



Nuclear and plastid DNA data confirm that *Sedum tosaense* (Crassulaceae) has a disjunct distribution between Pacific mainland Japan and Jeju Island, Korea

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Abstract

Our molecular phylogeographic analyses based on the nrDNA ITS and cpDNA *trnLF* of *Sedum tosaense* populations in the Shikoku District, Japan and Jeju Island, Korea suggested a disjunct distribution. Plants of *S. tosaense* from the two regions comprised a well-supported clade consisted of plants from Kochi (Shikoku District) and those from Jeju Island; we estimated a divergence time of 0.61 Ma between the Kochi and Jeju populations based on the ITS and partial *trnLF*. We conclude that: 1) *S. tosaense* has a disjunct distribution between Kochi and Jeju Island, and 2) plants of this species might have dispersed between Kochi and Jeju Island over water, but not via a land bridge, which flooded before subclade divergence.

Keywords: Disjunct distribution, ITS, Japan, Jeju, Kochi, Korea, *Sedum*, *trnLF*

Introduction

The term “disjunct distribution” is applied to a distribution pattern in which a species or species lineage occurs in two or more areas, but is absent from the intermediate areas (e.g., Gray 1878; Raven 1963; Thorne 1972). Several disjunct distributions in seed plants have been confirmed by molecular evidence, such as between North and South America (e.g., Spalik *et al.* 2010; Popp *et al.* 2011), between East Asia and North America (e.g., Huang *et al.*, 2013), between Japan and Australia (e.g., Nakamura *et al.* 2012; Kokubugata *et al.* 2012), and between the Japanese Mainland and Taiwan (e.g., Mitsui *et al.* 2008).

Jeju Island is a volcanic island located off the southern coast of the Korean Peninsula. It is 73 km long (east–west), 31 km wide (north–south), and 1847 km² in area. Mount Halla is the highest mountain on the island (Woo *et al.* 2013). The coastline is bathed by the Kuroshio Current, which transports warm seawater from tropical Asia. Jeju Island is believed to have received floristic elements from mainland Korea and China and from the southern part of Japan; tropical species occur in the lowlands (e.g., Chung *et al.* 2013) and temperate species are distributed at higher elevations (e.g., Kong 2000; Dolezal *et al.* 2012).

The genus *Sedum* Linnaeus (1753: 430) (Crassulaceae) includes about 420 species that are distributed widely in both the Old and New Worlds; it is the largest and most widespread genus in the Crassulaceae (Thiede & Eggli 2007). According to Ohba (2001), there are 24 species and subspecies of *Sedum* in Japan; there are 16 in South Korea (Korea National Arboretum 2011). *Sedum tosaense* Makino (1901: 35) (Fig. 1), the target species of this study, was formally described based on a type specimen collected from Kochi Prefecture, Shikoku District, Japan; the entity had been previously reported as a *nomen nudum* (Makino 1892). The species comprises perennial rosette herbs that are diminutive in winter. Alternate leaves with retuse apices are the most distinctive features, and it occurs on rocky slopes (Ohba 2001). Given its rarity, the species is treated as critically endangered; it has been included in the Japanese Plant Red List (Japanese Ministry of the Environment, 2012). *Sedum tosaense* occurs in Kochi (Ohba 2001; Kobayashi 2009; Akiyama 2011) and Tokushima (Abe 1990) Prefectures in the southern part of Shikoku District, on the Pacific-

facing side of the Japanese Mainland. In addition, Song *et al.* (2004) reported this species growing on the rocky slope of a volcanic crater on Jeju Island, Korea (Fig. 2), as a first record for Korea. However, there have been no molecular phylogenetic comparisons of the Japanese and Korean populations. Therefore, we performed molecular phylogenetic analyses using the internal transcribed spacer (ITS) region of nrDNA and part of an intron of the cpDNA gene *trnLF* of *S. tosaense* plants collected from the two regions to test for the presence of a disjunct distribution pattern between geographic regions.

