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Taxonomic reexamination of *Portulaca okinawensis* (Portulacaceae) in the Ryukyu Archipelago of Japan based on molecular and morphological data

GORO KOKUBUGATA[1*], KOH NAKAMURA[2], YUMIKO HIRAYAMA[1] & MASATSUGU YOKOTA[3]

[1]*Department of Botany, National Museum of Nature and Science, Tsukuba, Ibaraki 305-0005, Japan; e-mail: gkokubu@kahaku.go.jp*
[2]*Biodiversity Research Center, Academia Sinica, Nangang, Taipei 115, Taiwan.*
[3]*Laboratory of Ecology and Systematics, Faculty of Science, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903-0213, Japan.*
[*] *author for correspondence*

Abstract

We used molecular phylogenetic and morphological data to reexamine the taxonomic status of *Portulaca okinawensis* (≡ *P. pilosa* subsp. *okinawensis*) endemic to the central Ryukyu islands, southwestern Japan. Our molecular analyses showed that *P. okinawensis* is monophyletic, sister to the clade of *P. psammotropha* and *P. tuberosa* and it is not closely related to *P. pilosa* subsp. *pilosa*. Two subclades, one comprising plants from the Okinawa Islands and the other from the Amami Islands, were recognized. The plants from the Okinawa Islands had more than 20 stamens, orange-yellow nallowly obovate to oblanceolate petals, and reddish-green stems (as the holotype), while the plants from the Amami Islands had less than 20 stamens, lemon-colored obovate petals, and bright-green stems. The molecular and morphological data support a taxonomic treatment of Walker & Tawada (1951) regarding *P. okinawensis* as a separate species, also suggesting that the plants from the Amami and Okinawa islands should be treated as different taxa. A new variety *Portulaca okinawensis* var. *amamiensis* was here described.

Key words: ITS phylogeny, Japan, morphological data, new taxon, *Portulaca pilosa*, Ryukyu Islands

Introduction

Portulacaceae Juss. is a monotypic family including only *Portulaca* Linnaeus (1753: 445) (Nyffeler & Eggli 2010). *Portulaca* includes over 100 species of annuals and perennials and is distributed worldwide (mostly in the tropics and subtropics) with the center of species diversity in South America and Africa (Ocampo & Columbus 2012). The Ryukyu Archipelago (the Ryukyus) is located between the Kyushu Island (Japan) and Taiwan, and comprises about 140 islands (Fig. 1). Three native *Portulaca* species have been reported in the Ryukyus (Walker 1976, Shimabuku 1997): the pantropical *P. oleracea* Linnaeus (1753: 445) from most of the islands, *P. quadrifida* Linnaeus (1767: 328) distributed in tropical Asia and Africa but collected only once in Ikei-jima Island being an islet of the Okinawa Islands, and *P. okinawensis* Walker & Tawada (1951: 138) [≡ *P. pilosa* Linnaeus (1753:445) subsp. *okinawensis* (Walker & Tawada) Geesink (1969: 298)]. *P. okinawensis* was described by Walker & Tawada (1951) on a specimen collected from Okinawa Island. This taxon rarely occurs on coastal rocky slopes in the Amami Islands and the Okinawa Islands, and is a critically threatened species in a Japanese red list (Japanese Ministry of Environment 2012).

Portulaca okinawensis is considered to be endemic to the central Ryukyus that consisted of the Okinawa and Amami islands (Fig. 1; Hotta 2004, Shinjo & Shinzato 2006). Geesink (1969) recognized *P. okinawensis* as subspecies of *P. pilosa* based on the leaf morphology. *Portulaca pilosa* subsp. *pilosa* is native to South America and widely naturalized in the tropics and subtropics (PIER 2013) including the Ryukyus (Hatusima 1975). The taxonomic concept of Walker & Tawada (1951) was accepted by Momiyama (1982) and Akiyama

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(2006), while that of Geesink (1969) was accepted by Hatusima (1975) and Hatusima & Amamo (1994). Here, we tentatively used the name *P. okinawensis* and later discuss its taxonomy and proper name based on our resultant phylogeny.

Aiming to clarify the taxonomic status of *P. okinawensis,* we carried out molecular phylogenetic analyses of nuclear ribosomal DNA (ITS). Furthermore, we studied intraspecific taxonomy of *P. okinawensis* based on a morphological approach and the ITS-sequence polymorphisms to properly understand the floristic biodiversity of the Ryukyus and to benefit conservation activities for this critically endangered species.

Materials and Methods

Molecular analyses

DNA sample collection

Samples of *P. okinawensis* from 12 localities (two samples per locality) in six central Ryukyus islands were collected and analyzed: Amami Island (4 localities) and Tokuno-shima Island (1) in Amami Islands; Okinawa Island (4), Geruma Island (1), Aka Island (1) and Tonaki Island (1) in Okinawa Islands (Fig. 1; Table 1). A collection locality in Onna of Okinawa Island (OK2 in Table 1 and Fig. 1) is the locus classicus of *P. okinawensis*.

