 525 bp and 931 bp, respectively (without indels). The phylogenetic relationship indicated that the individuals of *B. boninensis* consisted of a monophyletic group with a high bootstrap value (COI: Fig. 3; 28S rRNA: Fig. 4). Furthermore, all individuals of the *B. boninensis* species had identical sequences of COI of the mtDNA and 28S rRNA of the nrDNA although they were collected from various populations in Chichi-jima Island. COI had high substitution rates and was well studied for intraspecific phylogeny (Hebert et al. 2003; Wishart and Hughes 2003; Dohrmann et al. 2008); however, no autapomorphic characters were observed among the individuals of *B. boninensis*. Therefore, it is likely that *B. boninensis* had undergone a recent, rapid expansion in its distribution, because of the lack of nucleotide mutation accumulations in this gene. In addition, the phylogenetic tree of COI indicated that *B. boninensis* had single colonization history into Chichi-jima Island; however, our results could not infer where this species had originated from, because our phylogenetic analysis did not include the closely related species of *B. boninensis*.

Recombination, which occurs when two molecules of DNA exchange pieces of their genetic material with each other in the nrDNA, is a very important source of genetic variation between individuals of sexually reproducing species and is the driving force for the process of natural selection. In contrast, asexual reproduction methods, including parthenogenesis, exhibit reduced genetic diversity due to the lack of recombination (Ironsides 2013). In addition to the results of COI, the 28S rRNA phylogenetic tree indicated that there was no genetic

