Phylogeography of the Laniatorid Harvestman Pseudobiantes japonicus and Its Allied Species (Arachnida: Opiliones: Laniatores: Epedanidae)

YOSHIMASA KUMEKAWA,1 KATSURA ITO,1 NOBUO TSURUSAKI,2 HIROSHI HAYAKAWA,1 KYOHEI OHGA,1 JUN YOKOYAMA,3 SHIN-ICHI TEBAYASHI,1 RYO ARAKAWA,1 AND TATSUYA FUKUDA1,4

ABSTRACT To clarify the phylogenetic relationships of Pseudobiantes japonicus Hirst, 1911 and its allied species, Epedanellus tuberculatus Roewer, 1911 and Kilungius insulanus (Hirst, 1911) (Arachnida: Opiliones: Laniatores: Epedanidae), we conducted morphological and nucleotide sequence analyses of this complex. Sequencing of the cytochrome c oxidase subunit I gene of 179 samples recovered six clades comprised of four allopatric lineages within P. japonicus and two monophyletic groups of E. tuberculatus and K. insulanus, respectively, related as follows: ([Clades A + B of P. japonicus] [Clade C of P. japonicus]) (K. insulanus [Clade D of P. japonicus + E. tuberculatus]). This phylogenetic relationship implies that “Pseudobiantes japonicus” is paraphyletic or polyphylectic, unless mitochondrial introgression between species is postulated. Moreover, Clades C and D of P. japonicus were also separable by the combination of a few morphological characters and from Clades A and B in P. japonicus. These results suggest that a thorough taxonomic revision is needed for the classification of P. japonicus and allied species.

KEY WORDS Epedanellus tuberculatus, Kilungius insulanus, phylogenetic relationship, phylogeography, Pseudobiantes japonicus

Harvestmen (Class Arachnida: Order Opiliones) are a diverse group of arachnids with >6,000 living species and are commonly found in many terrestrial habitats of every continent except Antarctica (Machado et al. 2007, Kury 2008). Because of low vagility, they often show high geographic differentiation both in external morphology and chromosomes even in a single species (Tsurusaki 2007). Furthermore, because of their general lack of resistance to desiccation, most harvestmen tend to be limited to forests with moderate moisture and several groups, such as Sabacon (Sabaconidae), Caddo (Caddidae), and Triaeononychidae, show typical patterns of disjunct distribution as relicts (Suzuki et al. 1977, Schönhofer et al. 2013). Their limited occurrence and enormous geographic variability have hampered phylogenetic analyses based on morphological characters because of the paucity of reliable external characters.

Recent molecular phylogenetic studies have revealed new aspects for our understanding of the relationships in various groups of harvestmen (Giribet et al. 1999; Thomas and Hedin 2008; Sharma and Giribet 2009, 2011; Derkarabetian et al. 2010, 2011; Giribet et al. 2010; Hedin and Thomas 2010; Schönhofer and Martens 2010; Burns et al. 2012; Hedin et al. 2012; Sharma 2010; Sharma et al. 2012; Schönhofer et al. 2013). In this study, as a first trial of the application of molecular phylogenetics for Japanese laniatorids, we focused on the phylogenetic relationships of various populations of the laniatorid Pseudobiantes japonicus Hirst, 1911 (Epedanidae) and its allied species of the same family.

P. japonicus is a common species of harvestman that occurs widely in the western part of the main islands of Japan (Fig. 1). The species is 3.5–4.0 mm in body length (BL) and lives in humid places such as crevices beneath stones and fallen twigs, or in forest floor litter. This species shows geographic variation in some external characters such as armaments on the second scutal area (Suzuki 1973a,b). The species also shows male dimorphism in cheliceral size, and the ratio of males with large chelicerae to males with small chelicerae varies geographically (Tsurusaki and Fujikawa 2004).

The distributional range of P. japonica mostly overlaps with that of Epedanellus tuberculatus Roewer, 1911 (Fig. 1). E. tuberculatus is ecologically and morphologically very similar to P. japonica, though E. tuberculatus, whose adult body size reaches ≈4.5–7.0 mm, is clearly larger than P. japonicus (Fig. 1). These two species have two geo-
graphical counterparts that are presumed to be allopatric sister species: *Kilungius insulanus* (Hirst 1911) in the middle part of the Ryukyu Islands (Island Amami-Ōshima, Island Yoron, Island Okinawa, etc.) and *Kilungius bimaculatus* Roewer, 1915 in the Sakishima Islands (the southernmost part of the Ryukyu Islands, which includes Island Ishigaki, Island Iriomote, Island Yonaguni, etc.; Fig. 1).

Tsurusaki (2006) indicated that these four species constitute a closely related monophyletic group based on their general morphological similarity (Suzuki 1973a,b; Suzuki and Tsurusaki 1999) and their distributional pattern, though they have so far been treated under three different genera in the existing system of classification (e.g., Roewer 1923).

To resolve phylogenetic relationships of *P. japonicus* and its allied species, we performed phylogenetic analysis of the group (excluding *K. bimaculatus*, as samples were unavailable) using the cytochrome *c* oxidase subunit I (COI) gene.

