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Takuro Ito[1,2*], Ren Chen[3], Qin-er Yang[3], Yukiko Saito[4], Masatsugu Yokota[5] and Goro Kokubugata[2,1*]: **Taxonomic reexamination of *Sedum formosanum* (Crassulaceae) in Japan, Taiwan, and the Philippines based on molecular data**

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Abstract

We conducted molecular phylogenetic analyses of plants treated as *Sedum alfredii* or *S. formosanum* collected from Japan, Taiwan, and the Philippines (Batan Island) with other 19 *Sedum* species primarily in East Asia using the internal transcribed spacer region of nuclear DNA to reexamine the taxonomic status of *S. formosanum*. Our results indicate that 15 plants treated as *S. alfredii* or *S. formosanum* included a clade with two Taiwanese endemic species, but were not sister to *S. alfredii* from Guangdong, China. In conclusion, this study supports the taxonomic treatment of Hatusima (1975), Ohba (1984, 2001), and Tang and Huang (1993) of *S. formosanum* as a separate species from *S. alfredii*.

Key words : ITS, molecular phylogeny, *Sedum alfredii*, taxonomy.

Introduction

Sedum formosanum N.E. Br. (Crassulaceae; Fig. 1 A to E) was described based on a type specimen collected from Taiwan (Brown 1885), and is an annual herb occurring on seasides and rarely inland rocky slopes (Hatusima 1975). Following Hatusima (1975) and Ohba (1984), it is thought that *S. formosanum* is distributed from south Kyushu, Japan, to Taiwan through the Ryukyu Archipelago, and extending to Batan Island, the Philippines. In Japan, this taxon is rare and is a threatened species (the category of Near Threatened) on the Japanese Red List (Japanese Ministry of Environment 2012).

There are two different treatments for this taxon: some taxonomists treat *S. formosanum* as an independent species (Hatusima 1975; Walker 1976; Ohba 1984; Tang and Huang 1993; Ohba 2001), while others treat it as a synonym of *S. alfredii* Hance (Fig. 1F) described based on a type specimen collected from Guangdong, China (Liu and Chung 1977; Shimabuku 1997). To reveal their taxonomic entity, therefore, we performed molecular phylogenetic analyses based on nuclear DNA sequences, using samples of *S. alfredii* or *S. formosanum* from Kyushu, the Ryukyus, Taiwan, and Batan Island, the Philippines, and *S. alfredii* from Guangdong, China.

Materials and Methods

DNA sample collection

We collected 15 plants treated as *Sedum alfredii* or *S. formosanum* from southern Kyushu (one plant from locality), the Ryukyus (eight plants from eight islands), Taiwan (two plants from two localities), and Batan Island, the Philippines (four plants from three localities); and three plants of *S. alfredii* from a locality in Guangdong, China (Table 1). We also collected ten other *Sedum* species from Japan and Taiwan (Table 1). Voucher specimens for our collections have been deposited in the herbaria