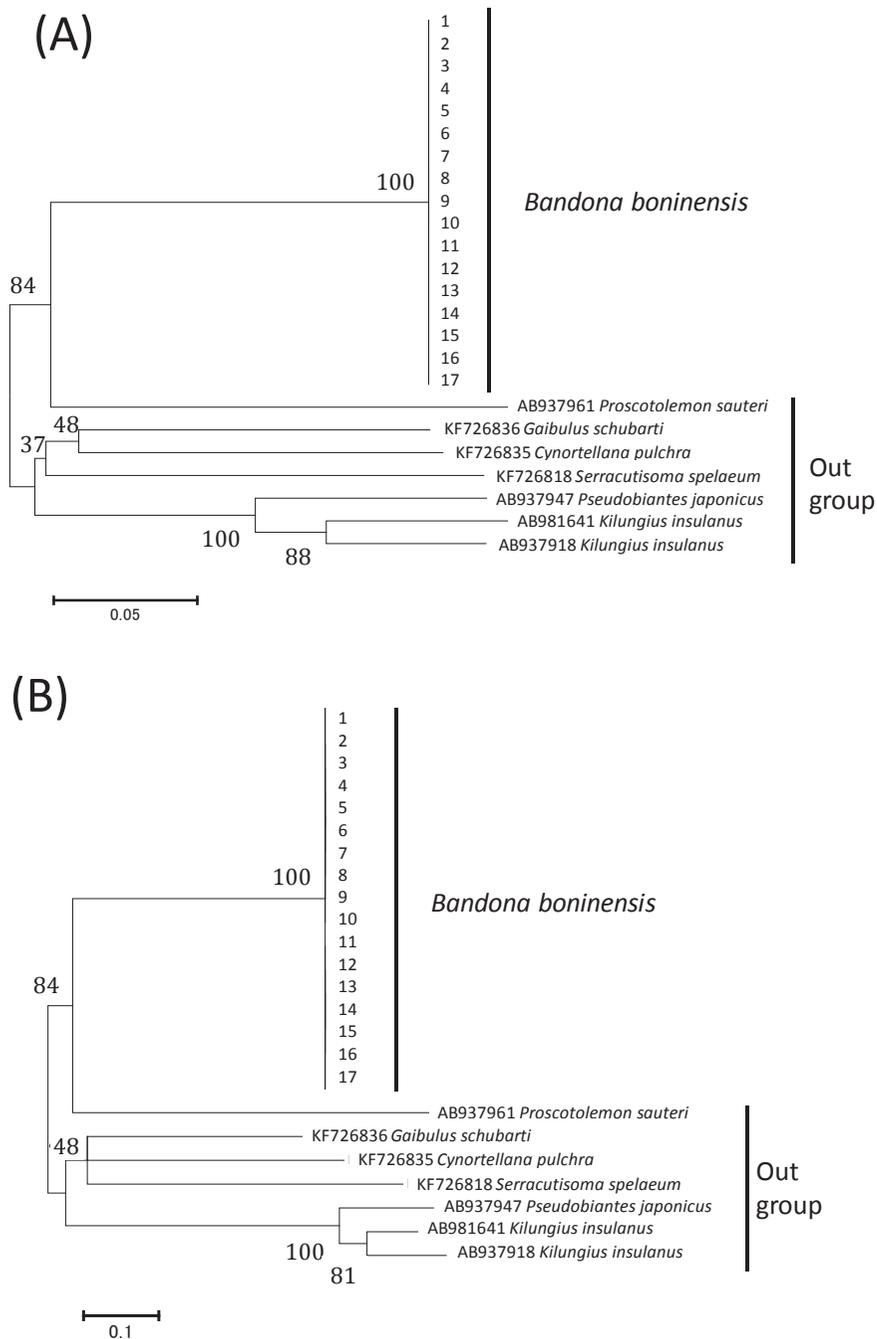


Fig. 3 Phylogenetic tree of COI (mtDNA) of *Bandona boninensis* and close related species using the neighbor-joining (NJ) (A) and maxim-likelihood (ML) methods (B). The numbers below the branches indicate the bootstrap value. For abbreviations, see Table 1.

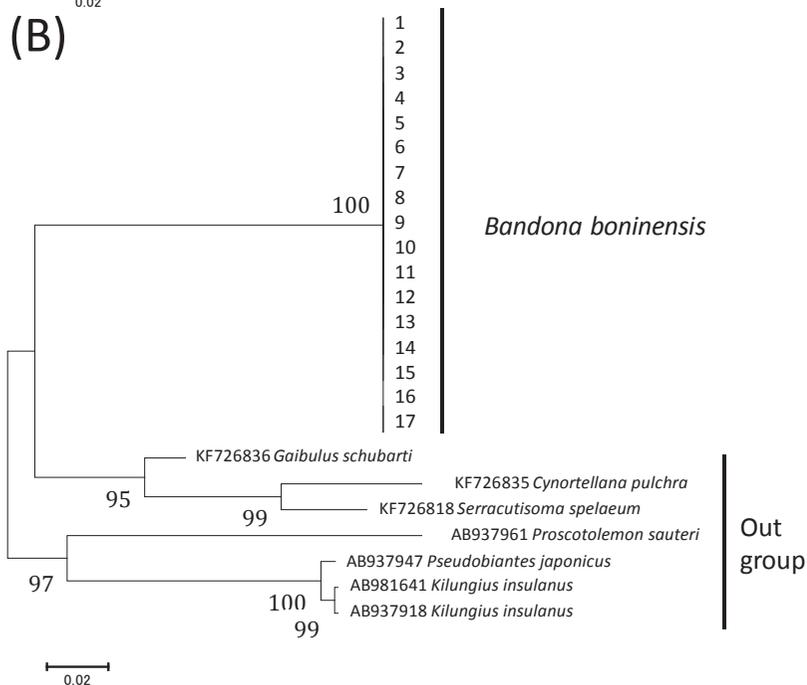
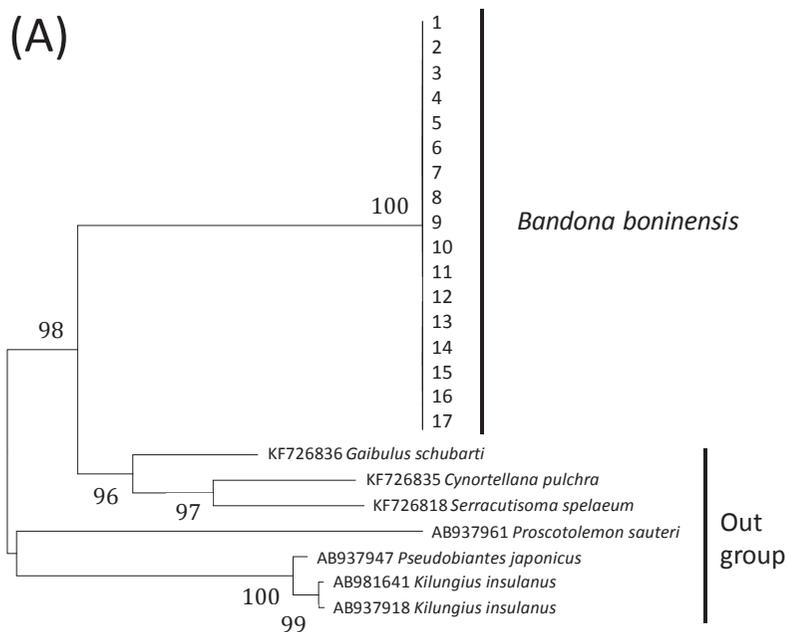