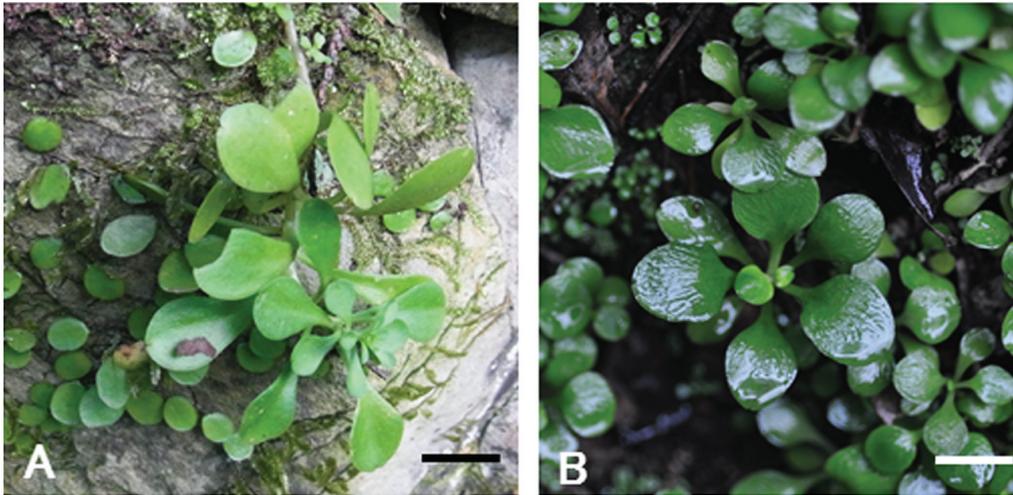


FIGURE 1. Habit of *Sedum tosaense*. A. Plant in Kochi Prefecture, Japan (8 December 2012). B. Plant on Jeju Island, Korea (6 July 2013). Bars = 3 cm.

Materials and Methods

DNA sample collection

For the molecular analyses, *Sedum tosaense* was collected from two localities in Kochi Prefecture, Shikoku District, Japan, and from one locality on Jeju Island, Korea (Table 1 and Fig. 2). We collected three *S. tosaense* plants each from one of the two Kochi localities and Jeju Island, and one from the other locality in Kochi (Table 1). We also collected six other *Sedum* species from East Asia for inclusion in our molecular phylogenetic analysis (Table 1). To test the phylogenetic relationships of *S. tosaense* samples from Kochi and Jeju Island, it was necessary for us to incorporate many other congeners in our analyses. Accordingly, we included sequences of the ITS and partial *trnLF* region reported in a previous molecular study of the genus by Mayuzumi & Ohba (2004) and stored in GenBank (Table 2). Our outgroup data comprised ITS and partial *trnLF* information for *Aeonium castello-paivae* Bolle (1859: 240), *A. gomerense* Praeger (1929: 473), *A. viscatum* Bolle (1859: 241), and *Greenovia aizoon* Bolle (1859: 242) that had been determined by Mort *et al.* (2002) and stored in GenBank (Table 2). We included 23 accessions of 17 *Sedum* species as ingroup members and four accessions of four species as outgroup member in our molecular analyses (Tables 1 and 2). Our taxonomic treatment of *Sedum* species primarily followed Mayuzumi & Ohba (2004), and it followed the treatment of Tang & Huang (1993) for Taiwanese endemic species. Voucher specimens for our collections have been deposited in the herbaria of the National Institute of Biological Resources, Korea (KB) and the National Museum of Nature and Science, Japan (TNS).

DNA extraction, amplification, and sequencing

We used the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) for DNA extraction following the manufacturer's protocol. Amplifications were conducted by the polymerase chain reaction (PCR) using an iCycler (Bio-Rad, Hercules, CA, USA) using 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) for the ITS region (ITS1, 5.8S rDNA, and ITS2), and the forward primer *trnLFa* (5'-CAT TAC AAA TGC GAT GCT CT-3') and reverse primer *trnLFf* (5'-ATT TGA ACT GGT GAC ACG AG-3') for the *trnLF* region (Taberlet *et al.* 1991). Amplifications were performed using Takara EX Taq polymerase (Takara, Otsu, Japan) with Ampdirect Plus buffer (Shimadzu, Kyoto, Japan) or EmeraldAmp PCR Master Mix dye (Takara, Otsu, Japan). After an initial 3-min denaturing at 94°C, the PCR profile comprised 35 cycles of 30 s at 94°C, 30 s at 50°C for the ITS sequence or 55°C for the *trnLF* sequence, and 1.5 min at 72°C. The PCR products were checked by electrophoresis before purification with an ExoStar clean-up kit (USB, Cleveland, OH, USA).