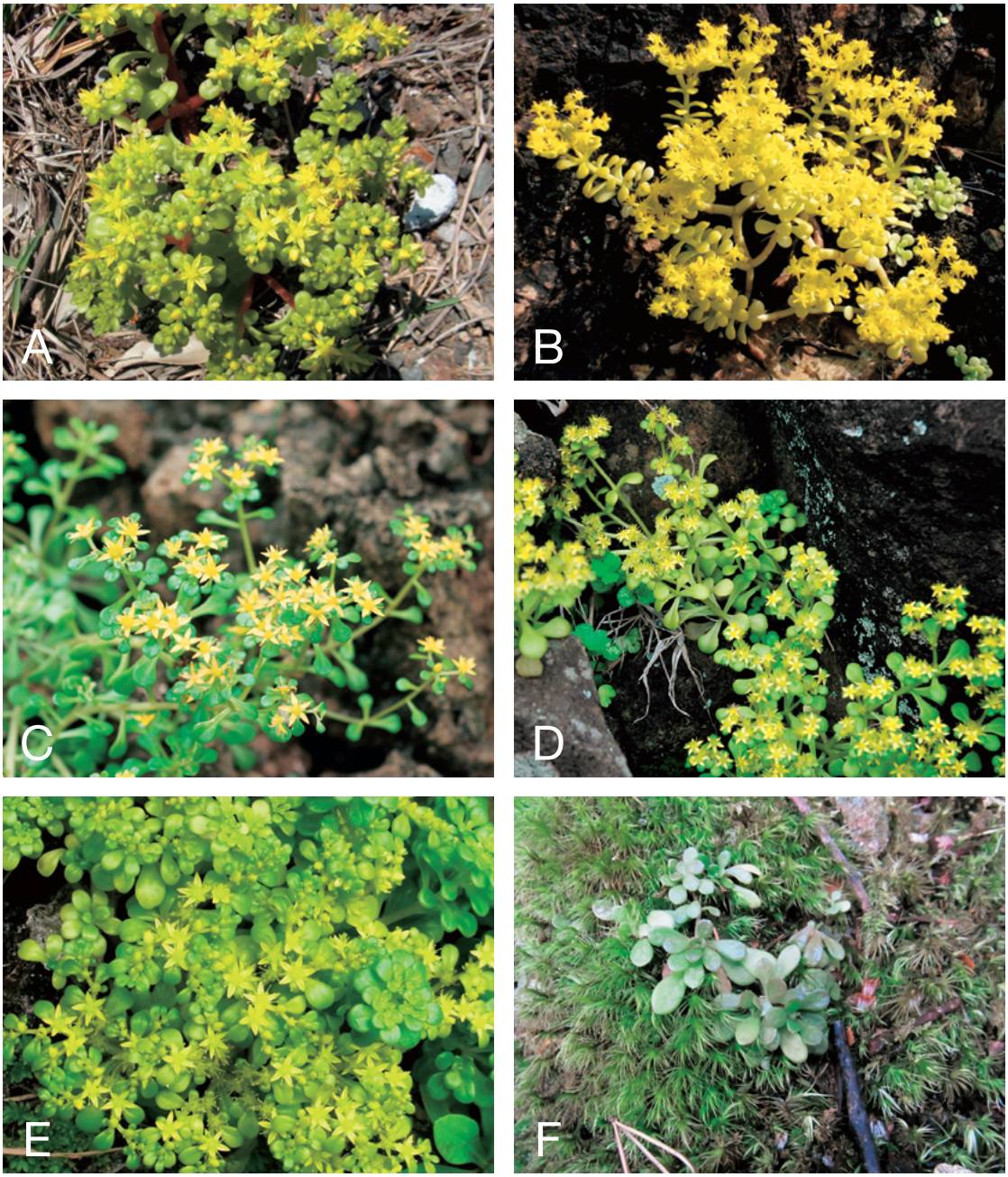

Fig. 1. Natural habit of *Sedum* plants treated as *Sedum alfredii* or *S. formosanum* in Japan, Taiwan and the Philippines, and *S. alfredii* in China.
A-E. plants had been treated as *S. alfredii* or *S. formosanum* from Japan, Taiwan and the Philippines. **F.** *S. alfredii* in Guangdong of China. **A.** Amami Island, Japan (*GK16712*; April 28, 2013). **B.** Iheya Island, Japan (*GK10726*; May 26, 2008). **C.** Ishigaki Island, Japan (*GK11775*; March 27, 2008). **D.** Lanyu Island, Taiwan (*GK6132*; March 28, 2008). **E.** Batan Island, the Philippines (*GK15818*; March 6, 2013). **F.** Guangdong, China (*GK17190*; November 28, 2013).

