Bull. Natl. Mus. Nat. Sci., Ser. B, 47(1), pp. 45–50, February 22, 2021

Confirmation of the Occurrence and Phylogenetic Backgrounds of *Portulaca okinawensis* var. *amamiensis* (Portulacaceae) in Uke and Yoro Islands of the Ryukyus Archipelago

Goro Kokubugata[1,*], Yumiko Hirayama[1] and Masatsugu Yokota[2]

[1] Department of Botany, National Museum of Nature and Science, 4–1–1 Amakubo, Tsukuba, Ibaraki 305–0005, Japan
[2] Laboratory of Ecology and Systematics, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903–0213, Japan
*E-mail: gkokubu@kahaku.go.jp

(Received 9 November 2020; accepted 23 December 2020)

Abstract Occurrence of *Portulaca okinawensis* var. *amamiensis* is confirmed from Uke and Yoro Islands, which are islets of Amami Island in the Ryukyus. The ITS sequences of the plants of Kakeroma, Uke and Yoro Islands being geographically adjacent were identical, and were different from those of other islands in the central Ryukyus by having deletion of a nucleotide base pair. This study is the first to confirm the occurrence of *Portulaca okinawensis* var. *amamiensis* in Yoro Island.

Key words: Amami island group, ITS, *Portulaca okinawensis*, the Ryukyus.

Introduction

Portulaca okinawensis Walker et Tawada was described based on a type specimen collected from Okinawa Island of the Ryukyu Archipelago (hereafter the Ryukyus; Fig. 1) in Japan (Walker and Tawada, 1951). This species is endemically distributed in the central Ryukyus, which are composed of the Amami and Okinawa island groups (Walker and Tawada, 1951; Hatusima, 1975; Shinjo and Shinzato, 2006; Kokubugata *et al.*, 2013, 2016; Fig. 1). This species is rarely found on coastal rocky slopes, and is a critically threatened species on the Japanese red list (Ministry of Environment of Japan, 2020). Previously, Kokubugata *et al.* (2013) distinguished plants of *P. okinawensis* in Amami and Tokuno-shima 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 an independent variety, namely *P. okinawensis* var. *amamiensis* Kokub., Koh Nakam. et Yokota, based on a type specimen collected from Amami Island (Kokubugata *et al.*, 2013). The two intraspecific taxa were supported by molecular phylogenetic analyses using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (Kokubugata *et al.*, 2013, 2016).

In 2017, we had a field survey of Uke Island and Yoro Island, which are adjacent to Kakeroma Island of the Amami island group (Fig. 1), and found a population of plants that were identified as *P. okinawensis* by their morphological characters on the two islands. The objective of this study was to determine the taxonomic status of these plants collected from Uke and Yoro Islands based on morphological comparisons, and to elucidate their phylogenetic relationships with those from other islands in the central Ryukyus using molecular phylogenetic analysis of the ITS region of nuclear DNA.

