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SHORT COMMUNICATION

The phylogenetic position of an *Armillaria* species from Amami-Oshima, a subtropical island of Japan, based on elongation factor and ITS sequences

Yuko Ota · Mee-Sook Kim · Hitoshi Neda · Ned B. Klopfenstein · Eri Hasegawa

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Abstract An undetermined *Armillaria* species was collected on Amami-Oshima, a subtropical island of Japan. The phylogenetic position of the *Armillaria* sp. was determined using sequences of the elongation factor-1α (*EF-1α*) gene and the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA). The phylogenetic analyses based on *EF-1α* and ITS sequences showed that this species differs from known Japanese taxa of *Armillaria*. The sequences of this species and *A. novae-zelandiae* from Southeast Asia were contained in a strongly supported clade, which was adjacent to a well-supported sister clade containing *A. novae-zelandiae* from Australia and New Zealand.

Keywords *Armillaria fuscipes* · *Armillaria novae-zelandiae*

Y. Ota (✉) · H. Neda
Forestry and Forest Products Research Institute,
Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan
e-mail: yuota@ffpri.affrc.go.jp

M.-S. Kim
Department of Forestry, Environment, and Systems,
Kookmin University, Seoul 136-702, Republic of Korea

N. B. Klopfenstein
USDA Forest Service, RMRS, 1221 South Main Street,
Moscow, ID 83843, USA

E. Hasegawa
Kansai Research Center,
Forestry and Forest Products Research Institute,
68 Nagai-Kyutaro, Momoyama, Fushimi,
Kyoto 612-0855, Japan

Approximately 40 *Armillaria* species (Physalacriaceae, Agaricales, Basidiomycota) are distributed worldwide (Volk and Burdsall 1995), and some of these species cause root disease to plant species (Hood et al. 1991; Dai et al. 2007). More than 600 species of woody and nonwoody plants are hosts of *Armillaria* spp. (Shaw and Kile 1991; Fox 2000). In Japan, nine annulate species [*A. cepistipes* Velen., *A. gallica* Marxm. & Romagn., *A. jezoensis* J.Y. Cha & Igarashi, *A. mellea* subsp. *nipponica* J.Y. Cha & Igarashi, *A. nabsnona* T.J. Volk & Burds., *A. ostoyae* (Romagn) Herink (reported to be a synonym of *A. solidipes* Peck by Burdsall and Volk 2008), *A. sinapina* Bérubé & Dessur., *A. singula* J.Y. Cha & Igarashi, and one undescribed species (Nag. E)] and two exannulate species [*A. ectypa* (Fr.) Lamoure and *A. tabescens* (Scop.) Emel] have been reported (Nagasawa 1991; Cha et al. 1992, 1994; Cha and Igarashi 1997; Ota et al. 1998, 2009; Kudo and Nagasawa 2003). These 11 species occur in warm-temperate to boreal areas in Japan (Ota et al. 1998, 2009). However, little information is available on *Armillaria* in subtropical Japan.

Amami-Oshima (28°17′N 129°22′E) is a subtropical island in the Ryukyu Archipelago (Nansei Islands) located approximately 300 km north of Okinawa Island and 380 km south of Kyushu. The vegetation is typical for evergreen forests of this region, dominated by *Castanopsis sieboldii* (Makino) Hatus, *Quercus miyagi* Koidz, and *Distylium racemosum* Sieb. et Maxim., along with a secondary planted forest composed of *Pinus luchuensis* Mayr. In 1998, an *Armillaria* sp. was collected on Amami-Oshima. The morphological characteristics of the basidiocarp were distinct from all previously reported species from the warm-temperate to boreal regions of Japan. Recently, sequences of elongation factor-1 alpha (*EF-1α*) gene and ribosomal DNA (rDNA regions) [internal transcribed spacer (ITS) and intergenic spacer-1 (IGS-1)] have been

used for phylogenetic studies of *Armillaria* spp. (Maphosa et al. 2006; Antonín et al. 2009). More recently, Hasegawa et al. (2010) showed that the *EF-1α* gene was the most suitable region for identifying eight species of Japanese *Armillaria*. Advantages of using *EF-1α* compared with ITS and IGS-1 regions for phylogenetic analyses of *Armillaria* are: (1) it is easier to align the sequence data because indels are less frequent than those found in the ITS and IGS regions, and (2) *EF-1α* contains sufficient sequence variation to distinguish among closely related species.