Table 1. List of plant material collected 13 *Sedum* taxa including plants treated as *S. alfredii* or *S. formosanum* for sequenced in the present study with their respective locality, voucher and the DDBJ accession numbers

Taxon	Source locality	Abbreviation*	Voucher	Accession No.	ITS type
S. alfredii	China, Ghangdong. (C-GND)		*G. Kokubugata 17190* (IBSC)	AB930259	
			G. Kokubugata 17191 (IBSC)	AB930260	
			G. Kokubugata 17192 (IBSC)	AB930261	
S. bulbiferum	Japan: Kyusyu, Nagasaki, Tsushima.		*T. Ito 416* (TNS)	AB930281	
S. erythrospermum	Taiwan, Kaohsiung, Taoyuan.		*C. Tsutsumi 504* (TNS)	AB906473	
S. formosanum	Japan: Kyusyu, Kagoshima, Minamisatsuma.	K-KGS	*G. Kokubugata 16768* (TNS)	AB930262	a
(or *S. alfredii*)	Japan: Ryukyus, Kagoshima, Tanegashima Island.	R-TNG	*G. Kokubugata 15602* (TNS)	AB930265	b
	Japan: Ryukyus, Kagoshima, Amami Island.	R-AMM	*G. Kokubugata 16712* (TNS)	AB930264	b
	Japan: Ryukyus, Okinawa, Iheya Island.	R-IHY	*G. Kokubugata 10726*(TNS)	AB930267	c
	Japan: Ryukyus, Okinawa, Izena Island.	R-IZN	*G. Kokubugata 12224* (TNS)	AB930266	c
	Japan: Ryukyus, Okinawa, Tonaki Island.	R-TNK	*G. Kokubugata 13049* (TNS)	AB930268	b
	Japan: Ryukyus, Okinawa, Kume Island.	R-KMJ	*G. Kokubugata 12755* (TNS)	AB930269	b
	Japan: Ryukyus, Okinawa, Ishigaki Island.	R-ISG	*G. Kokubugata 11775* (TNS)	AB906474	d
	Japan: Ryukyus, Okinawa, Iriomote Island.	R-IRO	*T. Ito 598* (TNS)	AB930270	b
	Taiwan: New Taipei City, Ruifang.	T-TPI	*G. Kokubugata 16446* (TNS)	AB930272	a
	Taiwan: Taitung, Lanyu Island.	T-LNY	*G. Kokubugata 6132* (TNS)	AB930271	e
	Philippines: Batanes, Batan Island 1.	P-BTN1	*G. Kokubugata 15715* (TNS)	AB930273	f
	Philippines: Batanes, Batan Island 2.	P-BTN2	*G. Kokubugata 15731* (TNS)	AB930275	f
	Philippines: Batanes, Batan Island 3.	P-BTN3	*G. Kokubugata 15818* (TNS)	AB930276	f
			G. Kokubugata 15821 (TNS)	AB930274	f
S. hakonense	Japan: Chubu, Yamanashi, Narisawa.		*T. Ito 623* (TNS)	AB930278	-
S. japonicum	Japan: Kyusyu, Nagasaki, Nagasaki.		*G. Kokubugata 16749* (TNS)	AB906475	-
S. makinoi	Japan: Kanto, Tochigi, Mt. Iwafune.		*T. Ito 626* (TNS)	AB930280	-
S. nokoense (A)	Taiwan: Kaohsiung, Taoyuan.		*G. Kokubugata 10831* (TNS)	AB906477	-
S. nokoense (B)	Taiwan: Hualien, Nenggao.		*G. Kokubugata 10426* (TNS)	AB906478	-
S. oryzifolium	Japan: Tokai, Shizuoka, Izu.		*T. Ito 628* (TNS)	AB930258	-
S. subtile	Japan: Kanto, Tokyo, Mt. Mitake.		*T. Ito 624* (TNS)	AB930277	-
S. tosaense	Japan: Shikoku, Kochi, Kochi.		*G. Kokubugata 16726* (TNS)	AB906483	-
S. yabeanum	Japan: Kyusyu, Nagasaki, Tsushima.		*T. Ito 406* (TNS)	AB930279	-

*Abbreviation used for Fig. 1 and Fig. 3.