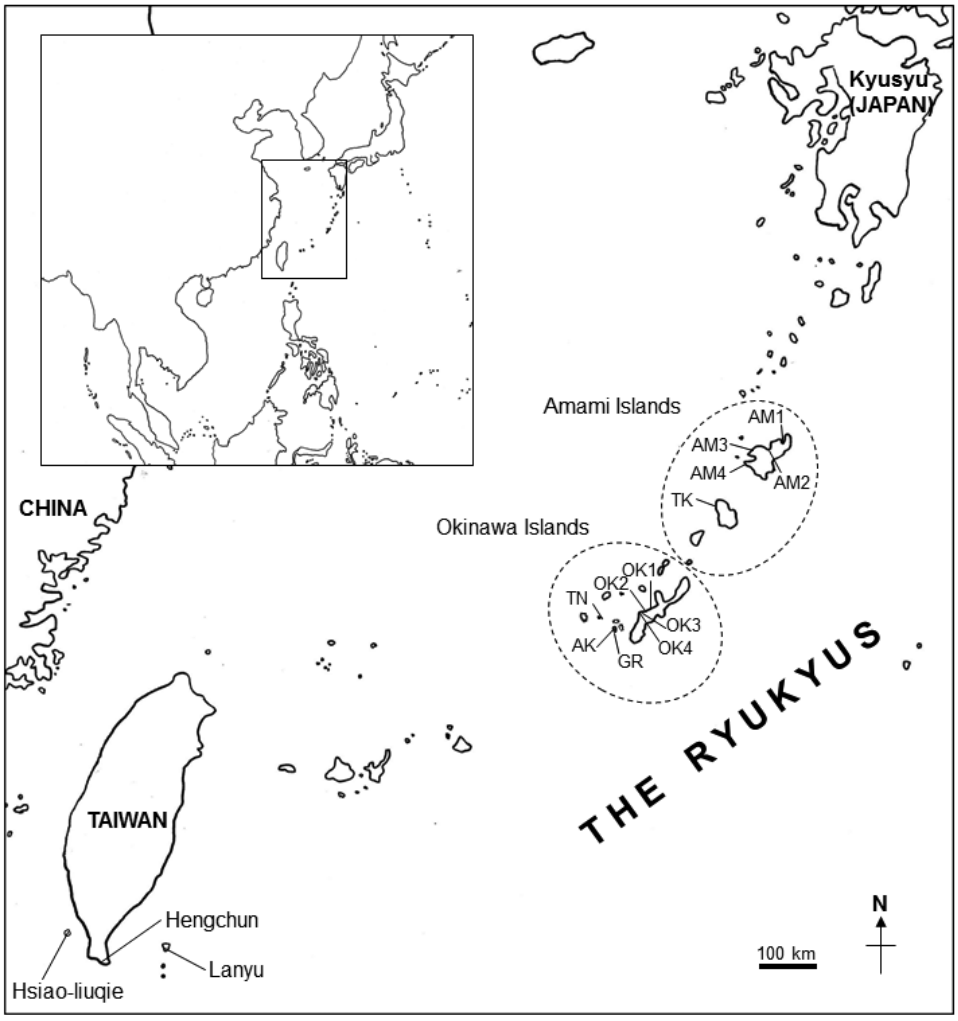

FIGURE 1. Map of the Ryukyus showing 12 localities on six islands of *Portulaca okinawensis* and four localities of *P. psammotropha* in Taiwan. Broken lines circle the islands of the Amami Islands and the Okinawa Islands (see Table 1 for abbreviations for collection localities).

TABLE 1. List of sampled three taxa from 16 localities with their respective vouchers and DDBJ Accession numbers (*abbreviations in parentheses refer to the localities reported in Fig. 1; **alphabets in parentheses indicate the ITS types).

Taxon	Collection number (Herbarium)	Locality*	ITS DDBJ no. **
Portulaca okinawensis Walker & Tawada	*G.Kokubugata15434* (TNS)	Japan, Ryukyus, Amami Group, Amami Isl., Amami (AM1).	AB823823 (A)
	G.Kokubugata15435 (TNS)		AB823824 (A)
	G.Kokubugata11096 (TNS)	Japan, Ryukyus, Amami Group, Amami Isl., Amami (AM2).	AB823825 (A)
	G.Kokubugata11097 (TNS)		AB823826 (A)
	G.Kokubugata15197 (TNS)	Japan, Ryukyus, Amami Group, Amami Isl., Yamato (AM3).	AB823827 (A)
	G.Kokubugata15198 (TNS)		AB823828 (A)
	G.Kokubugata11081 (TNS)	Japan, Ryukyus, Amami Group, Amami Isl., Uken (AM4).	AB823829 (A)
	G.Kokubugata11082 (TNS)		AB823830 (A)
	G.Kokubugata12141 (TNS)	Japan, Ryukyus, Amami Group, Tokuno-shima Isl., Amagi (TK).	AB823831 (B)
	G.Kokubugata12142 (TNS)		AB823832 (B)
	G.Kokubugata12873 (TNS)	Japan, Ryukyus, Okinawa Group, Okinawa Isl., Onna (OK1).	AB823833 (C)
	G.Kokubugata12874 (TNS)		AB823834 (C)
	G.Kokubugata9988 (TNS)	Japan, Ryukyus, Okinawa Group, Okinawa Isl., Onna (OK2).	AB823835 (C)
	G.Kokubugata9989 (TNS)		AB823836 (C)
	G.Kokubugata12887 (TNS)	Japan, Ryukyus, Okinawa Group, Okinawa Isl., Onna (OK3).	AB823837 (C)
	G.Kokubugata12888 (TNS)		AB823838 (C)
	G.Kokubugata12868 (TNS)	Japan, Ryukyus, Okinawa Group, Okinawa Isl., Yomitan (OK4).	AB823839 (C)
	G.Kokubugata12869 (TNS)		AB823840 (C)
	G.Kokubugata15162 (TNS)	Japan, Ryukyus, Okinawa Group, Aka Isl. (AK).	AB823841 (C)
	G.Kokubugata15164 (TNS)		AB823842 (C)
	G.Kokubugata15167 (TNS)	Japan, Ryukyus, Okinawa Group, Geruma Isl. (GR)	AB823843 (C)
	G.Kokubugata15168 (TNS)		AB823844 (C)
	G.Kokubugata13068 (TNS)	Japan, Ryukyus, Okinawa Group, Okinawa Isl., Tonaki Isl. (TN)	AB823845 (D)
	G.Kokubugata13069 (TNS)		AB823846 (D)
Portulaca oleracea L.	*K.Nakamura21011273* (HAST)	Taiwan, Taitung, Lanyu Isl. (1).	AB823847
Portulaca psammotropha Hance	*K.Nakamura10456* (HAST)	Taiwan, Pingtung, Hengchun.	AB823848
	K.Nakamura10457 (HAST)		AB823849
	G.Kokubugata13338 (TNS)	Taiwan, Miaoli, Hsiao-liuqie Isl.	AB823850
	G.Kokubugata13340 (TNS)		AB823851
	K.Nakamura21011269 (HAST)	Taiwan, Taitung, Lanyu Isl. (2).	AB823852
	K.Nakamura21011271 (HAST)		AB823853