The objective of this study was to clarify the phylogenetic position of an *Armillaria* sp. collected on Amami-Oshima, a subtropical island of Japan, using sequences of *EF-1α* and ITS region. Two isolates of *Armillaria* sp. were collected from Amami-Oshima, Kagoshima Prefecture, from basidiocarp tissue and multiple spores and established in culture (Table 1). For comparisons, *A. ectypa* from Japan was also sequenced (Table 1). Two isolates of *Tricholoma matsutake* and *T. magnivelare* (Table 1) were analyzed as outgroups in the preliminary analysis. The examined isolates are deposited in the culture banks of the Forestry and Forest Products Research Institute (FFPRI) in Tsukuba, Ibaraki, Japan, and the Microbial Ecology Lab of FFPRI; Japanese voucher specimens have been deposited in the Mycological Herbarium of FFPRI (TFM).

Isolates were grown on liquid MYG medium [2% (w/v) malt extract, 0.2% (w/v) yeast extract, 2% (w/v) glucose] at 25°C in the dark, and mycelia were harvested after 10 days in culture. DNA was extracted from cultured mycelia using a DNeasy extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Primers EF595F/EF1160R (Maphosa et al. 2006) and ITS1-F/ITS4-B (Gardes and Bruns 1993) were used to amplify a portion of the *EF-1α* gene and the ITS region, respectively. Each 20-μl reaction mixture contained 10 ng of template DNA (or no DNA template for a negative control), 10 mM Tris–hydrochloride (HCl) (pH 8.3), 50 mM potassium chloride (KCl), 1.5 mM magnesium chloride ($MgCl_2$), 0.1–0.2 mM of each primer, 2–2.5 mM of each deoxyribonucleotide triphosphate (dNTP), and 0.5 U Takara *Taq* or Ex*Taq* (Takara, Tokyo, Japan). The polymerase chain reaction (PCR) condition was as follows: 94°C for 1 min, 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min. The PCR products were purified with MicroSpin Columns and Sephacryl S-300 (GE Healthcare, Piscataway, NJ, USA) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in both directions to ensure accuracy. The sequences are deposited in the DNA Data Bank of Japan (accession numbers: AB558990–AB559004 and AB36893, Table 1).

The sequence data reported by Hasegawa et al. (2010) was used for eight known Japanese species (*A. cepistipes*, *A. gallica*, *A. mellea*, *A. nabsnona*, *A. ostoyae*, *A. sinapina*, *A. tabescens*) and Nagasawa's E (Nag. E). Nag. E is a Japanese *Armillaria* species reported by Nagasawa (1991) without valid description. *Armillaria jezoensis* and *A. singula* were not used for DNA analysis because cultured isolates could not be found. Some sequences from GenBank, which showed the highest similarities with the examined isolates according to a BLAST search, were also used for the analyses. Sequence alignments were examined using Clustal X (Jeanmougin et al. 1998) followed by visual editing and alignment using BioEdit (Hall 1999). All gaps and ambiguously aligned regions were removed for analyses. The sequence alignments were deposited in TreeBase as submission no. S10511. Phylogenetic analyses were conducted using PAUP* ver. 4.0b10 (Swofford 2002) for the neighbor-joining (NJ) method, and PhyML (Guindon and Gascuel 2003) was used for the maximum likelihood (ML) method. NJ trees were generated using p, K2P, and HKY85 distances. The confidence for the internal branches of the resulting trees were tested statistically using bootstrap analysis (Felsenstein 1985), with 1,000 bootstrap replications through a FastStep search using the default settings. For ML trees, the initial tree was constructed by BioNJ; the HKY85 substitution model was used with an estimated proportion of invariant sites (0.515 for