### Materials and Methods

**Sampling of *P. japonicus* and Allied Species.** To obtain a comprehensive phylogenetic sample of *P. japonicus* and its allied species, 146 *P. japonicus*, 7 *E. tuberculatus*, and 15 *K. insulanus* were sampled from across the ranges of these species (Fig. 1; Table 1). We failed to obtain samples suitable for DNA extraction for *K. bimaculatus*. As outgroup taxa, three *Proscotolemon sauteri* Roewer, 1916 (formerly Phalangodidae but now incertae sedis) and two *Metanippononychus daisenensis* Suzuki, 1974 (Triaenonychidae) were collected from the wild, and sequence data for *Bishopella laciniosa* (Crosby & Bishop, 1924) (Phalangodidae) (EU162812: Thomas and Hedin 2008), *Metanonychus setulus* (Briggs, 1971) (Triaenonychidae) (HM056732: Derkarabedian et al. 2011), *Sclerobunus* sp. (Triaenonychidae) (HM056742: Derkarabedian et al. 2011), and *Fumontana deprehendor* Shear, 1977 (Triaenonychidae) (EU162773: Thomas and Hedin 2008) were obtained from the DNA Data Bank of Japan. All
## Table 1. Summary of *P. japonicus* and its outgroup taxa analyzed in this study