To test the phylogenetic relationship between *P. okinawensis* and *P. pilosa* subsp. *pilosa*, we need to incorporate many other congeners in phylogenetic analyses; we therefore utilized ITS (Internal Transcribed Spacer region of nuclear ribosomal DNA) data reported in a preceding molecular study of the genus (Ocampo & Columbus 2012). The ITS sequences of 54 *Portulaca* species, including *P. pilosa* subsp. *pilosa*, were obtained from GenBank (Table 2). This data set from GenBank also included *P. villosa* von Chammisso (1831: 565) [≡ *P. pilosa* subsp. *villosa* (Cham.) Geesink (1969: 297)], which Geesink (1969) recognized as a morphologically closely related species of *P. okinawensis*. In addition, our collections of *P. psammotropha* Hance (1852: 660), distributing in southern China, southern Taiwan and the northern Philippines (Chung *et al.* 2008), were included in the analyses, because Walker & Tawada (1951) recognized that this species is morphologically most similar to *P. okinawensis* but differs in having axillary hairs. We collected a total of 6 samples of *P. psammotropha* in three islets of Taiwan (total three localities) (Table 1). Furthermore, our collection of one plant of *P. oleracea* from Taiwan was included in the analyses. For outgroups, we followed preceding studies (Ocampo & Columbus 2010, 2012) and used *Talinopsis frutescens* A. Gray (1852: 15; Anacampserotaceae), *Pereskia aculeata* Miller (1768: without pagination; Cactaceae), and *Talinum paniculatum* (von Jacquin 1760: 22) Gaertner (1791: 219; Talinaceae), whose ITS data were also cited from GenBank (Table 2).

Voucher specimens of our collection were deposited in the herbaria of Academia Sinica, Taipei (HAST) and the National Museum of Nature and Science, Japan (TNS).

DNA extraction, amplification, and sequencing

For DNA extraction, the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used following the manufacturer's protocols. The isolated DNA were deposited in the Molecular Biodiversity Research Center of the National Museum of Nature and Science (Japan).

The amplification of the ITS region (ITS1, 5.8S, and ITS2), was made by the polymerase chain reaction (PCR) using an iCycler (Bio-Rad, Hercules, CA, USA). The forward primer AB101 (5'-ACG AAT TCA AGG TCC GGT GAA GTG TTC G-3') and the reverse primer AB102 (5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3') (Douzery *et al.* 1999) were used for amplification. Amplifications were performed using Takara LA *taq* with GC buffer II (Takara, Otsu, Japan) and Ampdirect Plus buffer (Shimadzu, Kyoto, Japan). The PCR profile comprised 35 cycles of 1 min at 94°C, 30 s at 55°C, and 1.5 min at 72°C after an initial denaturing for 3 min at 94°C. The PCR products were checked by electrophoresis before purification with ExoSAP-IT (USB Corp., Cleveland, OH, USA).

The cycle sequencing was carried out with a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) using PCR primers listed above with the additional internal reverse primer N2 (5'-GGC GCA ACT TGC GTT CAA-3') and the forward primer N3 (5'-GCT CTC GCA GCA TCG ATG AAG-3') designated by T. Yukawa (TNS, personal communication). The Sanger sequencing products were then purified by ethanol precipitation. Automated sequencing was carried out with an Applied Biosystems 3130xl Genetic Analyzer. The electropherograms were assembled using ATGC ver. 4.01 software (Genetyx Co., Tokyo, Japan). Sequence data from this study were deposited in the DDBJ (DNA Data Bank of Japan) databases (since 1983).

Phylogenetic analyses

The DNA sequences were aligned using ClustalW 1.8 software (Thompson *et al.* 1994) and then manually adjusted. Phylogenetic analyses were based on a Bayesian approach using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford 2002). In the Bayesian phylogenetic analysis, the Hierarchical Likelihood Ratio Tests (hLRT) implemented in MrModeltest 2.2 (Nylander 2004) was used to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed, each with a random starting tree and four chains (one cold and

TABLE 2. Genebank accession numbers of ITS sequences referred from Ocampo & Columbus (2012).

