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Confirmation of the Occurrence of *Portulaca okinawensis* var. *amamiensis* (Portulacaceae) in Kakeroma Island of the Ryukyus Archipelago, Japan using Morphological and Molecular Data

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Abstract Plants of *Portulaca okinawensis* from Kakeroma Island, an islet adjacent to Amami Island, of the Ryukyus Archipelago were examined to determine the taxonomic status at the variety level. The present study based on molecular and morphological data indicated that the plants should be treated as *P. okinawensis* var. *amamiensis*. A new distribution record of the variety on Kakeroma Island was established.

Key words: ITS, *Portulaca okinawensis*, the Ryukyus, stem color.

Introduction

Portulaca okinawensis Walker et Tawada was described based on the type specimen collected from Okinawa Island of the Ryukyu Archipelago (the Ryukyus; Fig. 1) in Japan (Walker and Tawada, 1951). This species is endemic to central Ryukyus islands, which are composed of the Amami and Okinawa island groups (Hatusima, 1975; Shinjo and Shinzato, 2006; Kokubugata *et al.*, 2013; Fig. 1). Although some taxonomists have treated plants of the genus in those islands as *P. pilosa* L. var. *okinawensis* (Walker et Tawada) Geesink (Geesink, 1969; Hatusima, 1975), Kokubugata *et al.* (2013) concluded that they should be regarded as an independent species endemic to the Ryukyus, in agreement with Walker and Tawada (1951) and Hatusima and Amamo (1994). This is a rare species found on coastal rocky slopes in the two island groups and is a critically threatened species and listed on a Japanese red list (Japanese Ministry of Environment, 2012).

Previously Kokubugata *et al.* (2013) distinguished plants of *P. okinawensis* in Amami and Tokunoshima islands of the Amami island group from those in the Okinawa island group by the color of petals and stems. They treated the former as a new variety, namely *P. okinawensis* var. *amamiensis* Kokub., Koh Nakam. et Yokota (Kokubugata *et al.*, 2013). The two intraspecific taxa were supported by molecular phylogenetic analyses, using the internal transcribed spacer (ITS) region of nuclear ribosomal (nr) DNA (Kokubugata *et al.*, 2013). Shinjo and Shinzato (2006) reported that, in the Amami island group, *P. okinawensis* also distributed in Kakeroma Island, an islet adjacent to Amami Island (Fig. 1).

In 2016, we collected plants of *P. okinawensis* in Kakeroma Island, but their taxonomic status at the variety level was not determined because they did not have flowers whereas petal color is