| Species          | OTU name | Prefecture | City       | Town      | Clade | Sampling date | Reference | Accession no. |
|------------------|----------|------------|------------|-----------|-------|---------------|-----------|---------------|
| *P. japonicus*   | 1        | Chubu      | Shizuoka   | Sakuma    | C     | 18 Aug. 2010  | This study | AB937908      |
|                  | 2        | Chubu      | Shizuoka   | Toei      | C     | 18 Aug. 2010  | This study | AB937905      |
|                  | 3        | Chubu      | Shizuoka   | Haruno    | C     | 23 Oct. 2010  | This study | AB937907      |
|                  | 4        | Chubu      | Aichi      | Sanage-yama | C   | 23 Oct. 2010  | This study | AB937823      |
|                  | 5        | Chubu      | Aichi      | Tivohashi | C     | 20 May 2005   | This study | AB937896      |
|                  | 6        | Chubu      | Aichi      | Minamichita | C   | 23 Oct. 2010  | This study | AB937846      |
|                  | 7        | Kinki      | Mie        | Yoichi    | C     | 16 July 2010  | This study | AB937847      |
|                  | 8        | Kinki      | Mie        | Ujimazake | C     | 17 Aug. 2010  | This study | AB937848      |
|                  | 9        | Kinki      | Mie        | Ouchiya   | C     | 4 Sept. 2010  | This study | AB937849      |
|                  | 10       | Kinki      | Mie        | Funada    | C     | 25 Aug. 2010  | This study | AB937860      |
|                  | 11       | Kinki      | Nara       | Ogawa     | C     | 28 Aug. 2010  | This study | AB937890      |
|                  | 12       | Kinki      | Wakayama   | Katsuragi | C     | 17 Aug. 2010  | This study | AB937845      |
|                  | 13       | Kinki      | Wakayama   | Ichinono  | C     | 25 Aug. 2010  | This study | AB937851      |
|                  | 14       | Kinki      | Wakayama   | Terayama  | C     | 18 Aug. 2010  | This study | AB937854      |
|                  | 15       | Kinki      | Wakayama   | Oshima    | C     | 26 Aug. 2010  | This study | AB937833      |
|                  | 16       | Kinki      | Wakayama   | Shionomaki| C     | 31 Aug. 2010  | This study | AB937852      |
|                  | 17       | Chubu      | Fukui      | Oi        | B     | 25 Aug. 2010  | This study | AB937838      |
|                  | 18       | Chubu      | Fukui      | Hikasa    | B     | 18 Aug. 2010  | This study | AB937837      |
|                  | 19       | Chubu      | Fukui      | Katsura   | B     | 23 Oct. 2010  | This study | AB937840      |
|                  | 20       | Kinki      | Shiga      | Inazuma   | B     | 18 Aug. 2010  | This study | AB937839      |
|                  | 21       | Kinki      | Shiga      | Otsu      | B     | 30 May 2010   | This study | AB937840      |
|                  | 22       | Kinki      | Kyoto      | Fukuchiyama | B  | 3 Sept. 2010  | This study | AB937841      |
|                  | 23       | Kinki      | Kyoto      | Ochi      | B     | 24 Aug. 2010  | This study | AB937843      |
|                  | 24       | Kinki      | Kyoto      | Nantan    | B     | 26 Aug. 2010  | This study | AB937810      |
|                  | 25       | Kinki      | Kyoto      | Hirogawa  | B     | 26 Aug. 2010  | This study | AB937835      |
|                  | 26       | Chubu      | Hyogo      | Kani      | B     | 17 Aug. 2010  | This study | AB937859      |
|                  | 27       | Kinki      | Hyogo      | Kani      | B     | 31 Aug. 2010  | This study | AB937558      |
|                  | 28       | Kinki      | Hyogo      | Sano      | B     | 3 Sept. 2010  | This study | AB937836      |
|                  | 29       | Kinki      | Hyogo      | Yamakita  | B     | 26 Aug. 2010  | This study | AB937861      |
|                  | 30       | Kinki      | Hyogo      | Sakamoto  | B     | 17 Aug. 2010  | This study | AB937866      |
|                  | 31       | Kinki      | Hyogo      | Kobe      | B     | 22 Oct. 2010  | This study | AB937660      |
|                  | 32       | Kinki      | Hyogo      | Minamiwakai | B   | 25 Aug. 2010  | This study | AB937668      |
|                  | 33       | Kinki      | Hyogo      | Minamiwakai | B  | 30 Aug. 2010  | This study | AB937618      |
|                  | 34       | Chugoku    | Tottori    | Iwami     | B     | 30 Aug. 2010  | This study | AB937600      |
|                  | 35       | Chugoku    | Tottori    | Chizu     | B     | 5 July 2010   | This study | AB937877      |
|                  | 36       | Chugoku    | Tottori    | Nichinan  | B     | 4 July 2010   | This study | AB937907      |
|                  | 37       | Chugoku    | Okawama    | Nagi      | B     | 30 Aug. 2010  | This study | AB937861      |
|                  | 38       | Chugoku    | Okawama    | Koen      | B     | 22 Sep. 2010  | This study | AB937864      |
|                  | 39       | Chugoku    | Okawama    | Takano    | B     | 30 Aug. 2010  | This study | AB937862      |
|                  | 40       | Chugoku    | Hiroshima  | Miyoshi   | B     | 1 June 2005   | This study | AB937806      |
|                  | 41       | Chugoku    | Hiroshima  | Akita     | B     | 22 Sept. 2010 | This study | AB937579      |
|                  | 42       | Chugoku    | Hiroshima  | Hotsukaichi | B  | 30 Aug. 2010  | This study | AB937824      |
|                  | 43       | Shikoku    | Ehime      | Imabari   | B     | 5 June 2010   | This study | AB937821      |
|                  | 44       | Shikoku    | Ehime      | Tanbara   | B     | 5 June 2010   | This study | AB937820      |
|                  | 45       | Shikoku    | Ehime      | Fujinoshigoto | B  | 4 Nov. 2010   | This study | AB937903      |
|                  | 46       | Shikoku    | Ehime      | Toon      | B     | 25 May 2010   | This study | AB937895      |
| Species | OTU number | Prefecture | City | Town | Clade | Location | Sampling date | Reference | Accession no. |
|---------|------------|------------|------|------|-------|----------|---------------|-----------|--------------|
| *P. japonicus* | 47 | Shikoku | Ehime | Iyo | B | Saredani | 1 Oct. 2010 | This study | AB937597 |
| | 48 | Shikoku | Ehime | Iyo | B | Inuyose-toge | 15 July 2010 | This study | AB937599 |
| | 49 | Shikoku | Ehime | Matsuyama | B | Shukuno | 1 Oct. 2010 | This study | AB937558 |
| | 50 | Shikoku | Ehime | Matsuyama | B | Shukuno | 1 Oct. 2010 | This study | AB937558 |
