

# 奄美大島および沖縄本島におけるシマアカザトウムシ（クモ綱:ザトウムシ目:カマアカザトウムシ科）の分子系統学的研究

誌名	Edaphologia
ISSN	03891445
著者名	糸川,義雅 伊藤,桂 三浦,収 横山,潤 手林,慎一 荒川,良 福田,達哉
発行元	日本土壤動物研究会
巻/号	96号
掲載ページ	p. 1-7
発行年月	2015年3月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター  
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council  
Secretariat



## Molecular phylogeny of *Kilungius insulanus* (Arachnida: Opiliones: Epedanidae) in Amami-Oshima Island and Okinawa Island

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Received: 18 August 2014; Accepted 2 December 2014

**Abstract** The phylogeny of *Kilungius insulanus*, which is broadly distributed in the middle part of the Ryukyu Archipelago, was constructed based on the mitochondrial cytochrome oxidase c subunit I (COI) gene to evaluate the monophyletic hypothesis of this species. We collected *K. insulanus* samples from Amami-Oshima Island and Okinawa Island in the Ryukyu Archipelago. Our phylogenies demonstrated that the monophyly of *K. insulanus* was well supported with a relatively high bootstrap value. This monophyletic relationship was further confirmed by the Shimodaira-Hasegawa test. We found that there was a large genetic divergence between the populations in Amami-Oshima Island and Okinawa Island. Furthermore, there were genetically diverged lineages in Amami-Oshima Island. These results demonstrated that the populations of *K. insulanus* were genetically subdivided within and among islands, probably due to its limited dispersal ability. Future studies analyzing the populations in the other islands in the archipelago will clarify the origin and the diversification history of *K. insulanus*.

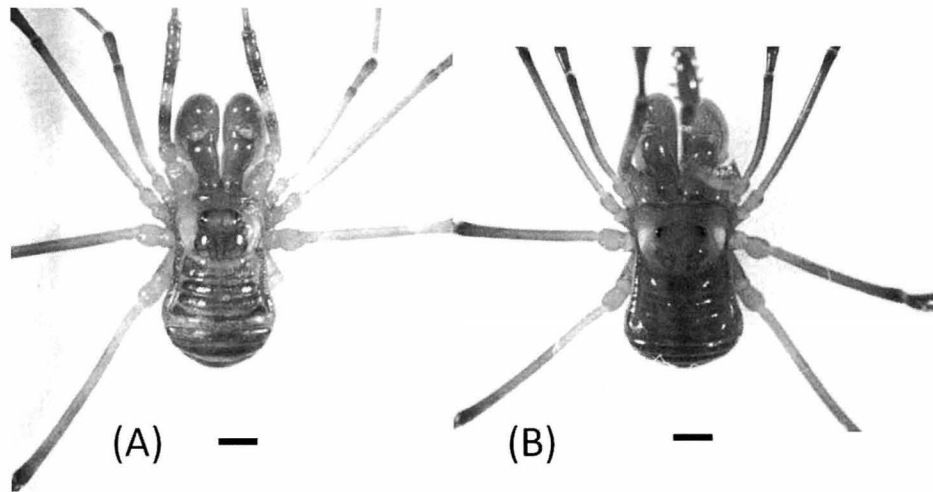
**Key words:** *Kilungius insulanus*, phylogeny, Amami-Oshima Island, Ryukyu Island

### Introduction

From evolutionary perspectives, a species is an assemblage of individuals descended from a common ancestor and thus should form a monophyletic group (Cracraft, 1983, 1989). However, recent advances in molecular biology and their application revealed that species level non-monophyly is prevalent in various groups of organisms (reviewed in Funk and Omland, 2003). It is therefore crucial to test the species-level monophyly to accurately estimate the evolutionary history and taxonomic position of study species.