Fig. 4 Phylogenetic tree of 28S rRNA (nrDNA) of *Bandona boninensis* and close related species using the neighbor-joining (NJ) (A) and maxim-likelihood (ML) methods (B). The numbers below the branches indicate the bootstrap value. For abbreviations, see Table1.

variation, and we could not detect the heterozygosity of this gene. Our data supports Suzuki's hypothesis which suggests that *B. boninensis* is parthenogenetic in nature (Suzuki 1974; 1978). However, our result is insufficiently detailed to facilitate the reconstruction of *B. boninensis* or to infer the parthenogenesis of this species because of the limited number of samples and molecular characteristics employed. Our molecular phylogeny analysis has provided some data for the parthenogenesis in *B. boninensis*. The two genes, COI and 28S rRNA analyzed in this study provide a robust phylogenetic hypothesis for the evolution of *B. boninensis*. This study, which is based on molecular information, is a timely contribution that allows unbiased interpretation of this species. Thus, further analyses including the closely related species will be necessary to confirm the evolutionary history of *B. boninensis*.

Adaptive radiation refers the evolution of many related species into different ecological zones within a relative short time (Futuyma 1986). In fact, some studies reported that various species experienced the rapid evolution of its morphologies in the Bonin Islands. For example, Chiba (1999) indicated that the morphologies of the land snail genus *Mandarina* (Bradybaenidae) occurred at an extremely high rate in this area. In addition, a few plant genera, *Pittosporum* (Pittosporaceae), *Crepidiastrum* (Asteraceae), *Symplocos* (Symplocaceae), and *Callicarpa* (Verbenaceae) were diversified in the Bonin Islands (Kawakubo 1986; Ito and Ono 1990; Kawakubo 1990; Soejima et al. 1994; Ito et al. 1997). However, Tsurusaki (1991) indicated that *B. boninensis* exhibited no morphological variations except for the slight size variation by studying samples collected from various environments including the stony ground, leaf litter and under the stones on the vegetated coral reef of Chichi-jima and Ani-jima Islands of the Bonin Islands. Therefore, we conducted morphological analyses using *B. boninensis* specimens (Table 2). Although our result of *B. boninensis* seems to minor morphological variations, it was unknown whether this data was supported to previous report of Tsurusaki (1991) because of collecting only from Chichi-jima. However, no genetic variation among the individuals collected from various populations in Chichi-jima Island was reflected Suzuki's hypothesis (Suzuki 1974; 1978) of the occurrence

Table 2. The measurement (average \pm standard deviation) of *Bandona boninensis*.

Measurement points	Results
Fe1L (μm)	2325.67 \pm 117.65
Fe2L (μm)	4499.03 \pm 278.06
Fe3L (μm)	3123.68 \pm 191.27
Fe4L (μm)	4681.22 \pm 286.04
BL (μm)	3229.14 \pm 444.52
CL (μm)	2378.54 \pm 158.84
CW (μm)	2028.11 \pm 116.78
PTL (μm)	588.44 \pm 29.64
Chel. L (μm)	1076.34 \pm 98.63

of parthenogenesis in *B. boninensis*. In the future, it needs to collect this species from Ani-jima in order to verify these previous reports.

Our sampling efforts were insufficient for a full revision of the previous studies for this species. Nonetheless, the present investigation provides a useful basis for future studies of *B. boninensis*. There is an urgent need for extensive survey of the harvestman, including conservation aspects in the Bonin Islands.

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Author Contributions

Y.K., K.I., J.Y., S.-I.T., R.A. and T.F. designed the study. Y.K., H.F. collected the samples, analysed the data and wrote the manuscript. O.M. analysed the data. O.M. and T.F. wrote the manuscript.

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小笠原諸島におけるムニンカケザトウムシ
の形態学および遺伝学的研究

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著者らは小笠原諸島の父島に生息するムニンカケザトウムシ *Bandona boninensis* の形態学および系統的分化を明らかにするために、父島の4地点から採集を行い、体長、触肢腿節の長さ、背甲長および背甲幅、鋏角長、第1~4脚の腿節長といった形態計測を実施し、ミトコンドリアDNAのCOI領域および核DNAの28SrRNA領域に基づく系統樹を作成した。その結果、ムニンカケザトウムシのCOIおよび28Sにおいて塩基置換は見られなかった。この結果は、ムニンカケザトウムシが父島内で系統的分化をほぼ起こしていないことを示す。また、採集を行った個体がすべて雌个体であったことから、これまでの報告の通り、父島においては単為生殖種として生息している可能性が高い。

キーワード：ザトウムシ、系統学、形態学、父島