Bull. Natl. Mus. Nat. Sci., Ser. B, **37**(2), pp. 79–86, May 22, 2011

The Phylogenetic Positions of Four Endangered *Vaccinium* Species in Japan

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(Received 15 February 2011; accepted 23 March 2011)

Abstract In total, 19 species and several varieties of *Vaccinium* are recognized in Japan. Five of them are evaluated to be endangered. In this study, molecular phylogenetic analyses for four of the five endangered species were performed using nuclear ITS and chloroplast *matK* regions in order to clarify whether they are recent derivatives, as suggested by morphological comparison. The result showed that *V. amamianum, V. boninense, V. sieboldii,* and *V. yakushimense* have the same or similar sequences to *V. emarginatum, V. wrightii, V. ciliatum,* and *V. hirtum* and relatives, respectively. Small genetic divergences between the endangered species and their respective relatives infer that the endangered species are likely neo-endemic.

Key words : endangered species, ITS, *matK*, molecular phylogeny, *Vaccinium*.

Introduction

Vaccinium L. (Ericaceae) consists of about 500 species distributed in worldwide except Australia (Vander Kloet and Dickinson, 2009). It is a morphologically diverse genus including terrestrial or epiphytic shrubs and lianas (Vander Kloet, 1990). It is divided into more than 30 sections (e.g., Sleumer, 1941; Vander Kloet and Dickinson, 2009), although the systematics is controversial (e.g., Sleumer, 1941; Stevens, 1969; Vander Kloet, 1996; Vander Kloet and Dickinson, 1992, 1999, 2009). Molecular phylogenetic studies of *Vaccinium* and Vaccinieae showed that Vaccinieae is monophyletic while *Vaccinium* is polyphyletic (Kron *et al.,* 1999, 2002; Powell and Kron, 2002). Kron *et al.* (2002) and Powell and Kron (2002) clarified several monophyletic sections in *Vaccinium,* although the other taxa within Vaccinieae remain unresolved.

Nineteen species and seven varieties of *Vaccinium* are recognized in Japan (Yamazaki, 1993). Among them, five species, i.e., *V. amamianum* Hatus. [treated as *V. emarginatum* Hayata in Yamazaki (1993)], *V. boninense* Nakai, *V. microcarpum* (Turcz. ex Rupr.) Schmalh., *V. sieboldii* Miq. and *V. yakushimense* Makino are endangered in the Red List of Japanese vascular plants (Environment Agency of Japan, 2000; Ministry of the Environment, Japan, 2007).

Vaccinium amamianum is an evergreen epiphytic shrub in evergreen forests and critically endangered (CR) according to modified IUCN conservation ratings. It occurs only on Amami-Oshima in Japan and is quite rare (Hotta, 2004). *Vaccinium amamianum* was considered to be close to, but distinct from *V. emarginatum* in Taiwan by the pubescent branches, small leaves and terminal racemose inflorescences (Hatusima, 1962, 2002). Yamazaki (1989, 1993), however, treated it as a synonym of *V. emarginatum* based on their morphological similarities.

Vaccinium boninense is an evergreen shrub and is vulnerable species (VU). It is distributed only on the Bonin Islands (Nakai, 1926; Toyoda, 1981; Yamazaki, 1993). It is morphologically similar to *V. wrightii* A. Gray distributed in the Ryukyu Islands and Taiwan, but distinct from it by the hairs on calyx and corolla, the narrow sepal, the short pedicel, and the short awn of anther (Yamazaki, 1993). *Vaccinium boninense,* along with *V. wrightii* and *V. bracteatum* Thunb., are included in sect. *Bracteata* (Sleumer, 1941; Stevens, 1969; Yamazaki, 1993), while Vander Kloet and Dickinson (2009) assigned *V. wrightii* and relatives to sect. *Eococcus*.