*Kilungius insulanus* (Hirst 1911) (Arachnida: Opiliones: Laniatores: Epedanidae) is a harvestman that has a body length of approximately 4.0 mm (Fig. 1) and lives in humid places such as crevices beneath stones and fallen twigs, or in evergreen forest floor litter (Suzuki, 1973). This species is distributed from Nakano-shima Island to Okinawa Island in the Ryukyu Archipelago (Suzuki, 1973). Interestingly,

the recent molecular phylogenetic study of *Pseudobiantes japonicus* Hirst, 1911 revealed that *K. insulanus* from Okinawa Island is closely related to one of the lineages of *P. japonicas*, though morphologically these species belong to the different genus according to the conventional Roewerian system of laniatorid classification (Kumekawa *et al.*, 2014). This incongruence between molecular and morphological data suggests that the taxonomical re-examination is required in these species. However, before the taxonomic revisions, it is necessary to confirm the monophyly of *K. insulanus* since above phylogenetic study used only individuals of *K. insulanus* from Okinawa Island (Kumekawa *et al.*, 2014), despite its wide distribution range in the Ryukyu archipelago. Several studies demonstrated that the populations of terrestrial animals in Amami-Oshima Island and Okinawa Island in the Ryukyu Archipelago are genetically diverged (Tominaga *et al.*, 2010; Kuramoto *et al.*, 2011; Nakamura *et al.*, 2012), suggesting that the populations of *K. insulanus*



**Fig. 1.** Males of *Kilungius insulanus* from Mt. Nishime-dake Okinawa Island (A) and Kasari-cho Amami-Oshima Island (B). Bar = 1 mm.

in these islands can have a large genetic divergence. Thus, it is possible that the diverged lineages of *K. insulanus* form a paraphyletic group in relation to the other close relatives such as *P. japonicus* and *Epedanellus tuberculatus* Roewer, 1911. To evaluate this possibility, we analysed the mtDNA variation of *K. insulanus* collected from Amami-Oshima and Okinawa islands and constructed the molecular phylogeny with its related species.

## Materials and Methods

### Sample collection, DNA Extraction, Amplification, and Sequencing

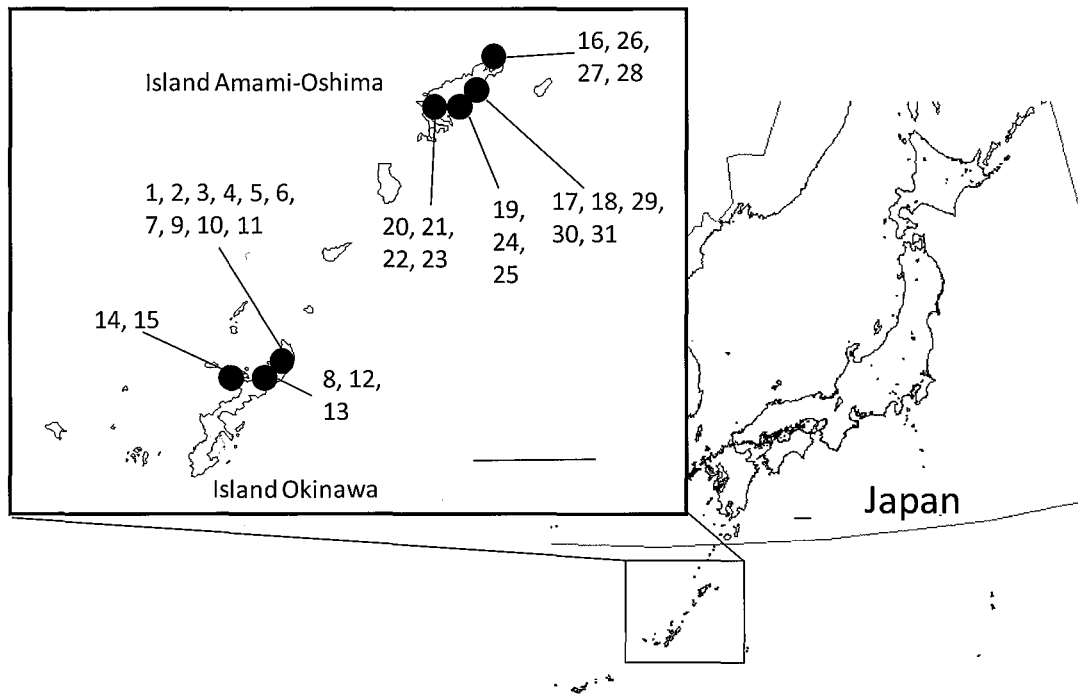
Figure 2 and Table 1 show the locations from which *K. insulanus* were collected in this study. All DNA extractions were performed using QIAGEN DNeasy kits (Qiagen, Valencia, CA), according to the manufacturer's protocol for animal tissue samples. The isolated DNA was resuspended in Tris-EDTA buffer and stored at  $-20^{\circ}\text{C}$  until use.