| | 51 | Shikoku | Ehime | Kochi | A | Nishinomiya | 15 July 2010 | This study | AB937591 |
| | 52 | Shikoku | Ehime | Kochi | A | Cokoya | 15 July 2010 | This study | AB937591 |
| | 53 | Shikoku | Ehime | Kochi | A | Shiroshigamachi | 15 July 2010 | This study | AB937591 |
| | 54 | Shikoku | Ehime | Kochi | A | Shiroshigamachi | 15 July 2010 | This study | AB937591 |
| | 55 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 56 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 57 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 58 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 59 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 60 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 61 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 62 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 63 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 64 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 65 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 66 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 67 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 68 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 69 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 70 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 71 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 72 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 73 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 74 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 75 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 76 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 77 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 78 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 79 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 80 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 81 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 82 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 83 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 84 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 85 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 86 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 87 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 88 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 89 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 90 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 91 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 92 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| | 93 | Shikoku | Kochi | Shikoku | B | Usuki | 1 Oct. 2010 | This study | AB937592 |
| Species name | OTU name | Location | Town | Clade | Sampling date | Reference | Accession no. |
|--------------|----------|----------|------|-------|---------------|-----------|--------------|
| Shikoku Kochi | Kitagawa | Agura | A | 7 Aug. 2010 | This study | AB937914 |
| Shikoku Kochi | Kitagawa | Himabe | A | 13 June 2005 | This study | AB937915 |
| Shikoku Kochi | Kitagawa | Nogawa | A | 7 July 2010 | This study | AB937830 |
| Shikoku Kochi | Muroto | Hane | A | 17 Sept. 2010 | This study | AB937871 |
| Shikoku Kochi | Muroto | Hane | A | 17 Sept. 2010 | This study | AB937873 |
| Shikoku Kochi | Muroto | Hane | A | 17 Sept. 2010 | This study | AB937574 |
| Shikoku Kochi | Muroto | Hane | A | 7 July 2010 | This study | AB937575 |
| Shikoku Kochi | Muroto | Hane | A | 7 July 2010 | This study | AB937575 |
| Shikoku Kochi | Muroto | Murotomisaki | A | 10 July 2010 | This study | AB937814 |
| Shikoku Kochi | Muroto | Moto | A | 4 Nov. 2010 | This study | AB937815 |
| Shikoku Kochi | Tosashimizu | Nakanohama | A | 27 Oct. 2010 | This study | AB937811 |
| Kyushu Kumamoto | Oguni | Kurobuchi | D | 10 Sept. 2011 | This study | AB937889 |
| Kyushu Kumamoto | Oguni | Kurobuchi | D | 14 Sept. 2011 | This study | AB937890 |
| Kyushu Kumamoto | Kikuchi | Toyoma | D | 12 Sept. 2011 | This study | AB937888 |
| Kyushu Kumamoto | Nankan | Koimachi | D | 10 Sept. 2011 | This study | AB937887 |
| Kyushu Kumamoto | Nankan | Sekihigashi | D | 10 Sept. 2011 | This study | AB937886 |
| Kyushu Kumamoto | Misato | Abe | D | 10 Sept. 2011 | This study | AB937854 |
| Kyushu Kumamoto | Isuki | Ko | D | 12 Sept. 2011 | This study | AB937853 |
| Kyushu Kumamoto | Hitoyoshi | Kawanaya | D | 24 Sept. 2010 | This study | AB937947 |
| Nagasaki Unzen | Chijiwa | D | 9 Sept. 2011 | This study | AB937883 |
| Miyazaki Shiba | Shinohakura | D | 24 Sept. 2010 | This study | AB937829 |
| Miyazaki Nobeoka | Akamizu | D | 20 July 2010 | This study | AB937891 |
| Miyazaki Nobeoka | Kitakata | D | 9 Sept. 2011 | This study | AB937849 |
| Miyazaki Shiba | Shinohakura | D | 26 Sept. 2010 | This study | AB937943 |
| Miyazaki Nishimera | Kaunera | D | 9 Sept. 2011 | This study | AB937940 |
| Miyazaki Kohayashi | Sukinokohara | D | 23 Sept. 2010 | This study | AB937925 |
| Miyazaki Takaharu | Nishihimoto | D | 23 Sept. 2010 | This study | AB937958 |
| Miyazaki Takaharu | Kamamuta | D | 23 Sept. 2010 | This study | AB937956 |
| Miyazaki Kohayashi | Nojiri | D | 13 Sept. 2011 | This study | AB937960 |
| Miyazaki Miyakonojo | Takajo | D | 24 Sept. 2010 | This study | AB937955 |
| Miyazaki Nichinan | Kitagou | D | 12 Sept. 2011 | This study | AB937957 |
| Miyazaki Nichinan | Nango | D | 12 Sept. 2011 | This study | AB937930 |
| Miyazaki Kushima | Honjo | D | 23 Sept. 2010 | This study | AB937946 |
| Miyazaki Kushima | Toi | D | 24 Sept. 2010 | This study | AB937951 |
| Kagoshima Yusu | Yusui | D | 13 Sept. 2011 | This study | AB937950 |
| Kagoshima Ezumi | Takenoto | D | 14 Sept. 2011 | This study | AB937933 |
| Kagoshima Kirishima | Yokogawa | D | 11 Sept. 2011 | This study | AB937072 |
| Kagoshima Aira | Kajiki | D | 10 Sept. 2011 | This study | AB937858 |
| Kagoshima Kagoshima | Koriyama | D | 10 Sept. 2011 | This study | AB937832 |
| Kagoshima Kirishima | Kokubukawahara | D | 11 Sept. 2011 | This study | AB937845 |
| Kagoshima Kirishima | Fukuyama | D | 11 Sept. 2011 | This study | AB937859 |
| Kagoshima Hiioki | Hiyoshi | D | 24 Sept. 2010 | This study | AB937942 |
| Kagoshima Kagoshima | Kamifukuamu | D | 14 Sept. 2011 | This study | AB937837 |
| Kagoshima Minamisatsuma | Kinpo | D | 22 Sept. 2010 | This study | AB937944 |
| Kagoshima Makurazaki | Nishikaga | D | 13 Sept. 2011 | This study | AB937827 |
| Kagoshima Minamikyushu | Chiranchoi | D | 13 Sept. 2011 | This study | AB937827 |
Table 1. Continued