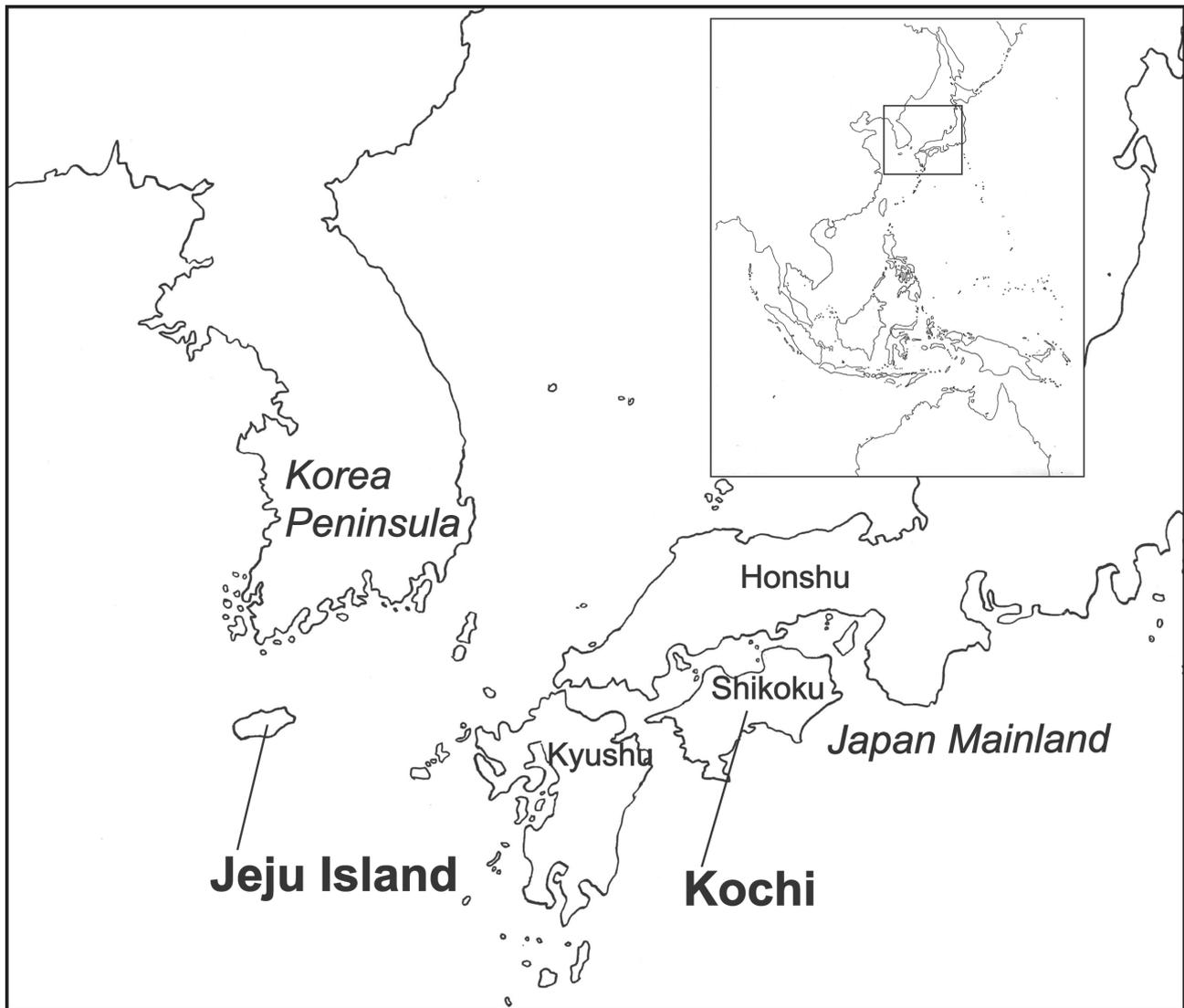


FIGURE 2. Map showing two distribution areas of *Sedum tosaense*: Kochi, Shikoku District, Japan and Jeju Island, Korea.