For all specimens, we amplified the COI gene, previously shown to be useful for revealing population-level relationships (Thomas and Hedin, 2008; Derkarabetian *et al.*, 2010), using the previously published primers of Kumekawa *et al.* (2014). DNA amplification was performed according to the method of Kumekawa *et al.* (2013). Briefly, samples were incubated at  $94^{\circ}\text{C}$  for 10 s followed by 45 cycles of incubation at  $94^{\circ}\text{C}$  for 1.5 min,  $48^{\circ}\text{C}$  for 2 min, and  $72^{\circ}\text{C}$  for 3 min, with a final extension at  $72^{\circ}\text{C}$  for 15 min. DNA was amplified by polymerase chain reaction (PCR) in a 50  $\mu\text{L}$  reaction volume containing 50 ng of total DNA, 10 mM Tris-HCl buffer

(pH 8.3) with 50 mM KCl and 1.5 mM  $\text{MgCl}_2$ , 0.2 mM of each dNTP, 1.25 U *Taq* DNA polymerase (TaKaRa, Tokyo, Japan), and 0.5  $\mu\text{M}$  of each forward and reverse primers. After amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gels for purification of amplified products. We sequenced the purified PCR products 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 manufacturer's instructions.

### Data Analysis

To construct phylogenetic trees for *K. insulanus* and its related species, *P. japonicus* and *E. tuberculatus*, sequences were aligned using ClustalW (Thompson *et al.*, 1994). *Bishopella laciniosa*, *Sclerobunus* sp., and *Metanonychus setulus* were selected for outgroups. We confirmed insertions or deletions using MEGA6 (Tamura *et al.*, 2013). In the neighbor-joining (NJ) method, the maximum composite likelihood model was used. Bootstrap resampling of 1,000 replications was performed in the NJ analysis. The maximum-likelihood (ML) method was reconstructed by MEGA 6 (Tamura *et al.*, 2013). The model-test function in MEGA was used to choose the best model for ML analysis. The reliability of branching patterns in ML trees was tested by bootstrapping (1,000 samples). TN93 + G + I models were used as the DNA sequence evolution models. We estimated nucleotide diversity



**Fig. 2.** Sampling locations of *Kilungius insulanus* in Okinawa Island and Amami-Oshima Island. Bar = 80 km. The numbers indicate the OTU name (Table 1).

**Table 1.** Summary of *Kilungius insulanus* analyzed in this study. OTU name correspond to our phylogenetic results (Figs. 2, 3).