| Species | OTU name | Location | Clade | Sampling date | Reference | Accession no. |
|---------|----------|----------|-------|---------------|-----------|---------------|
|         |          | Distinct | Prefecture | City | Town |            |
| 140 Kyushu | 140 Kyushu | Kagoshima | Minamikyushu | Kawanabe | D | 14 Sept. 2011 | This study | AB937936 |
| 141 Kyushu | 141 Kyushu | Kagoshima | Kinotsuki | Kitakata | D | 11 Sept. 2011 | This study | AB937939 |
| 142 Kyushu | 142 Kyushu | Kagoshima | Kinoko | Baha | D | 24 Sept. 2010 | This study | AB937934 |
| 143 Kyushu | 143 Kyushu | Kagoshima | Kanoya | Oara | D | 10 Sept. 2011 | This study | AB937941 |
| 144 Kyushu | 144 Kyushu | Kagoshima | Kanoya | Aira | D | 10 Sept. 2011 | This study | AB937928 |
| 145 Kyushu | 145 Kyushu | Kagoshima | Kinotsuki | Kishira | D | 11 Sept. 2011 | This study | AB937933 |
| 146 Kyushu | 146 Kyushu | Kagoshima | Kinoko | Kinko | D | 11 Sept. 2011 | This study | AB937926 |
| Pr. sauteri | Prs1 Kyushu | Miyazaki | Kobayashi | Nojiri | – | 13 Sept. 2011 | This study | AB937961 |
| Prs2 Kyushu | Prs2 Kyushu | Kagoshima | Kanoya | Kihoku | – | 12 Sept. 2011 | This study | AB937962 |
| Prs3 Kyushu | Prs3 Kyushu | Kumamoto | Hitoyoshi | Kawanai | – | 14 Sept. 2011 | This study | AB937963 |
| K. insulanus | Kii1 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937918 |
| Kii2 Ryukyu | Kii2 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937919 |
| Kii3 Ryukyu | Kii3 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937920 |
| Kii4 Ryukyu | Kii4 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937964 |
| Kii5 Ryukyu | Kii5 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937965 |
| Kii6 Ryukyu | Kii6 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937966 |
| Kii7 Ryukyu | Kii7 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937967 |
| Kii8 Ryukyu | Kii8 Ryukyu | Okinawa | Ogami | Taminoto | – | 11 July 2011 | This study | AB937921 |
| Kii9 Ryukyu | Kii9 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937965 |
| Kii10 Ryukyu | Kii10 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937969 |
| Kii11 Ryukyu | Kii11 Ryukyu | Okinawa | Kunigami | Nishine-dake | – | 11 July 2011 | This study | AB937970 |
| Kii12 Ryukyu | Kii12 Ryukyu | Okinawa | Ogami | Taminoto | – | 11 July 2011 | This study | AB937922 |
| Kii13 Ryukyu | Kii13 Ryukyu | Okinawa | Ogami | Taminoto | – | 11 July 2011 | This study | AB937971 |
| Kii14 Ryukyu | Kii14 Ryukyu | Okinawa | Nago | Katsu-dake | – | 6 July 2011 | This study | AB937923 |
| Kii15 Ryukyu | Kii15 Ryukyu | Okinawa | Nago | Katsu-dake | – | 6 July 2011 | This study | AB937924 |
| E. tuberculatus | Ept1 Shikoku | Ehime | Matsuyama | Shukun | – | 23 June 2005 | This study | AB937822 |
| Ept2 Kinki | Kinki | Kyoto | Nantan | Miyama | – | 17 Aug. 2010 | This study | AB937844 |
| Ept3 Kinki | Kinki | Hyogo | Shio | Yamasaki | – | 31 Aug. 2010 | This study | AB937865 |
| Ept4 Kinki | Kinki | Hyogo | Awaji | Kurokami | – | 4 Sept. 2010 | This study | AB937570 |
| Ept5 Shikoku | Shikoku | Ehime | Seiyo | Shirakawa | – | 1 Oct. 2010 | This study | AB937902 |
| Ept6 Chubu | Chubu | Aichi | Takara | Okubo | – | 23 Oct. 2010 | This study | AB937910 |
| Ept7 Kinki | Kinki | Nara | Gose | Takama | – | 24 Aug. 2010 | This study | AB937555 |
| Paranonychidae sp | Trs1 Chugoku | Tottori | Hino | Toyosaka | – | 4 July 2010 | This study | AB937972 |
| Trs2 Chugoku | Trs2 Chugoku | Tottori | Hino | Toyosaka | – | 4 July 2010 | This study | AB937973 |
| M. setulus | Mes | – | – | – | – | – | Derkarabetian et al. (2011) | HM056732 |
| S. embusus sp | Scs | – | – | – | – | – | Derkarabetian et al. (2011) | HM056742 |
| F. deprehendor | Fud | – | – | – | – | – | Thomas and Hedin (2008) | EU162773 |
| B. lactinosa | Bil | – | – | – | – | – | Thomas and Hedin (2008) | EU162512 |