We performed cycle sequencing with the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) using the PCR primers listed above, adding the 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') designed by T. Yukawa (TNS, personal communication) for the ITS sequence; and the forward primer *trnLFe* (5'-GGT TCA AGT CCC TCT ATC CC-3') and reverse primer *trnLFF* (Taberlet *et al.* 1991) for the partial *trnLF* sequence. 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 software (GENETYX, Tokyo, Japan). Sequence data from this study were deposited in the DNA Data Bank of Japan (DDBJ; extant since 1983).

Phylogenetic analysis

The DNA sequences were aligned using ClustalW 1.8 (Thompson *et al.* 1994) and then adjusted manually. The Bayesian phylogenetic analysis and molecular dating were based on ITS and partial *trnLF* sequence using multispecies coalescent analysis (Heled & Drummond 2010). A multispecies coalescent analysis estimates the species tree that is most probable given unlinked multi-locus sequence data (*i.e.*, the ITS and partial *trnLF* data). The analysis is applicable to any group of individuals that has no gene flow with other groups after divergence, and the term "species" here is a catch-all that can be replaced by diverging groups at any taxonomic rank or population (Heled & Drummond 2010). In our preliminary analysis, *S. tosaense* from Kochi and Jeju Island was separated into two distinct clades. Therefore, the samples from Kochi and Jeju Island were used as separate groups. The analysis was conducted using *BEAST (Heled & Drummond 2010) implemented in BEAST ver. 1.7.5 (Drummond *et al.* 2005, 2012). For the species tree prior, the Yule and Birth-Death models were applied in two separate runs for comparison. The piecewise linear and constant root population size model was used. A uniform distribution (lower bound = 0, upper bound = 1e100) was used for

the priors of the lineage birth rate in the Yule model and the mean growth and relative death rates in the Birth-Death model and for the hyperprior on the gamma-distributed population sizes. The most appropriate evolutionary model of nucleotide substitutions was estimated using the program Kakusan4 (Tanabe 2011). The GTR+G model was optimum for both the ITS and partial *trnLF* datasets based on the Bayesian information criterion (BIC), and was applied with empirical base frequency settings. The molecular clock hypothesis was tested using the likelihood ratio (LR) test (Felsenstein 1988) implemented in PAUP* ver. 4.0b10 (Swofford 2002) and rejected for ITS and partial *trnLF* at the $P = 0.0001$ significance level (ITS, $-\ln L_{\text{noclock}} = 4,530.48$, $-\ln L_{\text{clock}} = 4,832.20$, $\text{LR} = 603.44$, d.f. = 26, $P < 0.0001$; cpDNA, $-\ln L_{\text{noclock}} = 922.55$, $-\ln L_{\text{clock}} = 973.08$, $\text{LR} = 101.06$, d.f. = 26, $P < 0.0001$). Therefore, a relaxed-clock model was used. We applied an uncorrelated lognormal distribution model for rate variation among lineages. To calculate divergence time, we used substitution rates reported based on fossils of *Aichryson* Webb & Berthel., a herbaceous genus of Crassulaceae: 5.69×10^{-9} substitutions site⁻¹ year⁻¹ for ITS and 8.24×10^{-9} substitutions site⁻¹ year⁻¹ for *trnT-L* (Richardson *et al.* 2001). Since *Aichryson* and *Sedum* are distantly related in the family (Mort *et al.* 2001) and substitution rates should be used with some range, a normal distribution was applied to the ucl.d.mean prior: for ITS, mean value = 0.00569 substitutions per site per million years [SSMY], SD = 0.001, lower bound = 0.00373 SSMY, upper bound = 0.00765 SSMY; for *trnT-L*, mean value = 0.00824 SSMY, SD = 0.001, lower bound = 0.00628 SSMY, upper bound = 0.0102 SSMY. The unweighted pair-group method of arithmetic averages (UPGMA) was used to construct a starting tree. Default priors were used for the remaining parameters. Markov chain Monte Carlo (MCMC) chains were run for 30 million generations and sampled every 1,000 generations. We checked the convergence of all parameters using the program Tracer ver. 1.5.0 (Drummond & Rambaut 2007) and the first 3,000 of the 30,000 sampled trees were discarded as burn-in. The effective sample sizes of parameters in the log file were large enough (> 200) after the burn-in. A maximum clade credibility tree was estimated with a burn-in of 10% of the sampled trees and a posterior probability (PP) limit of 0.5 by TreeAnnotator ver. 1.5.4 (Drummond & Rambaut 2007), and visualized with FigTree ver. 1.3.1 (Drummond & Rambaut 2007).