OTU name	Sex		Location			Sampling date	Reference	Accession No.
			Island	City	Site			
1	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937918
2	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937919
3	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937920
4	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937964
5	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937965
6	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937966
7	♂	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937967
8	♀	Adult	Okinawa Island	Ogimi	Taminato	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937921
9	♀	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937968
10	♀	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937969
11	♀	Adult	Okinawa Island	Kunigami	Mt. Nishime-dake	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937970
12	♂	Adult	Okinawa Island	Ogimi	Taminato	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937922
13	♂	Adult	Okinawa Island	Ogimi	Taminato	11 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937971
14	♀	Adult	Okinawa Island	Nago	Mt. Nishime-dake	6 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937923
15	♀	Adult	Okinawa Island	Nago	Mt. Nishime-dake	6 Jul, 2011	Kumekawa <i>et al.</i> (2014)	AB937924
16	♂	Adult	Amami-Oshima Island	Amami	Kasari	26 Jul, 2014	This study	AB981633
17	-	Juv.	Amami-Oshima Island	Amami	Naze-hirata	26 Jul, 2014	This study	AB981634
18	♂	Adult	Amami-Oshima Island	Amami	Naze-oaza	26 Jul, 2014	This study	AB981635
19	♂	Adult	Amami-Oshima Island	Amami	Sumiyo	27 Jul, 2014	This study	AB981636
20	♂	Adult	Amami-Oshima Island	Amami	Setouchi	27 Jul, 2014	This study	AB981637
21	♀	Adult	Amami-Oshima Island	Amami	Setouchi	27 Jul, 2014	This study	AB981638
22	♀	Adult	Amami-Oshima Island	Amami	Setouchi	27 Jul, 2014	This study	AB981639
23	-	Juv.	Amami-Oshima Island	Amami	Setouchi	25 Jul, 2014	This study	AB981640
24	♀	Adult	Amami-Oshima Island	Amami	Sumiyo	27 Jul, 2014	This study	AB981641
25	♂	Adult	Amami-Oshima Island	Amami	Sumiyo	27 Jul, 2014	This study	AB981642
26	♀	Adult	Amami-Oshima Island	Amami	Kasari	26 Jul, 2014	This study	AB981643
27	♀	Adult	Amami-Oshima Island	Amami	Kasari	26 Jul, 2014	This study	AB981644
28	♀	Adult	Amami-Oshima Island	Amami	Kasari	26 Jul, 2014	This study	AB981645
29	♀	Adult	Amami-Oshima Island	Amami	Naze-oaza	26 Jul, 2014	This study	AB981646
30	♀	Adult	Amami-Oshima Island	Amami	Naze-oaza	26 Jul, 2014	This study	AB981647
31	♀	Adult	Amami-Oshima Island	Amami	Naze-oaza	26 Jul, 2014	This study	AB981648



**Fig. 3.** Phylogenetic tree of *Kilungius insulanus* and outgroups using the neighbour-joining (NJ) method and Maximum-likelihood (ML) method. Numbers near nodes are the support values for the clade from the different analyses in order: NJ/ML (values < 50% not shown). Black triangles represent the clades of *P. japonicus* (For the detailed phylogeny of *P. japonicus*, see Kumekawa *et al.* (2014)). For abbreviations, see Table 1.

for the populations on Amami-Oshima Island and on Okinawa Island using DNASP v. 5 (Librado and Rozas, 2009). We tested the monophyly of *K. insulanus* using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) using PAUP (Swofford, 1999). We constrained the individuals of *K. insulanus* to be monophyletic and tested it against the unconstrained (best) tree.

## Results and Discussions

We constructed phylogenetic trees based on 727 base pairs of the COI gene to investigate genetic relationships using 31 samples of *K. insulanus* (16 samples from 4 populations on Amami-Oshima Island and 15 samples from 3 sites on Okinawa Island). Branching patterns between the NJ and ML trees were mainly congruent (Fig. 3). The monophyly of *K. insulanus* was well supported with relatively high bootstrap values (78–83 %) in these phylogenies. Further, the SH test

favoured the monophyletic hypothesis of *K. insulanus* ( $P = 1.00$ ).

There was deep phylogenetic divergence between the populations in Amami-Oshima Island and Okinawa Island, demonstrating that the ocean is a major barrier for the dispersal of *K. insulanus*. This species is distributed in at least six islands (Is. Nakano-shima, Is. Takara-jima, Is. Kikai-jima, Is. Amami-Oshima, Is. Yoron-jima, Is. Okinawa) in the Ryukyu Archipelago (Suzuki, 1973) and our findings suggest that there can be phylogenetic divergence among the populations of *K. insulanus* in each island. Although we currently do not have the samples from the other four islands, these samples will enable us to investigate the origin and the history of population expansion of *K. insulanus* in the Ryukyu Archipelago.

Suzuki (1973) reported that *K. insulanus* in Amami-Oshima Island are diverse morphologically. For example, there are variations in the length of spine on the second scutal area, including the complete loss of the spine on this part. We found that the genetic diversity of the population in Amami-Oshima Island ( $P_i = 0.070$ ) was more than twice as high as that in Okinawa Island ( $P_i = 0.034$ ). Considering the genetic and morphological variations are often correlated, the morphological variations in Amami-Oshima Island may be partly explained by the high level of genetic diversity observed in this island.