OTU name and clade correspond to our phylogenetic results (Figs. 3–8).
samples were stored at $-30^\circ\text{C}$ until DNA could be extracted.

**Morphological Analyses.** For morphological analysis, the BL and eye–spine length (ESL; Fig. 2) were measured for each individual, and the morphology of the second scutal area was recorded (Fig. 2). We measured 146 *P. japonicus*, 7 *E. tuberculatus*, and 15 *K. insulanus*. Measurements of the BL and the ESL were made using a digital caliper.

**DNA Extraction, Amplification, and Sequencing.** 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 to reveal population-level relationships (Crosby and Bishop 1924, Thomas and Hedin 2008, Derkarabetian et al. 2011), was amplified using the previously published primers: LCO1490 (5’-GGT CAA CAA ATC ATA AAG ATA ATG G-3’; Folmer et al. 1994) and C1-N-2776-spider (5’-GGA TAA TCA GAA TAN CGN CGA GG-3’; Vink et al., 2005) adding to the following eight new primers: COI-Kume (5’-WAA YTT YAC MTC TTT YTT-3’), COI-Kume2F (5’-CTY CTY ACA GAC CGM AAT TT-3’), COI-Kume3F (5’-AAT ACC TYT ATT YGT HTG ATC-3’), Kume1–1 F (5’-ATD RSY TTY CCM CGD WTA AA-3’), Kume1–2 F (5’-AKR AGM TTY TGR YTD YTR CC-3’), COI-Yoshi (5’-TGY TGR TAW AGR ATD GGR TGY-3’), Yoshi2–1R (5’-TWG AYA TRG CTR ARA TTA TMC-3’), and Yoshi2–2R (5’-CCT ACD GTR AAT ATR TGR TG-3’). DNA was amplified by 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-μl reaction volume containing ≈50 ng of total DNA, 10 mM Tris–HCl buffer (pH 8.3) with 50 mM KCl and 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 U TaqDNA polymerase (TaKaRa, Tokyo, Japan), and 0.5 μM of each primer. After amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gels and purified using QIAGEN QuickSpin kits according to the manufacturer’s specifications. 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 manufacturers’ instructions.

**Data Analysis.** To construct phylogenetic trees for *P. japonicus* and its allied species, sequences were aligned using ClustalW (Thompson et al. 1994). Moreover, we confirmed insertions or deletions (indels) using MEGA5 (Tamura et al. 2011). In the neighbor-joining (NJ) method, the model maximum composite likelihood was used. Bootstrap resampling used 1,000 replications for the NJ analysis. In the maximum-likelihood (ML) method was reconstructed by MEGA program, version 5.05 (Tamura et al. 2011). The model test function in MEGA was used to choose models for ML analyses by the Bayes information criterion. The reliability of branching patterns in ML trees was tested by bootstrapping (1,000 samples). The following DNA sequence evolution models were used TN93 + G + I.

**Results**

**Phylogenetic Analyses of *P. japonicus* and Its Allied Species.** We reconstructed phylogenetic relationships using 179 samples of *P. japonicus* and its allied species including outgroup species. The length of the *P. japonicus* COI gene was 1,057 bp, with insertions of 6 bp compared with *P. sauteri*. Gaps in the sequences were eliminated in all of the following analyses, thus 1,051 bp were used in our study. We reconstructed phylogenetic trees based on the NJ method (Figs. 3–5).
phylogenetic trees indicated that *P. japonicus* was comprised of a paraphyletic assemblage and divided into four subgroups, denoted Clades A to D (Figs. 3–5). In addition, we reconstructed another phylogenetic tree based on ML method (Figs. 6–8). Branching patterns between NJ and ML trees were mainly congruence. It is surprising that both *E. tuberculatus* and *K. insulanus* were recovered with one *P. japonicus* group and the monophyletic *K. insulanus* was sister to remove Clade D of *P. japonicus* and *E. tuberculatus* (Figs. 5 and 8). The monophyly of each subgroup of *P. japonicus* and both *K. insulanus* and *E. tuberculatus* were supported by relatively high bootstrap values (94–100%); however, basal nodes had low support values in NJ and ML trees.

Figure 9 shows the geographical distribution of the four clades in *P. japonicus*. Clade A consisted of 27 individuals distributed in the eastern half of Shikoku and an isolated population (no. 78, Nakanohama in Tosashimizu City) at the southwestern end of Shikoku. In total, 60 individuals from the western half of Shikoku, northern part of Kinki District, and Chugoku and the northern part of Kyushu Districts were included in Clade B. Clade C consisted of 16 individuals from the southern Kinki and Chubu Districts. Clade D was composed of 44 individuals from the central and southern parts of Kyushu District.

**Morphological Analyses of *P. japonicus* and Its Allied Species.** BL, ESL, and the armaments of the second scutal area were analyzed and compared with mitochondrial clades (Figs. 3–5). Means with SDs of the BLs of males and females of Clades A, B, C, and D of *P. japonicus* were 3.67 ± 0.23 (male A), 3.83 ± 0.27 (female A), 3.56 ± 0.31 (male B), 3.65 ± 0.43 (female B), 3.81 ± 0.28 (male C), 3.98 (female C), 3.73 ± 0.26 (male D), and 3.79 ± 0.34 mm (female D). For *E. tuberculatus*, these values were 5.64 ± 0.05 (male) and 5.53 ± 0.16 (female), while for *K. insulanus* they were 4.23 ± 0.46 (male) and 4.01 ± 0.42 mm (female). Males and females of *E. tuberculatus* were significantly larger than those of any of other species. However, male *K. insulanus* were significantly larger than males in any of the other clades of *P. japonicus* (Fig. 10).
Means with SDs for the ESL of Clades A, B, C, and D of *P. japonicus* were 0.46 ± 0.10 (male A), 0.49 ± 0.06 (female A), 0.53 ± 0.07 (male B), 0.50 ± 0.11 (female B), 0.90 ± 0.19 (male C), 0.90 (female C), 1.09 ± 0.21 (male D), and 1.02 ± 0.17 mm (female D; Fig. 11). Those for males and females of *E. tuberculatus* were 0.96 ± 0.07 and 1.07 ± 0.08 mm, respectively, and those for males and females of *K. insulanus* they were 1.30 ± 0.20 and 1.12 ± 0.22 mm, respectively. Of these, male individuals of *K. insulanus* had the significantly longest ESL and Clades A and B had the shortest ESL of all examined samples (Fig. 11). There were no significant differences among male individuals of Clades C and D and *K. insulanus* (*P* value >0.05). Female ESL of Clades A and B were also significantly shorter than those of the remaining samples (*P* value <0.05). No significant differences were detected between Clades A and B in the ESL of either males and females (*P* value >0.05).