Table 1 Isolates used in this study

Species	Isolate no.	Original no.	Location	Host	Specimen no. (TFM)	EF accession no.	ITS accession no.
Armillaria sp.	00-13	S841	Amami, Kagoshima, Japan	Decayed wood	26911	AB558990	AB558998
Armillaria sp.	00-14	S842	Amami, Kagoshima, Japan	Decayed wood		AB558991	AB558999
A. ectypa	Je-2		Aomori, Japan	*Phragmites australis*	27105	AB558992	AB559001
	Je-4		Aomori, Japan	*Phragmites australis*	27109	AB558993	AB559002
	Je-7		Aomori, Japan	*Phragmites australis*	27212	AB558994	AB559003
	Je-9		Aomori, Japan	*Phragmites australis*	27213	AB558995	AB559004
Tricholoma matsutake	Tmm4	Tm029	Shiga, Japan	Unknown		AB558996	AB559005
T. magnivelare	Tmm27	Tp-C3	Canada	Unknown		AB558997	AB036893

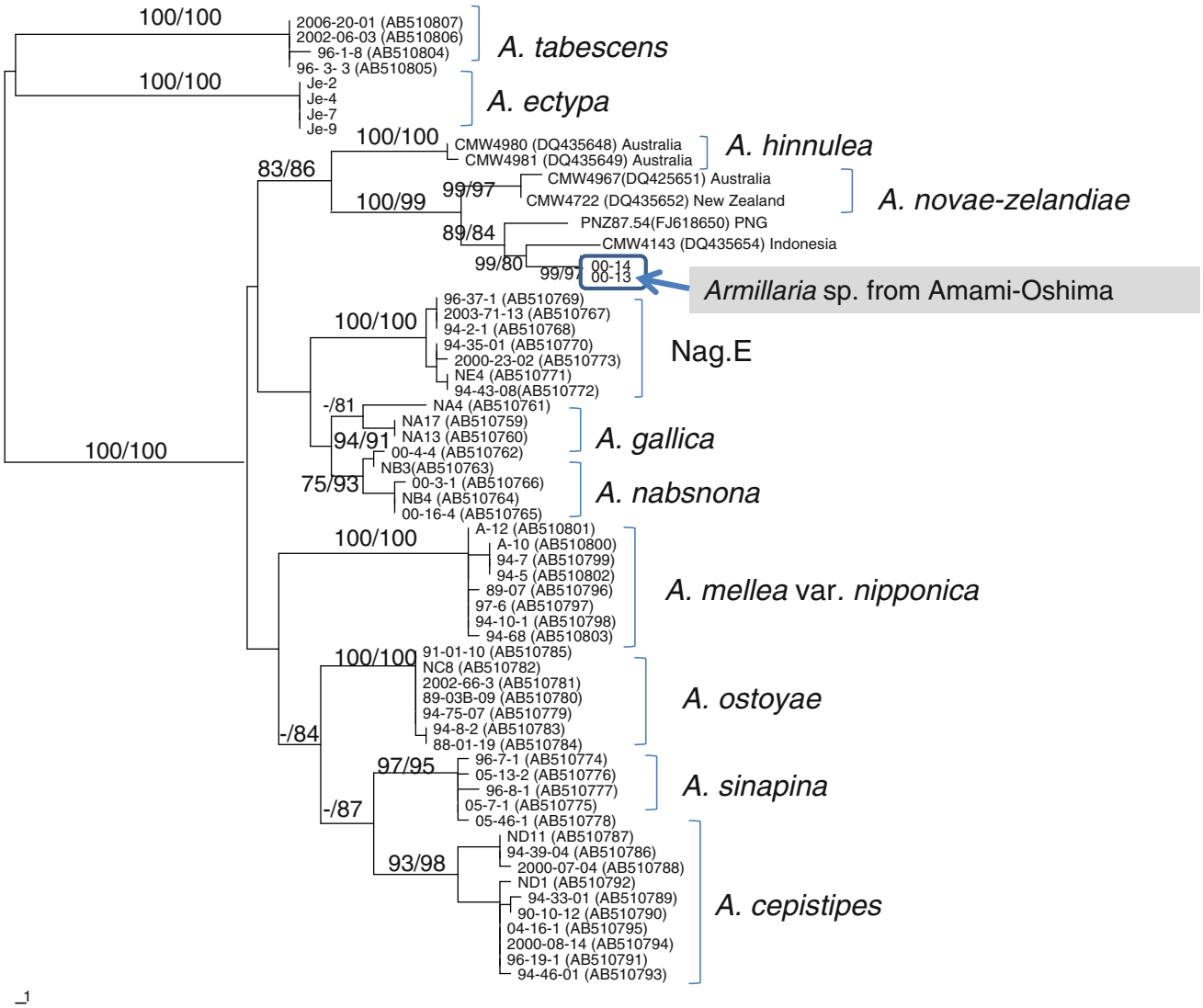

Fig. 1 Neighbor-joining tree based on elongation *factor-1α* gene sequences of known Japanese *Armillaria* species, the unknown isolates from Amami-Oshima, and other highly similar sequences from GenBank. Bootstrap values and reliability values are shown on the *nodes*

EF-1α gene and 0.446 for ITS region) and four gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 1.872 for *EF-1α* gene and 0.613 for ITS region). The reliability value of each internal branch was assessed using the aLRT test (SH-like; Anisimova and Gascuel 2006). This test is based on an approximation of the