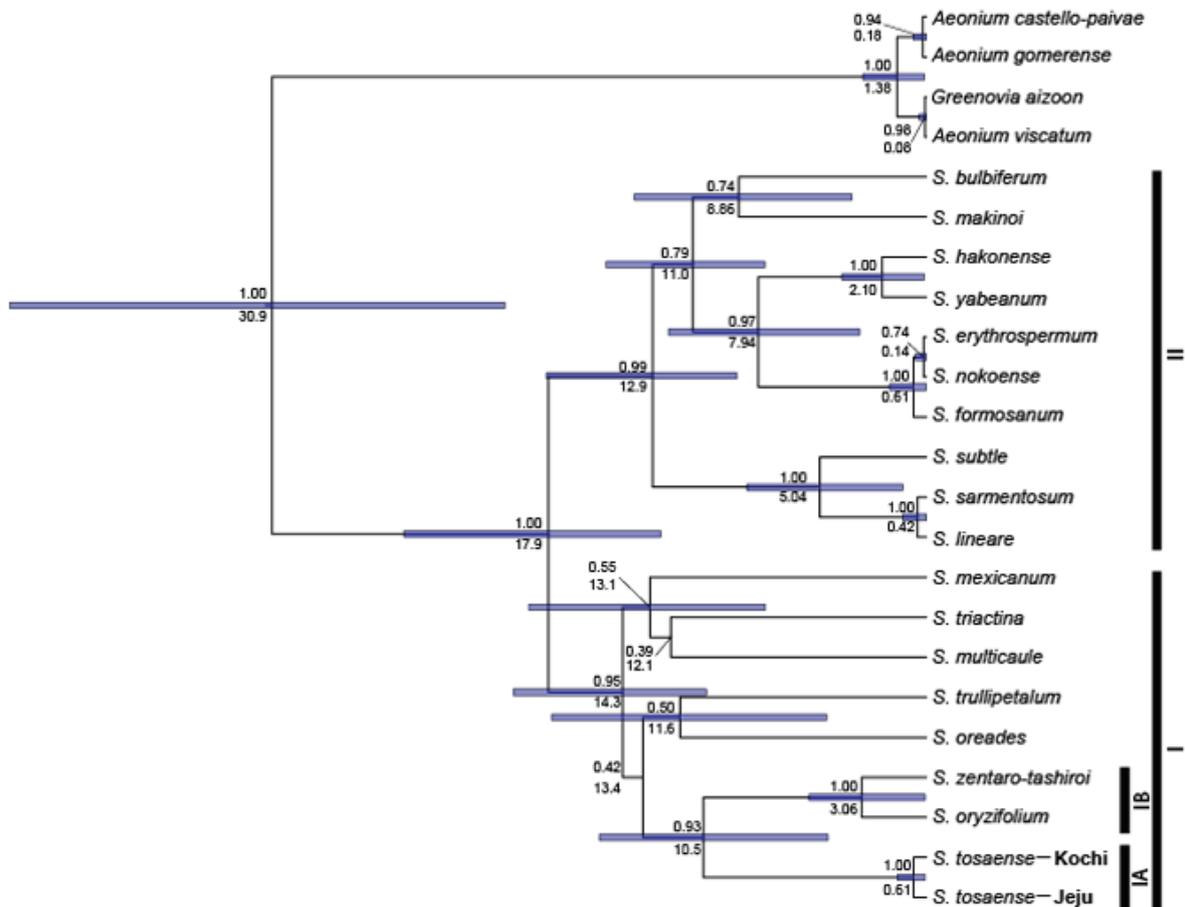


FIGURE 3. Maximum clade credibility tree using multispecies coalescent analysis based on ITS and cpDNA data. The numerals beside branches are Bayesian posterior probabilities (PP) (upper). Clade depth indicates the mean nodal age (million years) (lower) and nodes with $PP \geq 0.90$ are annotated with the 95% highest posterior density intervals for node ages by bars.

TABLE 2. GenBank accession numbers for the ITS and *trnL*F sequences determined by Mayuzumi & Ohba (2004) and Mort *et al.* (2002).