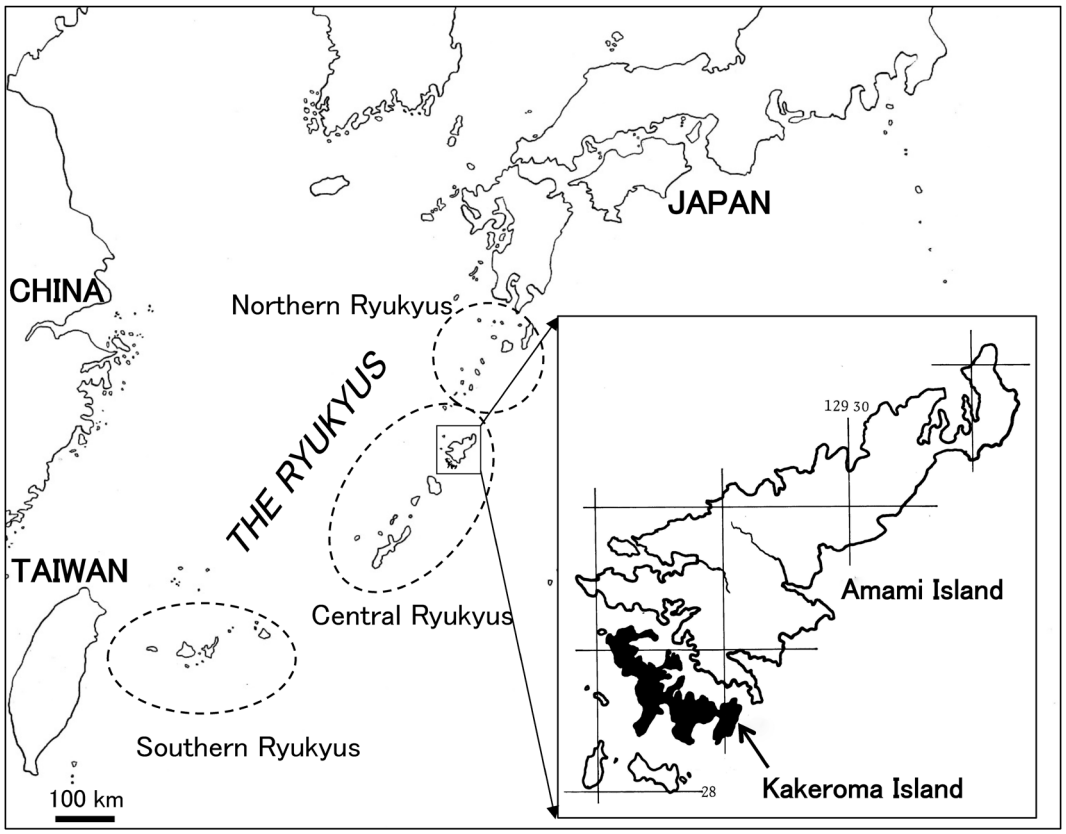

Fig. 1. Map showing Kakeroma Island and its adjacent area.

key characters to distinguish the two varieties. The aim of this study was to determine the taxonomic status of *P. okinawensis* in Kakeroma Island at the variety level, based on observation of the other key character, namely stem color and molecular phylogenetic analysis using the ITS region.

Materials and Methods

Plant material and morphological observation

In the Ryukyus, three *Portulaca* species are recognized: *P. okinawensis*, *P. oleracea* L. with a wide pantropic distribution (Geesink, 1969), and *P. pilosa* L. native to South America and introduced to the tropics and subtropics worldwide (PIER, 2013) including the Ryukyus (Hatusima, 1975). Among the three species, *P. okinawensis* is clearly distinguishable from the other two species in lacking axillary hairs (Walker and Tawada, 1951; Kokubugata *et al.*, 2013).

We collected eight plants identified as *P. okinawensis* based on the lack of axillary hairs from a coastal rocky slope in Kakeroma Island on January 11, 2016. The rocky habitat was very dry, as is typical for the species (Fig. 2). One of the eight plants was collected for the present molecular analysis, and was deposited (Kokubugata 18970) in the herbarium of National Museum of Nature and Science, Japan (TNS).

In the habitat of Kakeroma Island, we observed stem color of the plant of *P. okinawensis*.

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from silica gel-dried leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), following the

Fig. 2. *Portulaca okinawensis* on Kakeroma Island. Bar indicates 5 mm.

manufacturer's protocol. The ITS region was amplified by polymerase chain reaction (PCR) using an iCycler (Bio-Rad, Hercules, CA, USA) with the forward primer AB101 (5′-ACG AAT TCA AGG TCC GGT GAA TGT TTC G-3′) and reverse primer AB102 (5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′) (Douzery *et al.*, 1999). Amplifications were performed using TaKaRa Sapphire Amp Fast Master Mix (TaKaRa, Otsu, Japan). The PCR profile was 35 cycles for 5 s at 94℃, 5 s at 50℃, and 5 s at 72℃ after an initial denaturation step for 3 min at 94℃. PCR products were checked by electrophoresis before purification using the illustra ExoProStar (GE Healthcare, Tokyo, Japan). The cycle sequencing reaction was performed using the BigDye™ Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) with the same primers. The Sanger sequencing products were then purified by ethanol precipitation. Automated sequencing was performed on the 3130xl Genetic Analyzer (Applied Biosystems). The electropherograms were analyzed using ATGC ver. 4.01 (Genetyx Co., Tokyo, Japan). Sequence data from this study were deposited in the GenBank database (LC133271).

ITS data from DNA database

Kokubugata *et al.* (2013) reported four ITS types of *P. okinawensis*: two types of the variety *okinawensis* from Okinawa and Tonaki islands and the other two of the variety *amamiensis* from Amami and Tokunoshima islands. To elucidate the phylogenetic relationships among the *P. okinawensis* plants on Kakeroma Island and those on the other islands, the four ITS types of *P. okinawensis* previously provided by Kokubugata *et al.* (2013) were subjected to phylogenetic analyses (Table 1). For outgroup, we referred to Kokubugata *et al.* (2015) and hired *P. psammotropha* Hance from Bonin Islands of Japan and