Taxon	Collection number (Herbarium)	Collection area	ITS Genbank no.
INGROUP			
Portulaca amilis Speg.	*Ocampo et al. 1556* (RSA, SI)	Argentina	JF508527
P. australis Endl.	*Ocampo et al. 1747* (BRI, RSA)	Australia	JF508531
P. bicolor F.Muell.	*Ocampo et al. 1753* (BRI, RSA)	Australia	JF508532
P. cf. *bicolor*	*Ocampo et al. 1726* (BRI, RSA)	Australia	JF508530
P. biloba Urb.	*Carter 18168* (RSA)	USA	JF508533
P. californica D.Legrand	*Ocampo & Columbus 1529* (RSA)	Mexico	JF508534
P. canariensis Danin et Reyes-Bet.	*Reyes-Betancort s.n* (RSA, from seed)	Canary Isl.	JF508535
P. confertifolia Hauman	*Ocampo et al. 1619* (RSA, SI)	Argentina	JF508536
P. constricta M.G.Gilbert	*Kilian et al. 5916* (B)	Yemen	JF508537
P. cryptopetala Speg.	*Ocampo et al. 1540* (RSA, SI)	Argentina	JF508538
P. decipiens Poelln.	*Ocampo et al. 1758* (BRI, RSA)	Australia	JF508539
P. digyna F.Muell.	*Ocampo et al. 1749* (BRI, RSA)	Australia	JF508540
P. echinosperma Hauman	*Ocampo et al. 1638* (RSA, SI)	Argentina	JF508541
P. elatior Mart. ex Rohrb.	*Ocampo 1708cv* (RSA) (cult.)	Caribbean	JF508542
P. eruca Hauman	*Ocampo et al. 1645* (RSA, SI)	Argentina	JF508543
P. filifolia F.Muell.	*Ocampo et al. 1733* (BRI, RSA)	Australia	JF508544
P. foliosa Ker Gawl.	*Ocampo 1772cv* (RSA) (cult.)	Tropical Africa	JF508546
P. fulgens Griseb.	*Ocampo et al. 1636* (RSA, SI)	Argentina	JF508547
P. giliesii Hook.	*Ocampo et al. 1545* (RSA, SI)	Argentina	JF508548
P. grandiflora Hook.	*Ocampo et al. 1662* (RSA, SI)	Argentina	JF508549
P. grandiflora Hook. cv.	*Ocampo 1403cv* (RSA)	Cultivated	JF508550
P. guanajuatensis G.Ocampo	*Ocampo 1482* (RSA)	Mexico	JF508551
P. halimoides L.	*Ocampo 1474* (RSA)	Mexico	JF508552
P. hereroensis Schinz	*Roodt 223* (PRE)	Botswana	JF508554
P. howellii (D.Legrand) Eliasson	*Jaramillo 3332* (CDS)	Galápagos Isl.	JF508555
P. intraterranea J.M.Black	*Ocampo et al. 1748* (BRI, RSA)	Australia	JF508556
P. johnstonii Henrickson	*Columbus 5076* (RSA)	Mexico	JF508557
P. lutea Sol. ex G.Forster	*Morden 1575* (HAW)	Hawaii	JF508558
P. massaica S.M.Phillips	*Cruse-Sanders s.n.* (RSA)	Tanzania	JF508559
P. matthewsii G.Ocampo	*Ocampo 1425* (RSA)	Mexico	JF508560
P. mexicana P.Wilson	*Ocampo & Morales 1461* (RSA)	Mexico	JF508561
P. molokiniensis Hobdy	*Perlman 12643* (RSA)	Hawaii	JF508562
P. mucronulata D.Legrand	*Ocampo et al. 1598* (RSA, SI)	Argentina	JF508563
P. oblonga Peter	Mboya 877 (MO)	Tanzania	JF508564
P. obtusa Poelln.	Ocampo et al. 1591 (RSA, SI)	Argentina	JF508565
P. oligosperma F.Muell.	*Ocampo et al. 1751* (BRI, RSA)	Australia	JF508579
P. papulifera D.Legrand	*Ocampo et al. 1569* (RSA, SI)	Argentina	JF508580
P. perennis R.E.Fr.	*Ocampo et al. 1606* (RSA, SI)	Argentina	JF508581
P. pilosa L.	*Nortrup s.n.* (UNCC)	USA	JF508585
P. pusilla Kunth	*Gröger 927* (MO)	Venezuela	JF508587
P. quadrifida L.	*Cruse-Sanders s.n.* (RSA)	Tanzania	JF508588
P. retusa Engelm.	*Baker 16325* (ARIZ)	USA	JF508590
P. rotundifolia R.E.Fr.	*Ocampo et al. 1611* (RSA, SI)	Argentina	JF508591
P. rubricaulis Kunth	*Simá et al. 2433* (MO)	Mexico	JF508592
P. rzedowskiana G.Ocampo	*Ocampo 1124* (IEB)	Mexico	JF508593
P. sclerocarpa A.Gray	*Morden 1828* (HAW)	Hawaii	JF508594
P. smallii P.Wilson	*Herkenham s.n.* (UNCC)	USA	JF508595
P. suffrutescens Engelm.	*Ocampo & Columbus 1505* (RSA)	Mexico	JF508597
P. tingoensis J.F.Macbr.	*Ocampo et al. 1615* (RSA, SI)	Argentina	JF508598
P. trituberculata Danin, Domina et Raimondo	*Danin & Domina SC55* (PAL; RSA from seed)	Italy	JF508574
P. tuberosa Roxb.	*Ocampo et al. 1737* (BRI, RSA)	Australia	JF508599
P. villosa Cham.	*Perlman 13305* (PTBG)	Hawaii	JF508604
P. wightiana Wall. ex Wight et Arn.	*Burgoyne 3613* (PRE)	Namibia	JF508605
P. yecorensis Henrickson et T.Van Devender	*Columbus 5006* (RSA)	Mexico	JF508606
OUTGROUP			
Pereskia aculeata Mill. (Cactaceae)	*NA* (ZSS) (cult.)	Americas	JF508526
Talinopsis frutescens A.Gray (Montiaceae)	*Ocampo 1480* (RSA)	Mexico	JF508607
Talinum paniculatum (Jacq.) Gaertn. (Talinaceae)	*Ocampo & Morales 1458* (RSA)	Mexico	JF508608

three heated). The MCMCMC length was one million generations, and the chain was sampled every one hundredth generation from the cold chain. The first 2500 sample trees (25% of the total 10000 sample trees) were discarded as burn-in after checking that the average standard deviation of split frequencies (ASDSF) reached a stationary state at < 0.01 thereafter. As a guide to convergence, the potential scale reduction factors (PSRFs) were ascertained to be reasonably close to 1.0 for all parameters in an output table. A 50% majority consensus tree of the output tree file from MrBayes was generated by TREEVIEW (Page 1996).