Taxon	Collection country	Collection number (Herbarium)	Accession no.	
			ITS	<i>trnL</i> F
INGROUP				
<i>S. bulbiferum</i> Makino	Japan	<i>L. Niu 1999</i> (TI)	AB088628	AB089776
<i>S. hakonense</i> Makino	Japan	<i>S. Mayuzumi C00005</i> (TI)	AB088625	AB089777
<i>S. lineare</i> Thunb.	Japan	<i>S. Mayuzumi C00120</i> (TI)	AB088623	AB089773
<i>S. mexicanum</i> Britton	Japan	<i>S. Mayuzumi C00001</i> (TI)	AB088621	AB089783
<i>S. multicaule</i> Wall. ex Lindl.	Nepal	<i>F. Miyamoto et al. T19596136</i> (TI)	AB088631	AB089782
<i>S. oreades</i> Raym.-Hamet	Nepal	<i>F. Miyamoto et al. T19420140</i> (TI)	AB088632	AB089788
<i>S. sarmentosum</i> Bunge	Japan	<i>S. Mayuzumi C00008</i> (TI)	AB088624	AB089774
<i>S. subtile</i> Miq.	Japan	<i>A. Shimizu et al. 1999</i> (TI)	AB088622	AB089775
<i>S. triactina</i> A.Berger	Nepal	<i>F. Miyamoto et al. T19596091</i> (TI)	AB088629	AB089780
<i>S. trullipetalum</i> Hook.f. et Thomson	Nepal	<i>F. Miyamoto et al. T19420132</i> (TI)	AB088630	AB089781
<i>Sedum zentaro-tashiroi</i> Makino	Japan	<i>H. Ohba 1998</i> (TI)	AB088619	AB089785
OUTGROUP				
<i>Aeonium castello-paivae</i> Bolle	Canary	<i>M.E. Mort 1519</i> (WS)	AY082127	AY082236
<i>Aeonium gomerense</i> (Praeger) Praeger	Canary	<i>M.E. Mort 1454</i> (WS)	AY082133	AY082242
<i>Aeonium viscatum</i> Webb ex Bolle	Canary	<i>M.E. Mort 1432</i> (WS)	AY082154	AY082299
<i>Greenovia aizoon</i> Bolle	Canary	<i>M.E. Mort 1425</i> (WS)	AY082112	AY082229

Results

After alignment, we obtained a matrix of 640 bp for the ITS sequence, and 286 bp for the partial *trnLF* sequence. We found three ITS types (*a*, *b*, and *c*) and three *trnLF* haplotypes (*a'*, *b'*, and *c'*) in *S. tosaense*: types *a* and *a'* in one of the two localities in Kochi; types *b* and *b'* in the other locality in Kochi; and types *c* and *c'* in the single locality on Jeju Island (Table 1).

A maximum clade credibility tree estimated using multispecies coalescent analysis based on the ITS and partial *trnLF* sequence is depicted in Fig. 3. The analyses based on the Yule and Birth-Death models had the same topology and almost the same *PP* values. The result based on the Birth-Death model is shown. In the following, we consider clades supported by *PP* \geq 0.90.

In the Bayesian tree plot (Fig. 3), the 21 ingroup members were divided into two clades (clades I and II). Clade I including a clade consisting of *S. tosaense* studied herein, *S. oryzifolium* Makino (1891a: 2), *S. zentaro-tashiroi* Makino (1910: 125), (*PP* = 88%). Within the clade composed by these three *Sedum* species, two clades were recognized: one comprised two groups of *S. tosaense* collected from Kochi and Jeju Island (clade IA; *PP* = 1.00) and the second comprised *S. zentaro-tashiroi* and *S. oryzifolium* (clade IB; *PP* = 1.00). In clade IA, the estimated divergence time of *S. tosaense* from Kochi and Jeju Island was 0.61 Ma (95% highest posterior density [HPD] interval = 1.34–0.05 Ma).

Discussion

A disjunct Sedum tosaense distribution pattern between Kochi and Jeju Island

Our molecular analysis revealed that *Sedum tosaense* from Kochi and Jeju Island belonged to a strongly supported clade (clade IA in Fig. 3). This concurs with the report by Song *et al.* (2004), who found *S. tosaense* on Jeju Island. Therefore, we have shown a disjunct distribution pattern for *S. tosaense* between the two regions through an analysis of the ITS and partial *trnLF* sequences.