Table 2. Locality, voucher and Accession numbers of ITS sequences of *Sedum* and *Aeonium* and *Greenovia* species referred from the DDBJ/ENA/NCBI database

Taxon	Country	Voucher	Accession No.	Reference*
INGROUP				
Sedum alfredii	China	*Z. Wang IBK194562* (IBK)	FJ919952	a
	China	*Z. Wang IBK194562* (IBK)	FJ919953	a
Sedum lineare	Japan	*S. Mayuzumi C00120* (TI)	AB088623	b
Sedum mexicanum	Japan	*S. Mayuzumi C00001* (TI)	AB088621	b
Sedum multicaule	Nepal	*F. Miyamoto et al. TI9596136* (TI)	AB088631	b
Sedum oreades	Nepal	*F. Miyamoto et al. TI9420140* (TI)	AB088632	b
Sedum sarmentosum	Japan	*S. Mayuzumi C00008* (TI)	AB088624	b
Sedum triactina	Nepal	*F. Miyamoto et al. TI9596091* (TI)	AB088629	b
Sedum trullipetalum	Nepal	*F. Miyamoto et al. TI9420132* (TI)	AB088630	b
Sedum zentaro-tashiroi	Japan	*H. Ohba 1998* (TI)	AB088619	b
OUTGROUP				
Aeonium castello-paivae	Canary	*M.E. Mort 1519* (WS)	AY082127	c
Aeonium gomerense	Canary	*M.E. Mort 1454* (WS)	AY082133	c
Aeonium viscatum	Canary	*M.E. Mort 1432* (WS)	AY082154	c
Greenovia aizoon	Canary	*M.E. Mort 1425* (WS)	AY082112	c

*a: directly submitted by Z. H. Wang and W. S. Shu in 2009; b: Mayuzumi and Ohba (2004); c: Mort et al. (2002);

of the National Museum of Nature and Science, Japan (TNS) and South China Botanical Garden (IBSC).

For the molecular phylogenetic analyses, we also obtained internal transcribed spacer (ITS) sequence data for eight other *Sedum* species, including *S. alfredii*, in East Asia reported in a molecular study of the genus by Mayuzumi and Ohba (2004; Table 2) and ITS sequence data for two accessions of *S. alfredii* submitted directly to the DDBJ/ENA/NCBI database by Z. H. Wang and W. S. Shu in 2009 (Table 2).

As outgroups, we followed Mort et al. (2002) and used *Aeonium castello-paivae* Bolle, *Aeonium gomerense* Praeger, *Aeonium viscatum* Bolle, and *Greenovia aizoon* Bolle, using ITS data from the DDBJ/ENA/NCBI database (Table 2).

In total, 43 operational taxonomic units consisting of 39 ingroup and 4 outgroup members were included in the phylogenetic analyses.

DNA extraction, amplification, and sequencing

DNA was extracted from dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's protocols. The ITS region of nuclear ribosomal DNA was used for the phylogenetic analysis. The ITS region (ITS1, 5.8S rDNA, and ITS2) was amplified via polymerase chain reaction (PCR) using an iCycler (Bio-Rad, Hercules, CA, USA). The forward primer ITS1 (5- TCC GTA GGT GAA CCT GCG G -3) and reverse primer ITS4 (5- TCC TCC GCT TAT TGA TAT GC -3) (White et al. 1990) were used to amplify the ITS region using Takara EX *Taq* polymerase (Takara, Otsu, Japan) with Ampdirect Plus buffer (Shimadzu, Kyoto, Japan) or EmeraldAmp PCR Master Mix dye (Takara, Otsu, Japan). The PCR profile consisted of an initial 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. The PCR products were checked by electrophoresis before purification with an ExoStar clean-up kit (USB, Cleveland, OH, USA).