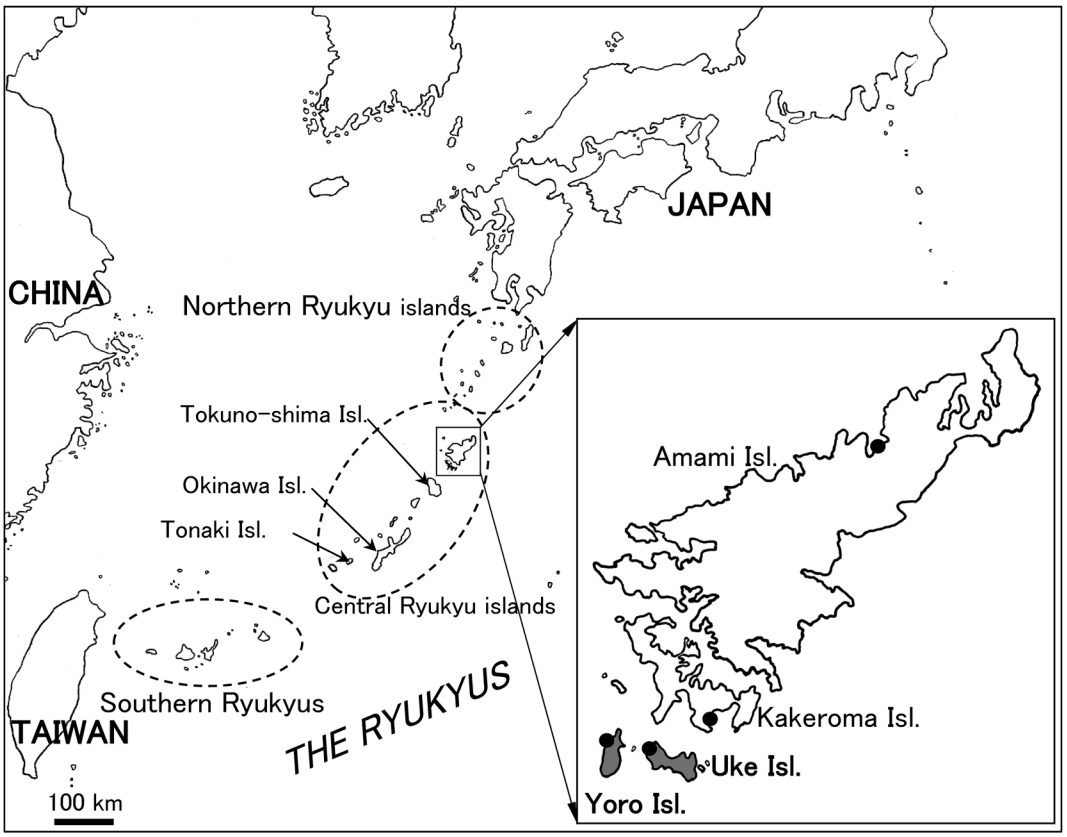

Fig. 1. Map of Uke and Yoro Islands and adjacent areas. Solid circles indicate the locations of plants investigated on Amami, Kakeroma, Uke, and Yoro Islands.

Materials and Methods

Field surveys and plant materials

We conducted a field survey of Uke Island on September 18 and Yoro Island on September 20, 2017. Two individuals of plants identified as *P. okinawensis* were collected from each population of both islands (*GK20228* and *20229* from Uke Island; *GK20305* and *20308* from Yoro Island) for detailed taxonomic analysis following the method of Kokubugata *et al.* (2013) and molecular phylogenetic analysis using the ITS region. Voucher specimens for the present study were deposited in the herbarium of National Museum of Nature and Science, Japan (TNS).

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 manufacturer's protocol. The ITS region of nuclear ribosomal DNA was amplified pantropic by polymerase chain reaction (PCR) using an iCycler (Bio-Rad, Hercules, CA, USA) and 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) with Sapphire Amp (TaKaRa). The PCR profile was 35 cycles for 5 s at 94°C, 5 s at 50°C, and 5 s at 72°C after an initial denaturation step for 3 min at 94°C. PCR products were checked by electrophoresis before purification using the Illustra ExoProStar (GE

Table 1. GenBank accession numbers of the ITS sequences of Portulaca plants used for phylogenetic analysis

Taxon	Collection locality	Collection number (herbarium)	GenBank accession no.*
Ingroup			
P. okinawensis var. *okinawensis*	Japan, central Ryukyus, Okinawa island group, Okinawa Isl., Onna.	*G. Kokubugata 12873* (TNS)	AB823837[a]
	Japan, central Ryukyus, Okinawa island group, Tonaki Isl.	*G. Kokubugata 17300* (TNS)	AB823845[a]
var. *amamiensis*	Japan, central Ryukyus, Amami island group, Amami Isl., Amami.	*G. Kokubugata 15434* (TNS)	AB823823[a]
	Japan, central Ryukyus, Amami island group, Kakeroma Isl., Akitoku.	*G. Kokubugata 18970* (TNS)	LC133271[b]
	Japan, central Ryukyus, Amami island group, Tokuno-shima Isl., Amagi.	*G. Kokubugata 12141* (TNS)	AB823831[a]
Outgroup			
P. psammotropha	Japan, Bonin, Chichi-jima Isl.	*G. Kokubugata 13338* (TNS)	LC018836[c]
	Taiwan, Pingtung, Liuqiu Shiang.	*G. Kokubugata 13338* (TNS)	AB823850[a]

* ITS sequences registered in the DNA Data Bank of Japan (DDBJ): [a]Kokubugata *et al.* (2013); [b]Kokubugata *et al.* (2016); [c]Kokubugata *et al.* (2015).