standard likelihood ratio test and is much faster to compute than the usual bootstrap procedure. Similar trees were derived from both methods; branch supports were generally highly correlated (Dereeper et al. 2008).

Amplification of the partial *EF-1α* gene yielded an approximately 600-bp product for isolates of Japanese *A. ectypa* and isolates from Amami-Oshima, which is in the expected size range (Hasegawa et al. 2010; Maphosa et al. 2006). *EF-1α* sequences from the isolates from Amami-Oshima showed the highest similarity (97%) to that of the isolate from Indonesia (isolate no. CMW4143) referred to as *A. novae-zelandiae*. The sequence similarities between the isolates from Amami-Oshima and *A. novae-zelandiae* from Australia, New Zealand, and Papua New Guinea, and *A. hinnulea* ranged from 92 to 95%. In the preliminary *EF-1α* sequence analysis, which used *Tricholoma matsutake* and *T. magnivelare* as the outgroup, *A. ectypa* and *A. tabescens* formed the basal clades for the *Armillaria* spp. (data not shown). Therefore, *A. ectypa* and *A. tabescens* were used as the outgroup for subsequent analyses. An *EF-1α* data set consisting of 61 sequences was analyzed.

Topologies of the NJ and ML trees based on the *EF-1α* sequences showed some minor differences, but both trees showed generally consistent relationships among the