Cycle sequencing was performed using the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA), and the PCR primers listed above with the internal reverse primer N2 (5- GGC GCA

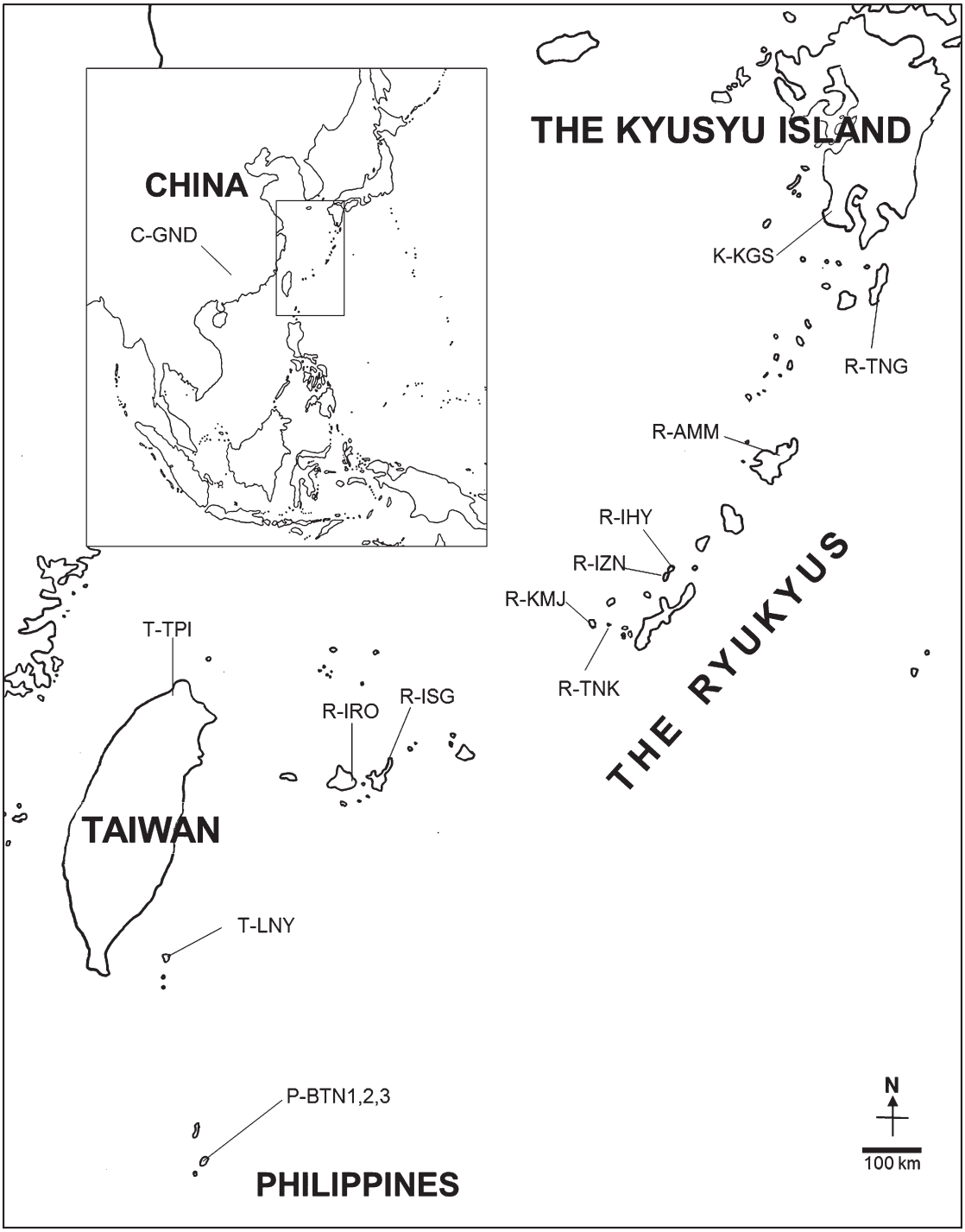

Fig. 2. Map of plants treated as *Sedum alfredii* or *S. formosanum* in Japan, Taiwan and the Philippines, and *S. alfredii* in China (see Table 1 for abbreviations for collection localities).