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). Primers used for sequencing were AB101 and AB102. 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 DNA Data Bank of Japan (DDBJ) (LC593637 and LC593638 for *GK20228* and *20229* from Uke Island; LC593639 and LC593640 for *GK20305* and *20306* from Yoro Island, respectively).

ITS data from the DNA database

Kokubugata *et al.* (2013, 2016) reported five ITS types of *P. okinawensis*: two types of *P. okinawensis* var. *okinawensis* from Okinawa and Tonaki Islands in the Okinawa island group, and three types from Amami, Kakeroma, and Tokuno-shima Islands in the Amami island group. To elucidate the phylogenetic relationships among the plants of *P. okinawensis* on Uke and Yoro Islands and those on other islands of the central Ryukyus, the five ITS types of *P. okinawensis* previously provided by Kokubugata *et al.* (2013, 2016) were subjected to the present molecular analyses as ingroup members (Table 1). We used *P. psammotropha* Hance from Bonin Island of Japan and Taiwan as outgroup members (Table 1) following Kokubugata *et al.* (2015). Finally, eleven operational taxonomic units (OTUs), nine ingroup and two outgroup members, were obtained for the present phylogenetic analysis.

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, indels were treated as missing data, characters were treated as unordered, 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

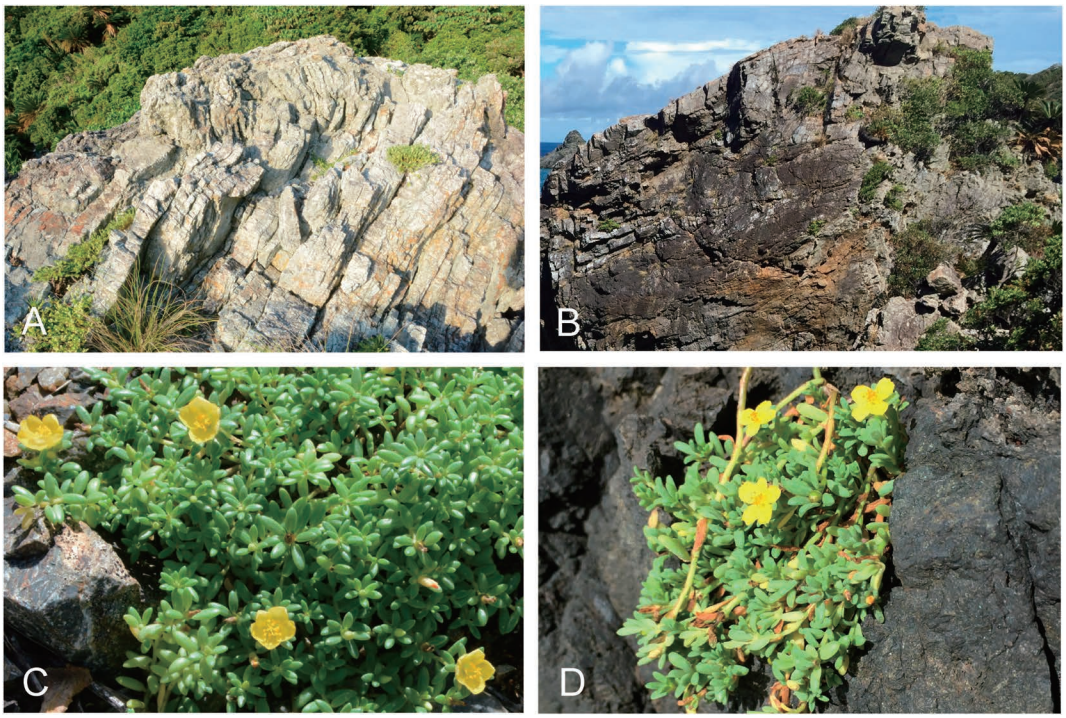

Fig. 2. *Portulaca okinawensis* and its habitats on Uke and Yoro Islands. A, B. Habitat. C, D. Plant. A, C. Uke Island. B, D. Yoro Island.

swapping, and MULTREES and STEEPEST DESCENT options on. Statistical analysis of each clade was performed using bootstrap analysis (Felsenstein, 1985). One thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap values with 10,000 repeats. The MP tree was generated using TreeView (Page, 1996).