In the MP phylogenetic analysis, indels were treated as missing data. The characters were treated as unordered, and the character transformations were equally weighted. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 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 support for each clade was assessed by 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).

Intraspecific morphological comparison

The following morphological characters of *P. okinawensis* were examined: number of stigma lobes and stamens, color and shape of petals, and color of stems. The number of stigma lobes and stamens was studied fixing the materials in the FAA solution, while the color and shape of the petals and stems were observed in living plants. A total of 46 samples were examined: 11 plants from Amami Island (4 localities), 5 plants from Tokuno-shima Island (1), 22 plants from Okinawa Island (4 including the locus classicus of *P. okinawensis*), 3 plants from Aka Island (1), 3 plants from Geruma Island (1), and 2 plants from Tonaki Island (1) (Table 3). The equality of variances for the number of the stigma lobes and stamens between the Amami Islands and the Okinawa Islands was firstly evaluated using the *F*-test. If the *F*-test shows equal variances, the Student's *t*-test is applied; if *F*-test shows no equal variances, the Student's t-test with Welch's correction is applied.

The color of the stems was examined in 79 samples from Amami Island (31 plants from 4 localities) and Tokuno-shima Island (5 from 1), Okinawa Island (28 from 4, holotype included), Aka Island (5 from 1), Geruma Island (5 from 1), and Tonaki Island (5 from 1). The on-line herbarium database of the National Museum of Natural History, Smithsonian (2013) was checked for the holotype.

Results

Phylogenetic relationships based on ITS

Four ITS types were found in *P. okinawensis*: type A in plants collected from Amami Island; type B in plants from Tokuno-shima Island; type C in plants from Okinawa Island, Aka Island, and Geruma Island; type D in plants from Tonaki Island. The ITS sequences were identical in the 6 plants of *P. psammotropha* collected from 3 localities of Taiwan, and differed from the four ITS types of *P. okinawensis*. The ITS sequence of *P. oleracea* was also different from those of *P. okinawensis* and *P. psammotropha*. For these three species, we used ITS types as operational taxonomic units (OUTs) in the Bayesian and MP phylogenetic analyses. Finally we obtained 63 OUTs, including six ITS types of the three species obtained herein, 54 accessions as ingroups and three outgroup accessions obtained from GenBank. After alignment of the 63 OUTs, we obtained a matrix of 650 bp.

The model of GTR+I+G was selected in the Bayesian analysis. The 50% majority rule consensus tree of all the post-burn-in trees is depicted with Bayesian posterior probabilities (PPs, Fig. 2). In the MP analysis, 233 of 350 variable characters were parsimony informative in the ITS matrix including the outgroup taxa. 3838 equally most parsimonious trees of 884 steps were obtained with a consistency index (CI) of 0.632, a retention index (RI) of 0.804, and a rescaled consistency index (RC) of 0.501. The topology of the strict consensus tree was highly compatible with that of the Bayesian tree, and thus bootstrap percentages (BPs) are plotted on the Bayesian tree (Fig. 2).