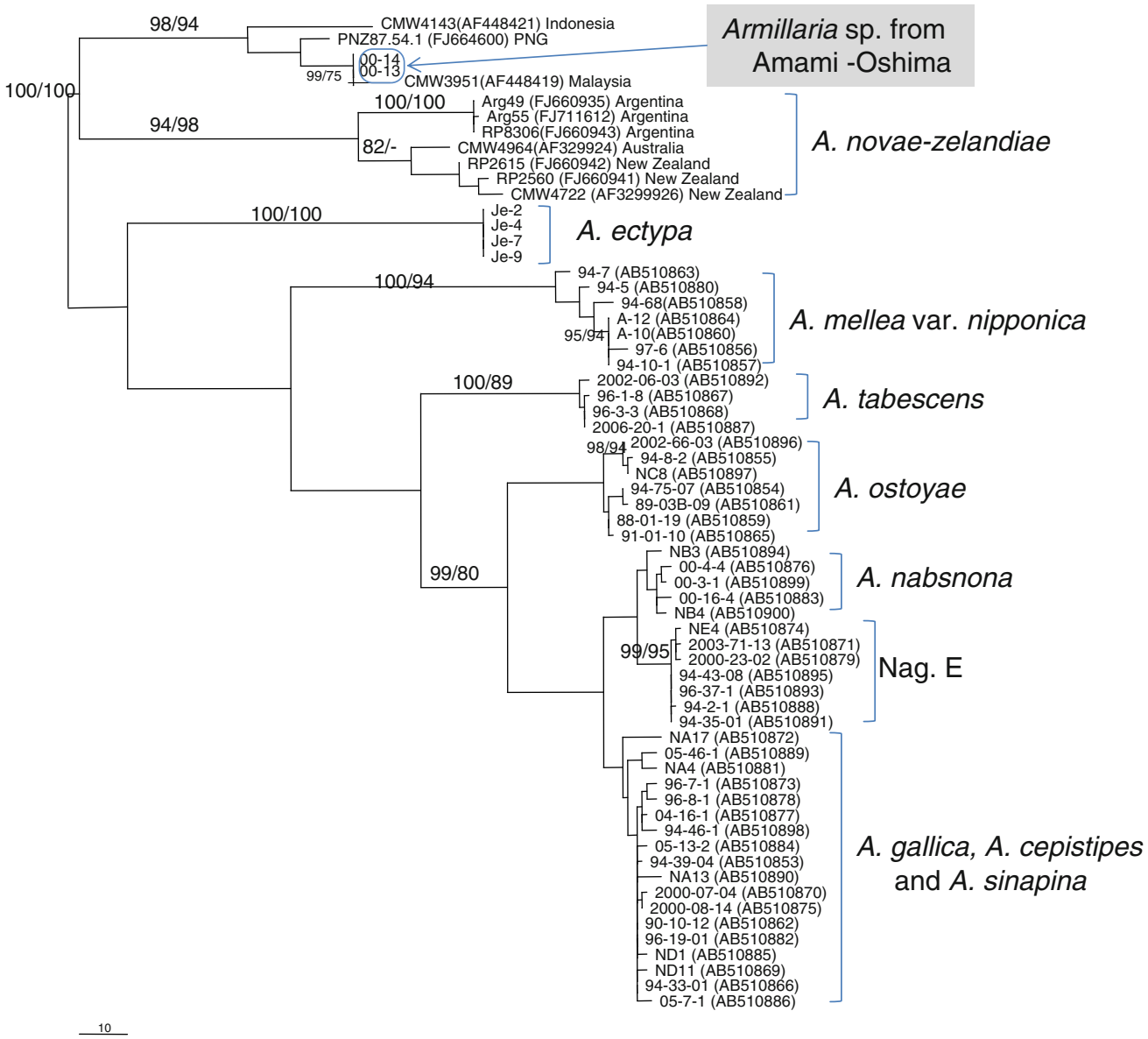

Fig. 2 Neighbor-joining tree based on internal transcribed spacer (ITS 1-5.8S-ITS 2) sequences of known Japanese *Armillaria* species, the unknown isolates from Amami-Oshima, and other highly similar sequences from GenBank. Bootstrap values and reliability values are shown on the *nodes*

strongly supported clades. The NJ tree based on *EF-1α* sequences is shown in Fig. 1. The *Armillaria* sp. sequences from Amami-Oshima isolates formed a strongly supported clade (bootstrap and reliability values >84%) with isolates from Indonesia and Papua New Guinea, *A. novae-zelandiae*. This Amami-Oshima isolate clade was adjacent to a well-supported sister clade (bootstrap and reliability values >97%) that comprised *A. novae-zelandiae* from Australia and New Zealand.