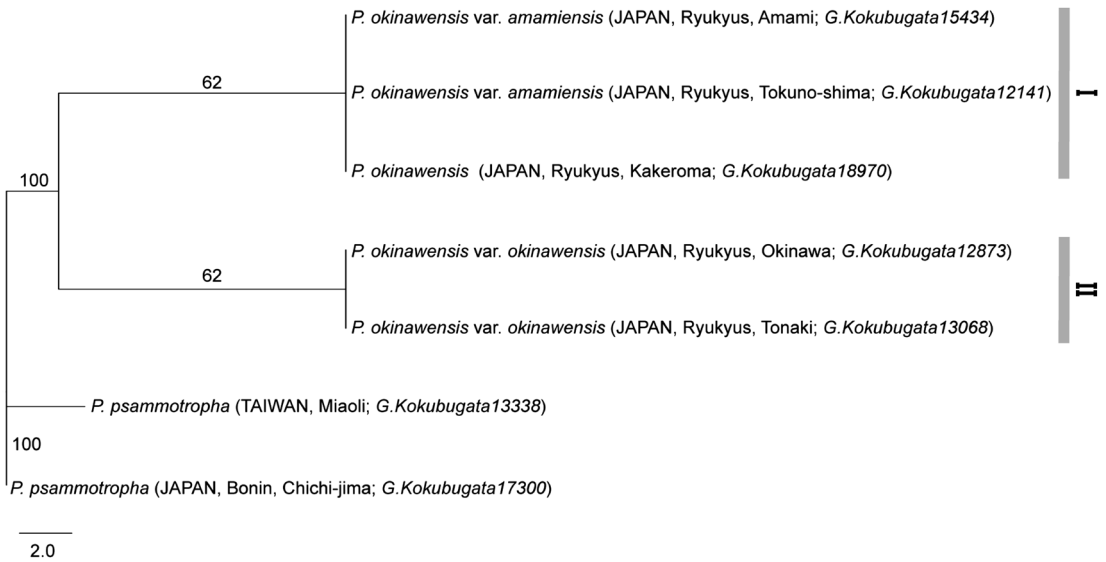

Fig. 3. A most parsimonious tree of *Portulaca* plants based on nrITS sequences. Numerals above branches indicate bootstrap percentages.

Xiao-liuchiu Island of Taiwan (Table 1).

Phylogenetic analyses

DNA sequences were aligned using ClustalW 1.8 (Thompson *et al.*, 1994) and then adjusted manually. Phylogenetic analyses were performed based on a maximum parsimony (MP) criterion, using PAUP* version 4.0b10 (Swofford, 2002).

For MP phylogenetic analysis, characters were treated as unordered, and character transformations were weighted equally. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed using 200 replicates of random additions of sequences, with ACCTRAN character optimization, tree bisection–reconnection (TBR) branch swapping, and MULTREES and STEEPEST DESCENT options on. Statistical analysis of each clade was performed using bootstrap analysis (Felsenstein, 1985). Ten thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap values (BS). The MP tree was generated using TreeView (Page, 1996).

Results and Discussion

In morphology, stem color of the plant of *P. okinawensis* from Kakeroma Island was light green (Fig. 2). As mentioned above, Kokubugata *et al.* (2013) used stem and petal color to distinguish plants of *P. okinawensis* in Amami and Tokunoshima islands as the new variety *amamiensis*, from those on the Okinawa island group. The former has light green stems and lemon-colored petals while the latter has reddish green stems and orange-yellow petals. The result of the present morphological observation suggests that the Kakeroma plant is likely to be *P. okinawensis* var. *amamiensis*.

The ITS sequence of the *P. okinawensis* plant from Kakeroma Island was different from those of the four ITS types of *P. okinawensis* reported by Kokubugata *et al.* (2013). After aligning the ITS sequences from the seven operational taxonomic units (OTUs; five ingroup and two outgroup members), a matrix of 598 bp was obtained. In the MP analysis, 11 of 15 variable characters were parsimony-informative, and a single most parsimonious tree of 15 steps was obtained (Fig. 2), with a consistency index of 1

The MP tree showed two major clades in the

ingroup (Clades I and II in Fig. 3). Clade I consisted of *P. okinawensis* from Kakeroma Island and two OTUs of *P. okinawensis* var. *amamiensis* from Amami and Tokunoshima islands. Clade II consisted of two OTUs of *P. okinawensis* var. *okinawensis* from Okinawa and Tonaki islands. This result is in agreement with the suggestion of the stem color that the Kakeroma plant is identified as *P. okinawensis* var. *amamiensis*.

Kokubugata *et al.* (2013) reported that petal color and stamen number are key characters to distinguish the two varieties. Although those characters were not examined this time because the plant from Kakeroma Island was not flowering, the present morphological and molecular data clearly indicated that the plants on Kakeroma Island should be treated as *P. okinawensis* var. *amamiensis*. This study establishes a new distribution record of the variety on the island.

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