Results

Populations of plants identified as *P. okinawensis* were found on dry and sunny seaside rocky slopes with typical seaside plants in the Ryukyus such as *Hedyotis strigulosa* (Bartl. ex DC.) Fosberg var. *parvifolia* (Hook. et Arn.) T.Yamaz. (Rubiaceae) on Uke and Yoro Islands (Fig. 1A, B). On both islands, these plants had light-green stems and light-yellow petals (Fig. 1C, D).

The ITS sequences of the plants from Uke and Yoro Islands were identical to that of *P. okinawensis* var. *amamiensis* from Kakeroma Island. The ITS sequences of the plants from the three islands were different from those of the plants from other islands of the central Ryukyus by having a deletion of adenine inserted at base pair 173 in the ITS sequences.

After aligning the ITS sequences from the 11 OTUs, a matrix of 598 bp was obtained. In the MP analysis, 11 of the 15 variable characters were parsimony-informative, and a single most parsimonious tree of 15 steps was obtained (Fig. 3), with a consistency index of 1.000, retention index of 1.000, and rescaled consistency index of 1.000.

The MP tree showed two major clades in the ingroup (Clades I and II in Fig. 3). Clade I consisted of three OTUs of *P. okinawensis* var. *amamiensis* from Amami, Tokuno-shima and Kakeroma Islands; and four OTUs of *P. okinawensis* from Uke and Yoro Islands evaluated herein. Clade II consisted of two OTUs of *P. okinawensis* var. *okinawensis* from Okinawa and Tonaki Islands.

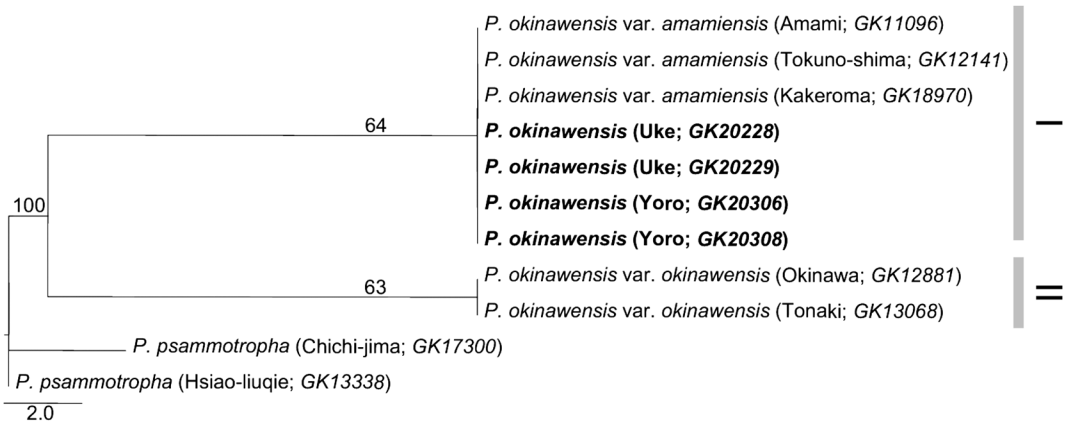

Fig. 3. Maximum parsimonious tree for plants of *Portulaca okinawensis* collected from the central Ryukyus based on ITS sequences. Numbers above branches indicate bootstrap percentages (lower, ≤50%). Bold indicates plants of *P. okinawensis* collected from Uke and Yoro Islands.

Discussion

As mentioned briefly, Kokubugata *et al.* (2013, 2016) reported that two varieties of *Portulaca okinawensis* were distinguishable according to stem and petal color: *P. okinawensis* var. *okinawensis* distributed in the Okinawa island group had reddish green stems and dark yellow petals, whereas *P. okinawensis* var. *amamiensis* distributed in the Amami, Kakeroma and Tokuno-shima Islands of the Amami island group had light green stems and lemon-colored petals. The present morphological comparison taxonomically concludes that plants collected from Uke and Yoro Islands should be treated as *P. okinawensis* var. *amamiensis*. The present MP analysis of the ITS sequences supports this morphological conclusion.