Amplification of the ITS region yielded an approximately 800-bp product for the Amami-Oshima and *A. ectypa* isolates. ITS sequences from the Amami-Oshima isolates showed the highest similarity (>92%) to the sequences of the isolates *A. novae-zelandiae* from Malaysia (isolate no. CMW3951). The sequence similarity between Amami-Oshima isolates and *A. novae-zelandiae* isolates from Australia, New Zealand, and Papua New Guinea ranged from 82 to 85%. The ITS 1 of *A. novae-zelandiae* from Australia, New Zealand, and Papua New Guinea contained more insertions than in the ITS 1 of the isolates from Amami-Oshima and Malaysia. Compared with known *Armillaria* spp. in Japan, the isolates from Amami-Oshima had a >100-bp insertion in ITS 1 and a >100-bp deletion in ITS 2.

In the way similar to that of the *EF-1α* gene, *A. novae-zelandiae* was used as the outgroup for subsequent analysis of ITS region. The ITS-based NJ tree that used a data set

consisting of 63 sequences is shown in Fig. 2. The isolates from Amami-Oshima formed a strongly supported clade with those of *A. novae-zelandiae* from Malaysia, Indonesia, and Papua New Guinea, with bootstrap and reliability values of 98% and 94%, respectively. In addition, the Amami-Oshima isolate clade and another clade containing *A. novae-zelandiae* from Australia and New Zealand formed a well-supported, larger clade with bootstrap and reliability values of 100%.

In this study, the undetermined *Armillaria* isolates from Amami-Oshima in subtropical Japan were shown to be distinct from previously reported *Armillaria* spp. in Japan. The phylogenetic trees based on *EF-1α* and ITS sequences revealed that the Amami-Oshima isolates were contained within a strongly supported clade that comprised the isolates *A. novae-zelandiae* from Southeast Asia and tropical areas of Oceania (Malaysia, Indonesia, and Papua New Guinea). In addition, Amami-Oshima isolate clade was adjacent to a well-supported sister clade that comprised *A. novae-zelandiae* from Australia and New Zealand. *A. novae-zelandiae* was originally described from New Zealand (Stevenson 1964) and occurs in the temperate rainforests of southeastern Australia, New Zealand (Hood et al. 1991), and South America (Coetzee et al. 2003; Pildain et al. 2010). Separation of the clade of Southeast Asia and that of Australia and New Zealand suggests that they could represent discrete taxa.

The morphological characteristics of the *Armillaria* sp. from Amami-Oshima appear indistinguishable from those of *A. fuscipes* based on a comparison with the type specimen originally reported from Sri Lanka (Neda 2009). The cream-colored pileus and dark brown to black stipe are the characteristics of *A. fuscipes*, which also correspond with the *Armillaria* sp. from Amami-Oshima. The phylogenetic position of the type specimen of *A. fuscipes* from Sri Lanka has not been determined because DNA analyses are not permitted on the type specimens of *A. fuscipes*. The GenBank sequences submitted for *A. fuscipes* from Africa (Ethiopia, Kenya, South Africa, Tanzania, Malawi, and Zimbabwe) are quite distinct from the analogous sequences from the Amami-Oshima isolates collected in this study. Micro- and macro-morphological differences between African *A. fuscipes* (Coetzee et al. 2000) and type specimen of *A. fuscipes* from Sri Lanka described by Petch (1909) suggest that *A. fuscipes* from Africa could be distinct from *A. fuscipes*, as described by Petch (1909). The relationship between '*A. novae-zelandiae*'' and '*A. fuscipes*'' has not been fully investigated so far. Further morphological and phylogenetic studies using these taxa are needed to identify the species of the undetermined taxa from Amami-Oshima.

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