ACT TGC GTT CAA -3) and forward primer N3 (5-GCT CTC GCA GCA TCG ATG AAG -3) designed by T. Yukawa (TNS, personal communication). The samples were purified by ethanol precipitation, and then electrophoresed on an Applied Biosystems 3130xl Genetic Analyzer. The electropherograms were assembled using ATGC ver. 6 (GENETYX, Tokyo, Japan). The sequence data obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ).

Phylogenetic analysis

The DNA sequences were aligned using ClustalW 1.8 (Thompson et al. 1994) and then adjusted manually. Phylogenetic analyses were conducted with a Bayesian approach using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford 2002). In the Bayesian phylogenetic analysis, we used the hierarchical likelihood ratio test (hLRT) implemented in MrModeltest 2.2 (Nylander 2004) to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model we had selected, we performed two separate runs of Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses, each with a random starting tree and four chains (one cold and three hot). The MCMCMC was 10 million generations long, and the chain was sampled every one-hundredth generation from the cold chain. The first 2500 sample trees (25% of 100,000 sample trees) were discarded as burn-in after first checking that the value of the average standard deviation of split frequency (ASDSF) was less than 0.01. As a guide to convergence, we determined that the potential scale reduction factors (PSRFs) were reasonably close to 1.0 for all parameters in the output table. The 50% majority-rule consensus tree of all of the post-burn-in trees was generated using TreeView ver. 1.6.6 (Page 1996).

Our MP phylogenetic analysis treated indels as missing data. The 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 with 200 replications of random additions of sequences with ACCTRAN character optimization, tree bisection-reconnection (TBR) branch swapping, and the MULTREES and STEEPEST DESCENT options switched on. Statistical support for each clade was assessed by bootstrap analysis (Felsenstein 1985). Ten thousand replicates of heuristic searches with TBR branch swapping switched on and the MULTREES options switched off were performed to calculate bootstrap (BS) values.

Results

Phylogenetic relationships based on ITS

The aligned ITS sequence length was 643 bp and six ITS types were recognized in samples of plants that had been treated as *Sedum alfredii* or *S. formosanum* from Japan, Taiwan, and the Philippines: type a in Kagoshima, Kyushu and New Taipei, Taiwan; type b on Tanegashima, Amami, Tonaki, Kume, and Iriomote Islands, the Ryukyus; type c on Iheya and Izena Islands, the Ryukyus; type d on Ishigaki Island, the Ryukyus; type e on Lanyu Island, Taiwan; and type f on Batan Island, the Philippines (Table 1).

In the Bayesian analyses, the GTR+G model was selected. The 50% majority rule consensus tree of all of the post-burn-in trees is depicted with Bayesian posterior probabilities (PPs) in Figure 3. In the MP analysis, 267 of 345 variable characters were parsimony-informative in the ITS matrix (including the outgroup taxa). Four most-parsimonious trees of 843 steps were obtained with a consistency index (CI) of 0.610, a retention index (RI) of 0.838, and a rescaled consistency index (RC) of 0.548. These were compatible with the Bayesian tree, and so bootstrap percentages (BPs) were plotted with Bayesian posterior probabilities (PPs) on the Bayesian tree (Fig. 3).

Both the Bayesian and MP analyses showed that plants that had been treated as *S. alfredii* or *S. formosanum* and the endemic Taiwan species *S. nokoense*, *S. erythrospermum*, and *S. morrisonense* formed a clade with high support (Clade I, PP/BP=1.00/100), while *S. alfredii* from Guangdong, China, formed another clade with high statistical support (Clade II, 1.00/100). Within Clade I, three polytomic clades were