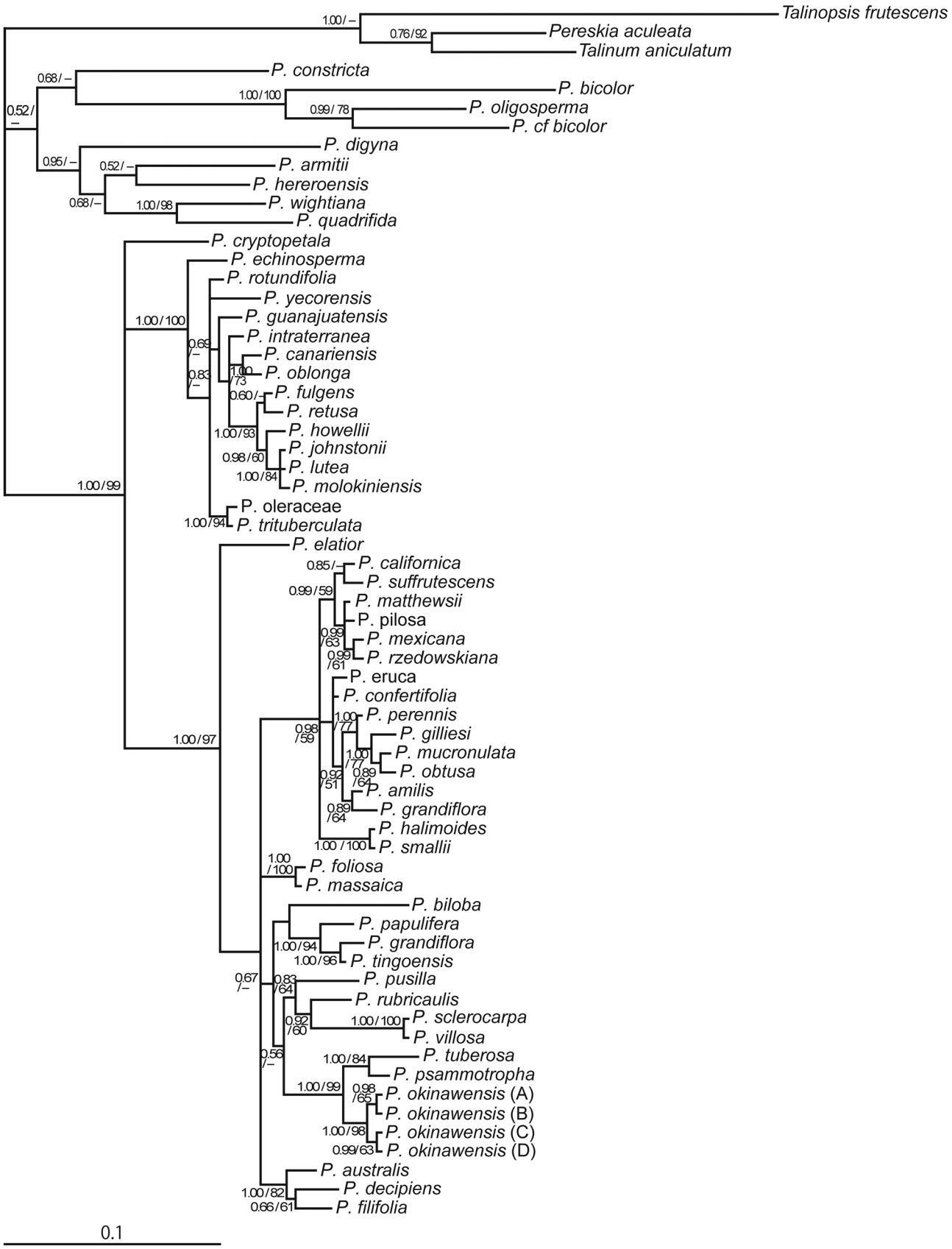

FIGURE 2. Bayesian phylogenetic tree for 60 OTUs of *Portulaca* with three outgroups based on internal transcribed spacer (ITS) sequence. The topology of the maximum parsimony (MP) strict consensus tree was highly compatible with the Bayesian tree. Bayesian posterior probabilities (left) and bootstrap percentages in the MP analysis (right) are shown [see the Table 1 for the localities collection of the four ITS types (A–D) of *P. okinawensis*].

Both the Bayesian and MP analyses (Fig. 2) demonstrated that four ITS types of *P. okinawensis* formed a well-supported clade (PP / BS = 1.00 / 98%) including two subclades: the first one comprises the type A (plants from Amami Island) and the type B (plants from Tokuno-shima Island) (0.98 / 65%), while the other group comprises the type C (plants from Okinawa, Aka, and Geruma Islands) and the type D (plants from Tonaki Island) (0.99 / 63%). A sister clade of *P. okinawensis* includes *P. psammotropha* and *P. tuberosa* Roxb. On the other hand, *P. okinawensis* and *P. pilosa* subsp. *pilosa* were intervened by many species in the phylogeny and they were not closely related with each other. *Portulaca okinawensis* and *P. villosa* (≡ *P. pilosa* subsp. *villosa*) were comparatively closely related but fell into two different well-supported clades.

Morphological characters

The number of stamens ranged from 12 to 32 in *P. okinawensis* (Table 3). In the plants from the Amami Islands, the stamens were 12–15, while in those from the Okinawa Islands were 14–32. The *F*-test showed that the variances in the number of the two island groups were not statistically equal ($p < 0.0001$), and the Student's *t*-test with Welch's correction was applied. The test showed that there was a significant statistic difference between the two island groups ($p < 0.0001$).

The number of stigma lobes was 3–4 in *P. okinawensis* (Table 3). The *F*-test showed that the variances of the two island groups were statistically equal, and the Student's *t*-test was applied. The test showed that there was no significant difference between the two island groups.

The plants collected from the Amami Islands had bright-green stems (Fig. 3A–C); while those from the Okinawa Islands had reddish-green stems (Fig. 3D–H). Petals of the plants from the Amami Islands were lemon-colored (Fig. 3A–C). Those from the Okinawa Islands were orange-yellow (Fig. 3D–H). Moreover, the plants collected from Amami (Fig. 3A & B), Tokuno-shima (Fig. 3C), Aka (Fig. 3H) and Geruma islands had the petal obovate, while the plants from Okinawa (Fig. 3D–F) and Tonaki (Fig. 3G) islands had nallowly obovate to oblanceolate petals.

The colors of stems and petals of the holotype (*S. Tawada 2221*) were degraded and not observable and not written on the description of *P. okinawensis* (Walker & Tawada 1951). However, there was a hand-written note indicating that petal color of the holotype was orange-yellow, and its stem color was reddish on the specimen sheet.

TABLE 3. Comparison of number of stigma lobes and stamens of *Portulaca okinawensis* from the Ryukyus of Japan.

Island group	Island	Locality abbreviation*	Plant sample number	Number of stigma lobe (avg.)	Number of stamen (avg.)
Amami Islands	Amami	AM1	4	4 (4.0)	12–15 (14.3)
		AM2	3	3 (3.0)	12–15 (14.0)
		AM3	2	3–4 (3.5)	12–15 (13.5)
		AM4	2	3 (3.0)	12 (12.0)
	Tokuno-shima	TK	5	4 (4.0)	12–15 (12.6)
Okinawa Islands	Okinawa	OK1	4	3–4 (3.3)	23–24 (24.7)
		OK2	3	3–4 (3.5)	24–26 (24.7)
		OK3	6	3–4 (3.3)	23–26 (24.0)
		OK4	9	3–4 (3.1)	15–24 (19.9)
	Aka	AK	3	3–4 (3.7)	14–20 (16.7)
	Geruma	GR	3	3 (3.0)	15–25 (18.3)
	Tonaki	TN	2	4 (4.0)	26–32 (29.0)

* Refer Table 1 and Fig. 1.