Importantly, our phylogenies exhibited that *K. insulanus* is ingroup taxa of *Pseudobiantes japonicus* which is broadly distributed in the Japanese mainland (Fig. 3). This result suggests that *K. insulanus* can be a subspecies of *P. japonicus*, or *P. japonicus* is actually a species complex composed of multiple cryptic species. Further phylogenetic studies with additional genes together with detailed morphological inspections are needed to evaluate the taxonomic status of *K. insulanus*.

The recent wealth of data from phylogenetic studies of harvestmen has provided material for new studies on the underlying direction of morphological evolution as well as the diversification histories of this species (Giribet *et al.*, 1999; Thomas and Hedin, 2008; Derkarabetian *et al.*, 2010; Giribet *et al.*, 2010; Hedin and Thomas, 2010; Sharma and Giribet, 2011; Burns *et al.*, 2012; Hedin *et al.*, 2012; Sharma *et al.*, 2012; Kumekawa *et al.*, 2014). However, major gaps in our understanding remain. For example, little is known about the phylogenetic position of the other confamilial species, such as *K. bimaculatus* and *Zepedanulus ishikawai* which can be

closely related to *K. insulanus* according to the morphological taxonomy (Suzuki, 1973). Data from wider geographical and taxonomic samplings and/or additional sequencing regions would help to fill the gaps, including the taxonomic status and the diversification history of species in the family Epedanidae.

### Acknowledgments,

We thank Drs N. Tsurusaki, Y. Minamiya and H. Hayakawa for discussion in this study, and C. Uemoto, Y. Ozaki, K. Yoshioka, Y. Kamakura, Y. Mori, Y. Nibuno, T. Miyashita, F. Maekawa, and H. Fujimoto for their field and laboratory assistance. This study was partly supported by a Student Research Project Creation Support Program from the United Graduate School of Agricultural Science, Ehime University (to R. A.) and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (25440213 to T. F. and 23370039 to J. Y.).

### Author Contributions

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

### 摘要

糸川義雅<sup>1,2</sup>・伊藤桂<sup>2</sup>・三浦収<sup>3</sup>・横山潤<sup>4</sup>・手林慎一<sup>2</sup>・荒川良<sup>2</sup>・福田達哉<sup>2</sup> (1 〒783-8502 高知県南国市物部乙200 愛媛大学大学院連合農学研究科・2 〒783-8502 高知県南国市物部乙200 高知大学農学部・3 〒783-8502 高知県南国市物部乙200 高知大学総合研究センター・4 〒990-8560 山形県山形市小白川町1-4-12 山形大学理学部)：奄美大島および沖縄本島におけるシマアカザトウムシ (クモ綱：ザトウムシ目：カマアカザトウムシ科) の分子系統学的研究。

著者らは、琉球列島に広く分布しているシマアカザトウムシ *Kilungius insulanus* の単系統性を検証するために、琉球列島における奄美大島と沖縄本島からシマアカザトウムシを採集し、ミトコンドリア DNA の COI 領域に基づく系統樹を作成した。その結果、シマアカザトウムシの単系統性は高いブートストラップ値で支持された。さらに、下平・長谷川検定を用いてこの単系統性が統計学的に支持されることを確認した。また、本研究により、奄美大島と沖縄本島のシマアカザトウムシが島間で顕著に遺伝的に分化していることが明らかとなった。さらに、奄美大島の島内においても遺伝的に分化した複数の系統があることを確認した。シマアカザトウムシは

分散能力が非常に低いことから、島内および島間で遺伝的に細分化されたことが考えられる。将来的に他の島の集団も含めた網羅的な遺伝的分析を進めることで、シマアカザトウムシの起源と多様化の歴史を明らかにできると考えられる。

キーワード：シマアカザトウムシ, 系統学, 奄美大島, 沖縄島

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Fig. 1 appears in color online and in grayscale in print.