Plants species that occur on both Jeju Island and in most regions of the Japanese mainland have been reported previously; *e.g.*, *Stellaria sessiliflora* Y. Yabe (1903: 194; Caryophyllaceae) (Akiyama 2006), *Lysimachiaacroadenia* Maxim. (1868: 70; Primulaceae) (Yamazaki 1993), and *Carpesium glossophyllum* Maxim. (1874: 475; Asteraceae) (Koyama 1995). Some species on Jeju Island occur in restricted western parts of the Japanese mainland, such as the Kyushu District, which is the Japanese territory closest to Jeju (Fig. 1); *e.g.*, *Adenophora tashiroi* (Makino et Nakai) Makino et Nakai (1911: 66; Campanulaceae) (Shimizu 1993). However, ours is the first report of a disjunct distribution between Kochi, a Japanese region bordering the Pacific Ocean (Fig. 1), and Jeju Island.

Although our molecular analyses clearly show that *S. tosaense* from Kochi and Jeju Island were phylogenetically closest among Asian *Sedum* species we investigated, the taxonomic affinity of the Kochi and Jeju populations remains unclear. Plants identified as *S. tosaense* were reported from Mt. Lin'an Xian area, situated in the northern part of Zhejiang Province, China (Fu & Ohba 2001), but the taxonomic status of the Chinese plants is not clear, because Fu & Rao (1998) treated them as a different subspecies, *S. tosaense* subsp. *sinense* Fu & Rao (1998: 121), and stated that this subspecies was morphologically different from var. *tosaense* in having aggregated leaves at the upper part of the sterile stem. For a comprehensive, global understanding of the taxonomic status of the species, morphological comparisons should be performed on collections from all three countries; the comparisons should include floral traits. Even if the Jeju and Zhejiang populations were subsequently shown to be taxonomically independent of *S. tosaense* in the Shikoku District of Japan, we have nevertheless shown that there is a disjunct distribution between Kochi and Jeju Island at the infraspecific level.

Dispersal of Sedum tosaense between Japan and Korea

Jeju Island was formed by volcanic activity, which started ~2.0 million years ago (Ma), and by repeated connections and separations to/from the Eurasian Continent (Woo *et al.* 2013). Recent biostratigraphic analyses indicate that the Eurasian Continent, including the Korean peninsula and Jeju Island, was connected to the Japanese mainland by a land-bridge that flooded 3.5–1.7 Ma, leading to the formation of the present-day Tsushima Strait (Kitamura & Kimoto 2006). Our molecular analysis indicated that the populations of *S. tosaense* in Kochi and on Jeju populations diverged ~0.61 (1.37–0.07) Ma. Therefore, divergence must have occurred much later than the formation of the Tsushima Strait. It is more likely that *S. tosaense* crossed the water body presently separating the two populations. Some *Sedum* species

have migrated to ocean islands; for example, *S. boninense* Yamamoto ex Tuyama (1936: 428) occurs on the Bonin Islands, Japan (Tuyama, 1936) and *S. formosanum* N.E. Br. (1885: 134) occurs on Lanyu Island, Taiwan (Tang & Huang, 1993). Presently, no relevant morphological data on the fruits and seeds of *S. tosaense* suggests a mechanism that would allow the crossing. These data are required to test any putative mechanisms for dispersal across the strait.

Phylogenetic relationships between *Sedum tosaense* and other species

Song *et al.* (2004) determined that *S. tosaense* is morphologically related to *S. bulbiferum* Makino (1891b: 2) and *S. oryzifolium*. Mayuzumi & Ohba (2004) analyzed a part of the chloroplast DNA *trnL-trnF* region and the internal transcribed spacer region of ribosomal DNA (ITS) to determine the phylogenetic relationships of 74 taxa of East Asian *Sedum*, and concluded that *S. tosaense* falls within a single clade with *S. japonicum*, *S. oryzifolium*, and *S. zentaro-tashiroi*. Our molecular phylogenetic analyses indicate that *S. tosaense* fits best within a clade comprising *S. oryzifolium* and *S. zentaro-tashiroi*, agreeing with the findings of Mayuzumi & Ohba (2004) and some of the morphological conclusions reported by Song *et al.* (2004).

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