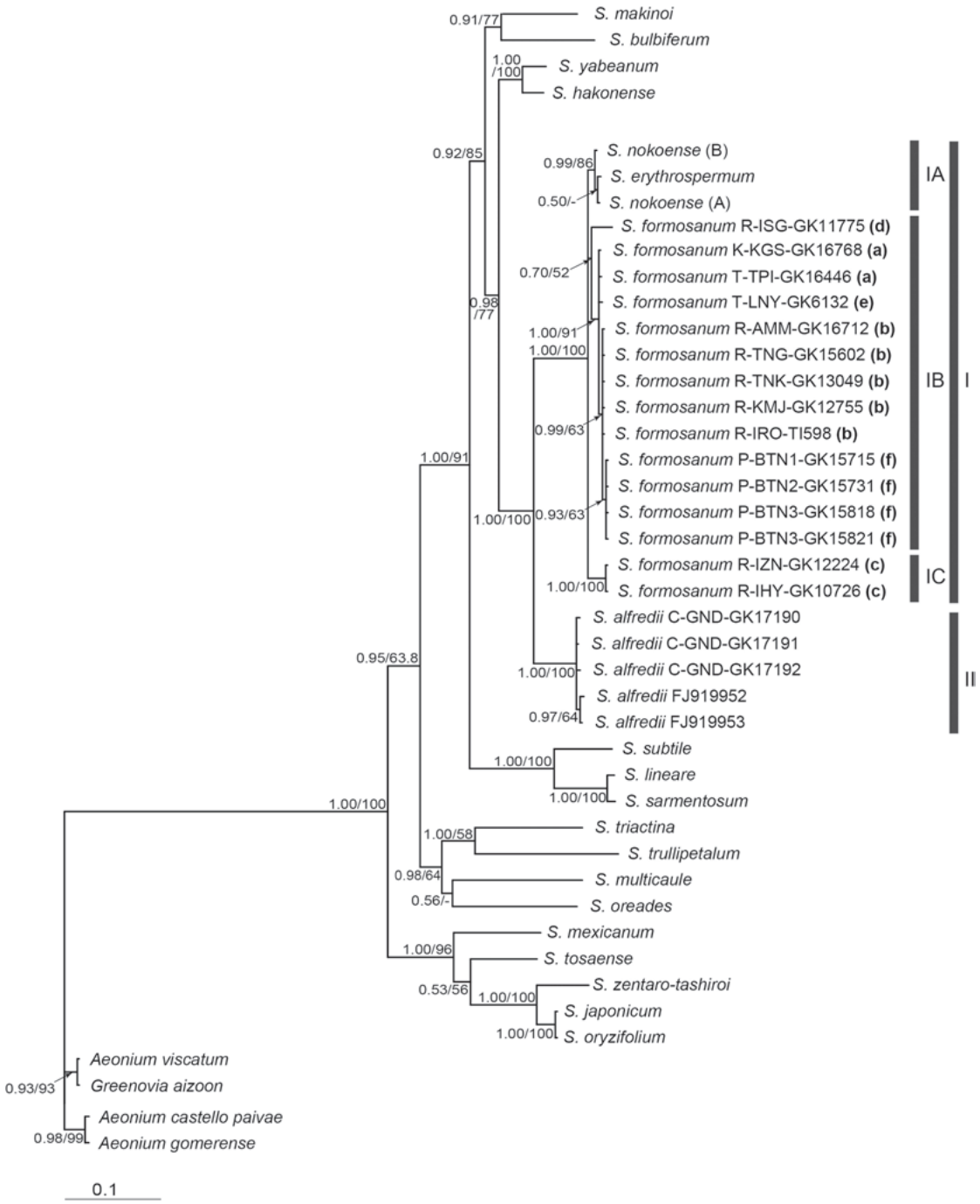

Fig. 3. The Bayesian 50% majority rule consensus tree of *Sedum* based on nrITS sequences.
The topology of the maximum parsimony strict consensus tree was compatible with the Bayesian tree. Alphabets in parentheses indicate nrITS types. Numerals above branches indicate Bayesian posterior probabilities (*left*) and bootstrap percentages in the maximum parsimony analysis (*right*; – < 50%).

recognized: the first comprised two Taiwanese-endemic species, namely *S. nokoense*, and *S. erythrospermum* (Clade IA, 0.99/86); the second comprised 13 plants that had been treated as *S. alfredii* or *S. formosanum* from Kyushu and the Ryukyus, Japan, Lanyu Island, Taiwan, and Batan Island, the Philippines (types a, b, d, e and f) (Clade IB, 0.70/52); and the third comprised plants that had been treated as *S. alfredii* or *S. formosanum* from Izena and Iheya Islands, the Ryukyus (type c) (Clade IC, 1.00/100).