Armaments on the second scutal area of *P. japonicus* and the allied species can be divided into two types: 1) second scutal area unarmed or only with rudimentary spines, and 2) second scutal area with a pair of short spines; and the results of the examination are shown in Table 2. Individuals of Clades A, B, and D had an unarmed second scutal area, except for two males of Clade A. However, Clade C was prominent in having spines on the second scutal area without exception. *E. tuberculatus* usually had two median granules on the second scutal area or lacked them (nonspined type). *K. insulanus* had a transverse row of granules on the same position (nonspined type).

**Discussion**

Phylogeographic Relationship Within *P. japonicus* and Possible Roles of the Ota and Asahi Rivers as Pleistocene Geographical Barriers. Ikeda et al. (2012) reported that the loss of flight promotes speciation in coleopteran insects. It is expected that genuinely flightless animals such as *P. japonicus* and related species would be bound to show more prominent geo-

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**Fig. 4.** Phylogenetic tree of *P. japonicus* of Clade B and its related forms with outgroups using the NJ method. The numbers above or below the branches indicate the bootstrap value (>90%). For abbreviations, see Table 1. (I) Complete tree topology. (II) Magnification of highlighted region.
Fig. 5. Phylogenetic tree of *P. japonicus* of Clade D and its related forms with outgroups using the NJ method. The numbers above or below the branches indicate the bootstrap value (>90%). For abbreviations, see Table 1. (I) Complete tree topology. (II) Magnification of highlighted region.
graphical population differentiation. In fact, our results indicated that individuals of *P. japonicus* are divided into four allopatric clades across their distribution.

The current distribution of biological diversity cannot be understood without information about how organisms responded to historical changes in geological and climatic conditions. There is no doubt that the glacial–interglacial cycles of the Quaternary period would have played an important role in shaping the distribution of biodiversity among current populations, even in warm temperate zones where the land was not covered with ice sheets (Minato and Ijiri 1976). During the Last Glacial Maximum, the mean annual temperature was ≈5–9°C cooler and the precipitation was lower than present levels (Tsukada 1984). Therefore, climatic cooling caused southward shifts and shifts to lower altitude in the geographical ranges of various species (Tsukada 1974). Palynological evidence indicates that broadleaved deciduous and evergreen forests in Japan were subjected to cold periods at least four times during the Quaternary (Tsukada 1974). Aoki et al. (2004) hypothesized that the pollen record indicates that refugial populations were sparsely distributed along the coasts of the Pacific Ocean, while refugia of broadleaved evergreen forests were limited to southern areas mainly at the southern end of Kyushu. Moreover, fossil evidence also indi-

![Fig. 6. Phylogenetic tree of *P. japonicus* of Clade B and its related forms with outgroups using the ML method. The numbers above or below the branches indicate the bootstrap value (>90%). For abbreviations, see Table 1. (I) Complete tree topology. (II) Magnification of highlighted region.](https://academic.oup.com/aesa/article-abstract/107/4/756/19102)
cates that these populations migrated northward from refugia after the Last Glacial Maximum (Tsukada 1984, Matsuoka and Miyoshi 1998). These studies suggest that populations of some forest-dependent insects and other arthropods responded to the environmental changes associated with the glacial cycles in a similar fashion to that of their associated forests.

Our studies indicated that *P. japonicus* consists of four clades occupying different geographical areas; Clade A (eastern Shikoku), Clade B (northern Kyushu, western Shikoku, Chugoku, Kinki, and the western part of Hokuriku Districts), Clade C (from southern Kinki to Tokai Districts), and Clade D (southern and middle part of Kyushu; Fig. 9). *P. japonicus* is widely distributed in western Japan from evergreen forests near sea level to Japanese beech *Fagus crenata* Blume forests, which usually grow in mountainous areas above ~800 m in altitude in western Japan. Palynological evidence shows distributional ranges of *F. crenata* moved southward and only grew in southern Kyushu, southern Shikoku, and the Kii Peninsula of the Kinki District as refugia during the last ice age 18,000–20,000 yr ago (Fujii et al. 2002). It is presumed that the Ota River that flows down from the western part of the Chugoku Mountains of Honshu ran southward through the present day Bungo Channel that separates Kyushu Island and Shikoku Island when the Seto Inland Sea regressed at that time. However, the Asahi River that originates from the Mt. Daisen–Hiruzen area in the eastern part of the Chugoku Mountains had run through the Kii Channel (Ota et al. 2004) until the Seto Inland Sea was reformed ~10,000 yr ago.

![Phylogenetic tree of *P. japonicus*](https://academic.oup.com/aesa/article-abstract/107/4/756/19102)
Thus, it is plausible that these two rivers (and the present day Bungo and Kii Channels) have acted as barriers to gene flow for land animals with very low vagility, such as *P. japonicus*.