In the Amami island group, this variety has been reported to occur on Amami, Kakeroma and Tokuno-shima Islands by Kokubugata *et al.* (2013, 2016), and on and Uke Islands by Shinjo and Shinzato (2006; as *P. okinawensis*). This study is the first to report the occurrence of *P. okinawensis* var. *amamiensis* on Yoro Island.

It is noteworthy that the plants from Kakeroma, Uke and Yoro Islands share the same ITS sequences. The three islands are geographically adjacent, being separated from each other by less than 5 km, and have similar flora (Suzuki *et al.*, 2020). The consistency of the ITS sequences among the three islands must be resulted from their geographical relationships. On the other hand, it is unexpected that the ITS sequences of the plants from the three islands are different from those from Amami Island. Previously, any specific biological barrier has been unknown between the three islands and Amami Island. The present study did not reveal phylogeographic relationship of the plants from Kakeroma, Uke and Yoro Islands with those from other islands of the Amami island group in detail, because indels on the ITS sequences were treated as missing data in the present MP analysis following Kokubugata *et al.* (2013, 2016). Further molecular phylogenetic analyses using other DNA sequences, including cpDNA, are required to better understand the phylogenetic relationships of *P. okinawensis* var. *amamiensis*.

Acknowledgments

We thank M. Tabata for providing valuable information, H. Sannokyo (Minorino-kai in Uke Island) and Setouchi Town Office for assisting field trip in Uke and Yoro Islands, and R. Matsuda for assisting DNA experiments. This study was partly funded by a Grant-in-Aid for Scientific Research (B) (JSPS KAKENHI Grant Number 25290085).

References

Douzery, E. J. P., Pridgeon, A. M., Kores, P., Linder, H. P., Kurzweil H. and Chase, M. W. 1999. Molecular phylogenetics of *Diseae* (Orchidaceae): A contribution from nuclear ribosomal ITS sequences. American Journal of Botany 86: 887–899.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791.

Hatusima, S. 1975. Flora of the Ryukyus, Added and Corrected. Okinawa Association of Biology Education, Naha (in Japanese).

Kokubugata, G., Ito, T. and Yokota, M. 2016. Confirmation of the occurrence of *Portulaca okinawensis* var. *amamiensis* (Portulacaceae) in Kakeroma Island of the Ryukyus Archipelago, Japan using morphological and molecular data. Bulletin of the National Museum of Nature and Science, Series B 42: 67–71.

Kokubugata, G., Nakamura, K., Hirayama, Y. and Yokota, M. 2013. Taxonomic reconsideration of *Portulaca okinawensis* (Portulacaceae) in the Ryukyus Archipelago based on morphological and molecular data. Phytotaxa 117: 11–22.

Kokubugata, G., Kato, H., Iamonico, D., Umemoto, H., Ito, T., Nakamura, K., Murakami, N. and Yokota, M. 2015. Taxonomic reexamination of *Portulaca boninensis* (Portulacaceae) in the Bonin (Ogasawara) Islands of Japan using molecular and morphological data. Phytotaxa 217: 279–287.

Ministry of Environment of Japan. 2020. Red List of Japan. http://www.env.go.jp/press/files/jp/114457.pdf (in Japanese) [Accessed 2 November 2020].

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in Bioscience 12: 357–358.

Shinjo K. and Shinzato, T. 2006. *Portulaca okinawensis* Walker et Tawada. In: Okinawa Prefecture (ed.), Red Data Okinawa, revised version, p. 72. Okinawa Prefecture, Naha (in Japanese).

Suzuki, E. 2020. Flora on Kakeroma, Uke, and Yoro Islands. Occasional Papers 61: 32–33 (in Japanese).

Swofford, D. L. 2002. PAUP: Phylogenetic analysis using parsimony, version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Walker, E. H. and Tawada, S. 1951. A new species of *Portulaca* from Okinawa. Journal of the Washington Academy of Sciences 41: 138.