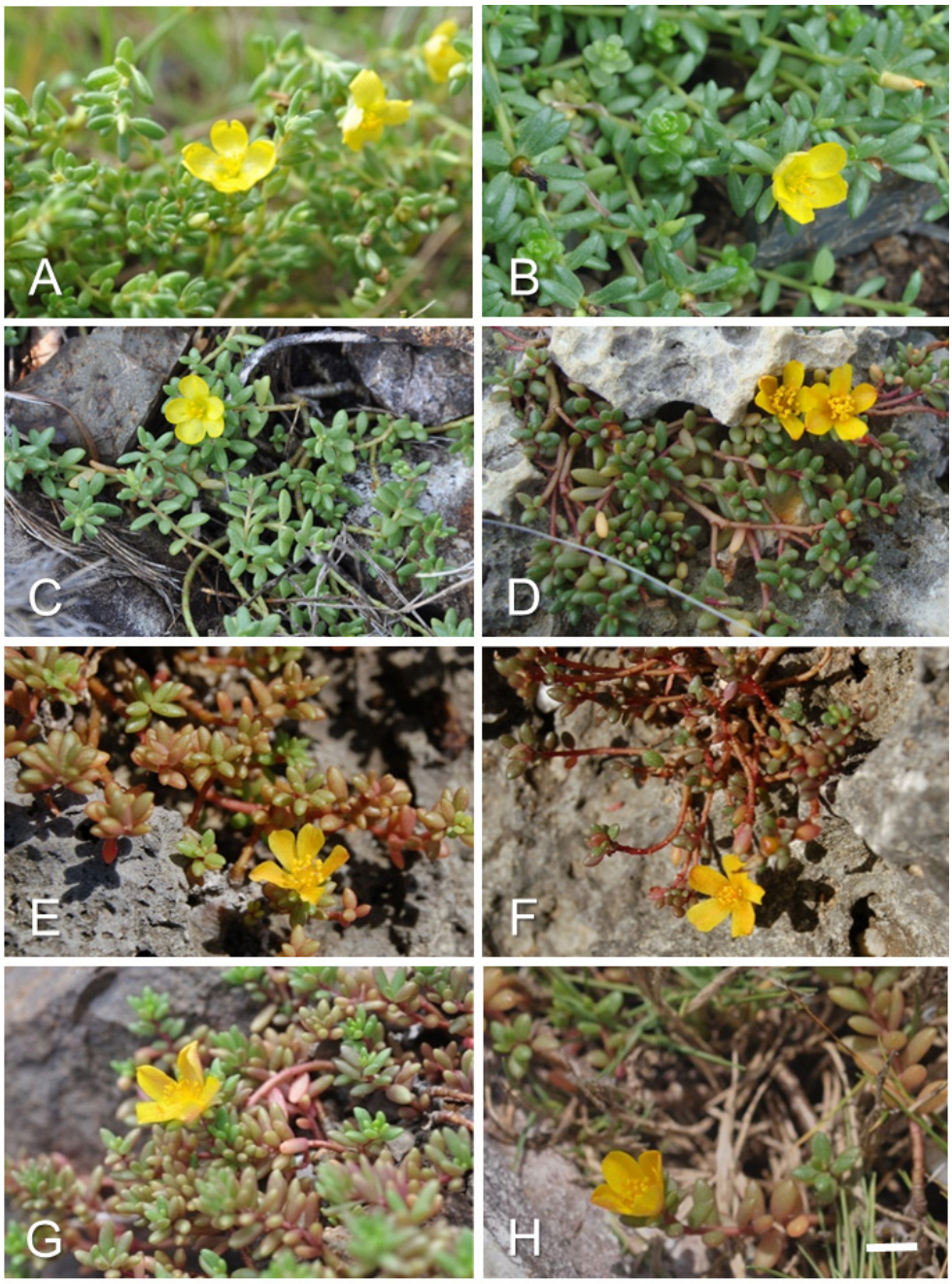

FIGURE 3. Flowering plants of *Portulaca okinawensis* from the Ryukyus. **A**. Amami Island (AM1). **B**. Amami Island (AM3). **C**. Tokuno-shima Island. **D**. Okinawa Island (OK1). **E**. Okinawa Island (OK2). **F**. Okinawa Island (OK4). **G**. Tonaki Island (TK). **H**. Aka Island (AK). Scale bar in H also applies to A–G, and indicates 5 mm (see the Table 1 for the localities abbreviations).

Discussion

Taxonomic reconsideration of Portulaca okinawensis

Our molecular analysis results disagree with Geesink (1969) showing that *P. okinawensis* are not closely related to *P. pilosa* subsp. *pilosa* and it cannot be treated at subspecies rank of *P. pilosa* and revealing that *P. okinawensis* and *P. villosa* (≡ *P. pilosa* subsp. *villosa*) are not conspecific.

Portulaca okinawensis is instead phylogenetically related to *P. psammotropha* (*sensu* Chung *et al.* 2008) collected from islets of southern Taiwan and *P. tuberosa* distributed from India, through Malesia, to Christmas Island of Australia (Puy *et al.* 1993). However, *P. okinawensis* is morphologically distinguishable from these

two species by the absence of axillary hairs (Puy *et al.* 1993, Chung *et al.* 2008). Therefore, the present molecular data and morphological difference support the taxonomic concept of Walker & Tawada (1951) treating *P. okinawensis* as an independent endemic species to the central Ryukyus.

Intraspecific taxonomy of Portulaca okinawensis

From the morphological point of view, the two subclades of *P. okinawensis* (from the Amami Islands, and from the Okinawa Islands), include plants clearly different. The plants from the Amami Islands have bright-green stems and lemon-colored petals, while plants from the Okinawa Islands have reddish-green stems and orangey-yellow petals as same as the holotype (*S. Tawada 2221*). Although the ranges of the number of stigmas were slightly overlapped between the two groups, the numbers of the former are always less than 20, and are significantly different from the other. The number of stamens is 25 in the holotype (Walker & Tawada 1951) and this is included in the ranged for the plants from the Okinawa Islands.

We conclude that the plants from the Amami and Okinawa islands should be treated as different taxonomic entities. A new variety is described below.