The distributional patterns of the four clades of *P. japonicus* strongly suggest that these clades had been restricted to evergreen forests along the coasts of the Pacific Ocean in the last glacial age and were differentiated under the influence of the ancient Ota River and ancient Asahi River, and have recently expanded their distribution independently from each refugium.

The comparison of intraspecific phylogeographic patterns with other taxa over the same area and the search for congruent geographic patterns of genetic variation play important roles in indicating the influence of common historical factors (Arbogast and Kenagy 2001, Hewitt 2004). Comparing the intraspecific phylogeographic patterns among different species dis-
tributed in a single vegetation zone should be more informative because a group of species living together in the present environment likely responded in a similar or possibly the same manner to past geological or climatic events (Griffin and Barrett 2004).

In this context, it is interesting that there are many other examples that show morphological and genetic differentiation between Kyushu and Shikoku or between Shikoku and the Kii Peninsula. For example, the six-spined form of the soil-dwelling harvestman _Sys-
tenocentrus japonicus_ (Sclerosomatidae) is distributed in Shikoku and Chugoku District except for Yamaguchi Prefecture, which is in contrast to the distribution of the five-spined form that occurs in Kyushu, Yamaguchi Prefecture, Kinki District, and eastward of Honshu (Suzuki and Tsurusaki 1981). A phylogeographic study based on the mitochondrial COI gene for the metallic blue earthworm _Metaphire sieboldii_ (Horst, 1883) showed that the Group III occurring in the eastern part of Shikoku was replaced by Group I in the Kii Peninsula (Minamiya et al. 2009). In Japanese beech, _F. crenata_, the Clade III chloroplast DNA haplotype that occurs in Kyushu, the western half of Chugoku District, and Shikoku is substituted by Clade II in the Kii Peninsula (Fujii et al. 2002). Furthermore, recent phylogeographical analyses of mammals such as the Japanese monkey _Macaca fuscata_ and Japanese deer _Cervus nippon_ have also shown that there are separate mitochondrial
clades between Shikoku and the Kii Peninsula (Kawanoto 2007, Nunome et al. 2010).

The arrangement of analyzed morphological characters of *P. japonicus* on our phylogenetic tree could reveal that each clade of this species was recognized by the combination of BL, ESL, and the spine type of the second scutal area (Fig. 12). For example, individuals of Clade C have the longest ESL and the spine type of second scutal area, but Clade D lacks of them despite having the longest ESL of all *P. japonicus*. Individuals of Clades A and B have a relatively short ESL and the granule and the loss types of second scutal area, except for two male individuals with the spine type in Clade A. Unfortunately, individuals of Clade A could not be distinguished with those of Clade B by morphological characters we examined. A remaining problem is the occurrence of two clades (Clade A and Clade B in eastern and western Shikoku, respectively) of *P. japonicus* in Shikoku. Additional molecular markers may lend support to the scenario inferred here, and multiple markers should be surveyed to find further variation within this species. Such research would help to validate existing COI data sets and facilitate new interpretations.

Historical hybridization between different genotypes could be most commonly identified by the heterogeneity of nuclear DNA (nrDNA) and the incongruence between mitochondrial DNA (mtDNA) and nrDNA phylogenies that may indicate different parental contributions to the hybrid genome (e.g., Funk and Omland 2003). In this study, it allows for the discussion about hybridization, introgression, and incomplete lineage sorting between phylogenetic groups of *P. japonicus* to add the nrDNA information. Previous studies indicated that polymorphisms in 28S rRNA in nrDNA had proven to be effective tools to clarify the relationship of closely related taxa in harvestmen (Wheeler and Hayashi 1998; Giribet et al. 1999; Sharma and Giribet 2009, 2011; Derkarabetian et al. 2010; Giribet et al. 2010; Hedin and Thomas 2010; Sharma 2010; Sharma et al. 2012). Therefore, the phy-

### Table 2. The morphology of second scutal area of *P. japonicus*, *E. tuberculatus*, and *K. insulanus* (number of individuals)

| Taxa           | Cladea | Type (male) | Type (female) |
|----------------|--------|-------------|---------------|
|                |        | Spine       | Non-spine     | Spine | Nonspine |
| *P. japonicus* | A      | 2           | 6             | 0     | 10       |
|                | B      | 0           | 34            | 0     | 22       |
|                | C      | 8           | 0             | 1     | 0        |
|                | D      | 0           | 23            | 0     | 20       |
| *E. tuberculatus* | –   | 0           | 2             | 0     | 4        |
| *K. insulanus* | –      | 0           | 8             | 0     | 7        |

*a Clade names are as indicated in Figs. 3–5.*

![Fig. 12. The arrangement of morphological characters on the simplified phylogenetic tree of *P. japonicus* and its allied species (Figs. 3–5).](image-url)

Table 2. The morphology of second scutal area of *P. japonicus*, *E. tuberculatus*, and *K. insulanus* (number of individuals)
logenetic study using both mtDNA and an additional nrDNA could provide an answer not only to reticulated evolution but also to cryptic speciation between genotypes in *P. japonicus*.

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