Discussion

As mentioned, Hatusima (1975), Walker (1976), Ohba (1984, 2001), and Tang and Huang (1993) treated *Sedum formosanum* (Fig. 1A to E) as an independent species, while Liu and Chung (1977) and Shimabuku (1997) treated it as a synonym of *S. alfredii* (Fig. 1F) described based on a type specimen collected from Guangdong, China (Hance 1870) by their leaf-morphological similarity. On the other hand, floral-morphologically, Ohba (1984) stated that *S. formosanum* was morphologically distinguishable from *S. alfredii* because the former had erect carpels when it matured, while the latter had horizontal or oblique carpels. In addition, Ohba (1984) mentioned petal length, thickness of the flowering stem, axial sterile branches while flowering, and the shape of cauline leaves as useful characters to distinguish the two species. As habitat, *S. formosanum* occurs on sunny rocky slopes near the seacoast (Ohba 2001), while *S. alfredii* occurs on shady rocky slopes under forest at 2000~3000 m elevation (Fu and Ohba 2001).

Our molecular analysis indicated that plants that had been treated as *S. alfredii* or *S. formosanum* were phylogenetically closer to three Taiwanese-endemic species (*S. nokoense* and *S. erythrospermum*) than to *S. alfredii* from Guangdong, China. The three Taiwanese-endemic species are known to occur at high elevations more than 1,000 m altitude differing from the plants treated as *S. alfredii* or *S. formosanum* at seasides (Tang and Huang 1993). Also they are apparently distinguishable from plants treated as *S. alfredii* or *S. formosanum* by leaf-morphological characters (Tang and Huang 1993). The present molecular analyses reveal that it is not appropriate to treat *S. formosanum* as a synonym of *S. alfredii*. In conclusion, this study supports the taxonomic concept of Hatusima (1975), Walker (1976), Ohba (1984, 2001), and Tang and Huang (1993) regarding *S. formosanum* as a separate species from *S. alfredii*.

The Clade I did not comprised of all of the *S. formosanum* plants from the Ryukyus together. Specifically, most of the plants from the Ryukyus formed a clade with those from Taiwan and the Philippines (Clade IB), but plants from Izena and Iheya Islands were positioned in a different clade (Clade IC). The inconsistency between phylogeny and phytogeography might suggest multiple migration events to the Ryukyus. Further study is necessary to discuss to clarify phylogenetic relationships of *S. formosanum* to the two Taiwanese-endemic species (Clade IA), and to phylogeographically consider how and when *S. formosanum* migrated to the Ryukyus using other DNA sequence and microsatellite DNA markers.

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日本，台湾およびフィリピンに分布が知られているハママンネングサの分類に関しては，中国を基準産地とする *Sedum alfredii* とする見解（Liu and Chung 1977; 島袋1997）と *S. alfredii* とは独立した *S. formosanum* とする見解（初島1975; Ohba 1984, 2001; Tang and Huang 1993）の2つがある。本研究ではハママンネングサの分類見解を再検討するため，東アジアに産するマンネングサ属他種19種を含めた核 DNAの ITS 領域を用いた分子系統解析（ベイズ法と最節約法）を行った。

解析の結果，日本（九州・琉球列島），台湾およびフィリピンから採集したハママンネングサは中国から採集した *S. alfredii* と同じクレードにはまとまらず，台湾に固有のマンネングサ属2種とクレードを形成した。

以上の結果から，ハママンネングサを *S. alfredii* から独立した *S. formosanum* とする見解（初島1975; Ohba 1984, 2001; Tang and Huang 1993）が支持された。

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