Taxonomic treatment

A diagnostic key of *Portulaca okinawensis* and the related species follows:

1. Stem 10–30 cm long, leaf 5–28 mm long 2
- Stem ca. 10 cm long, leaf 5–8 mm long 3
2. Leaf obovate to liner, axillary hair up to 12 mm long, petal white, pink, or pink with a white base, fruit 3–4 mm long ..... *P. villosa*
- Leaf elliptic to liner, axillary hair up to 6 mm long, petal reddish violet or pink, fruit 5–6 mm long. ..... *P. pilosa*
3. Axillary hairs present ..... *P. psammotropha*
- Axillary hairs absent 4
4. Number of stamens mostly more than 20, petals orange yellow, mostly obovate to nallowly obovate, stems reddish green ..... *P. okinawensis* var. *okinawensis*
- Number of stamens less than 20, petals lemon-colored, obovate to oblanceolate, stems bright green ..... *P. okinawensis* var. *amamiensis*

Portulaca okinawensis Walker & Tawada var. ***okinawensis***

Type:—JAPAN. The Ryukyus, the Okinawa Islands, Okinawa Island, Onna, 2 October 1949, *Tawada 2221* (holotype US!; available from: http://collections.mnh.si.edu/search/botany/?ti=3).

Description:—Herbs perennial, 3–5 cm tall. Stems not articulated, diffuse, branched basally, ca. 1 mm thick; basal stems woody, prostrate, upper stems herbaceous, upright, reddish green. Root fleshy, much-branched. Leaves spirally arranged to alternate, subsessile, without axillary hairs; leaf blade 2–3 mm thick, oblong to obovate, 5–8 mm long, base obtuse, apex obtuse or rounded. Flowers solitary, about 10–15 mm in diameter. Sepals 2, ovate-deltate, about 2 mm long. Petals 5, obovate to nallowly obovate, orangey yellow, mostly without margins overlapping. Stamens 12–15. Ovary ovoid. Stigma usually trilobed or tetralobed; capsule glossy, 2–4 mm long, 2–3 mm wide.

Distribution and habitat:—The Ryukyus, the Okinawa Islands, Okinawa, Aka, Geruma, Tonaki islands. Coastal rocky slopes, xeric, saline, and exposed to direct sunlight. *Portulaca okinawensis* has also been recorded from Aka, Geruma, Yakabi, Tonaki, Aguni, and Kume islands of the Okinawa Islands (Shinjo & Shinzato 2006). We have not collected samples from these islands. We examined a herbarium specimen of *P. okinawensis* collected from Yakabi Island (*Miyagi 8414*, RYU, collected in 1978) but we could not identify it at variety level because of the difficulty in counting of the number of stamens and the discoloring of the petals and stems.

Additional specimens examined:—JAPAN. **The Ryukyus, the Okinawa Islands**: Okinawa Island, Onna (OK1), 10 August 2010, *Kokubugata 12873–12878* (TNS); Okinawa Island, Onna (OK2), 20

September 2007, *Kokubugata 9988*, *9989*, *10018* (TNS); Okinawa Island, Onna (OK3), 1 September 2010, *Kokubugata 12886–12891* (TNS); Okinawa Island, Yomitan (OK4), 29 August 2010, *Kokubugata 12867–12872*, *12879–12885* (TNS); Aka Island, 30 August 2012, *Kokubugata 15161–15165* (TNS); Geruma Island, 30 August, 2012, *Kokubugata 15166–15170* (TNS); Tonaki Island, 31 August 2012, *Kokubugata 15179–15183* (TNS).

Portulaca okinawensis Walker & Tawada var. ***amamiensis*** Kokubugata, Koh Nakam. & Yokota, *var. nov.*

Type:—JAPAN. The Ryukyus, the Amami Islands, Amami Island, Yamato, 6 September 2012, *Kokubugata 15198* (holotype TNS!).

Diagnosis:—Differt a var. *okinawensi* staminibus 12–15, petalis citrinis, obovatis vel oblanceolatis, caulibus viridibus.

Description:—Herbs perennial, 3–5 cm tall. Stems not articulated, diffuse, branched basally, ca. 1 mm thick; basal stems woody, prostrate, upper stems herbaceous, upright, bright green. Root fleshy, much-branched. Leaves spirally arranged to alternate, subsessile, without axillary hairs; leaf blade 2–3 mm thick, oblong to obovate, 5–8 mm long, base obtuse, apex obtuse or rounded. Flowers solitary, about 10–15 mm in diameter. Sepals 2, ovate-deltate, about 2 mm long. Petals 5, obovate, emarginate, rotund to obtuse, lemon-colored, with margins overlapping. Stames 12–15. Ovary ovoid. Stigma usually trilobed or tetralobed; capsule glossy, 2–4 mm long, 2–3 mm wide.

Etymology:—The epithet refers to the Amami Islands, the locus classicus.

Distribution and habitat:—Endemic to the Amami Islands (the Ryukyus), in Amami and Tokuno-shima islands. Coastal rocky slopes, xeric, saline, and exposed to direct sunlight. In Kakeroma Island, being an islet situated in the Amami Island, *P. okinawensis* has been recorded. However, we could not obtain and investigate samples from the island.

IUCN Red list category:—It can be included in the Data Deficient (DD) category of IUCN Red List categories (IUCN 2010) as there are inadequate information to make a direct or indirect assessment of its risk of extinction based on its distribution and/or population status.

Additional specimens examined (paratypes):—JAPAN. **The Ryukyus, the Amami Islands**: Amami Island, Amami (AM1), 3 November 2010, *Kokubugata 12939–12942*, 7 September 2012, *Kokubugata 15434–15443* (TNS); Amami (AM2), 18 October 2008, *Kokubugata 11096–11100* (TNS); Yamato (AM3), 6 September 2012, *Kokubugata 15197–15202*; Uken (AM4), 18 October 2008, *Kokubugata 11081–11085*, *11102* (TNS); Tokuno-shima Island, Amagi (TK), 17 October 2007, *Kokubugata 12140–12144* (TNS).

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