Vaccinium microcarpum (VU) is an evergreen dwarf shrub distributed in Hokkaido and high mountains in Honshu, Japan, and also widely in cool regions of the northern hemisphere (Yamazaki, 1993). It is quite similar to *V. oxycoccus* L. Vander Kloet (1983b) treated *V. microcarpum* as conspecific with *V. oxycoccus* by the morphological similarities, while Yamazaki (1993) treated them as distinct species by the leaf size and presence/absence of hairs on the pedicel.

Vaccinium sieboldii (CR) is a deciduous erect shrub and distributed in very limited, 65 km wide in western Shizuoka and Aichi Prefecture, central Japan (Yamazaki, 1993; Takahashi, 2001; Hirai *et al.,* 2010). Morphologically it is quite similar to, but different from *V. ciliatum* Thunb. in the long pedicel and sparse hairs on the leaf beneath and pedicel (Yamazaki, 1993). It is assigned to sect. *Ciliata* including *V. ciliatum, V. oldhamii* Miq. and relatives (Sleumer, 1941; Yamazaki, 1993). A study on their genetic diversities and phylogenetic relationships also revealed that *V. sieboldii* is quite close to *V. ciliatum* (Hirai *et al.*, 2010).

Vaccinium yakushimense (VU) is one of endemic species in Yakushima Island (Yahara *et al.,* 1987). It is usually epiphytic at high elevations (>500 m alt.) (Yamazaki, 1993; Kawahara, 1995; Takahashi, 2001). Morphologically it is close to *V. smallii* s.l., and *V. hirtum* s.l. (Vander Kloet and Dickinson, 1992; Yamazaki, 1993). They had been placed in sect. *Cyanococcus* (Sleumer, 1941) and later they were transferred to sect. *Hemimyrtillus* including several European species (Stevens, 1969; Vander Kloet, 1983a; Vander Kloet and Dickinson, 1992). Molecular phylogeny, however, showed that *V. smallii* and *V. hirtum* in northeast Asia are neither closely related to sect. *Cyanococcus* nor sect. *Hemimyrtillus,* but are close to sections *Macropelma, Myrtillus, Oxycoccoides* and *Praestantia* (Powell and Kron, 2002).

As described above, the morphological similarities between the Japanese endangered *Vaccinium* species and the respective relatives are well recognized. However, their taxonomic treatments and systematics remain unclear. To determine the molecular phylogenetic positions of the species and recognize the most closely related species to them, I conducted molecular phylogenetic analyses for the endangered species and their morphological relatives, and measured molecular differences between the endangered species and its relatives. Furthermore, the endemism of the species is also discussed.

Materials and Methods

Leaf samples of 11 *Vaccinium* species from Japan and one species from Taiwan were collected in the field or from cultivated plants (Table 1). One of the five endangered species, *V. microcarpum,* was not analyzed in this study because material was not available. DNA was extracted from fresh or silica-gel-dried materials using a QUIAGEN DNeasy Mini Kit (QUIAGEN, Valencia, CA) following the manufacturer's instruction.

The internal transcribed spacer regions of 18S–26S nuclear ribosomal DNA (ITS), and *matK* (a maturase-encoding gene) were analyzed. Primers for amplification are 17SE (Sun *et al.*, 1994) and '26SE' (Topik *et al.,* 2005, modified 26SE in Sun *et al.,* 1994) in the ITS region, and trnK-3914F and trnK-2R (Johnson and Soltis, 1994) in *matK* with internal primers MK-F1 and MK-F2 (Koi *et al.,* 2008). PCR was performed using a DNA thermal cycler (Perkin-Elmer 9700, Applied Biosystems, Foster, CA) with *Ex Taq* DNA polymerase (TaKaRa Bio, Tokyo, Japan) and Ampdirect Plus (Shimadzu, Kyoto, Japan) in 35 denaturation, annealing, and elongation cycles (30 sec at 94°C, 30 sec at 50–55°C and 90 sec at 72°C) with a final elongation step (7 min at 72°C). The

Table 1. Species used in molecular phylogenetic analysis, sources and GenBank accession no. of ITS and *matK* regions

Species		Source	GenBank accession No. ITS	GenBank accession No. *matK*
V. amamianum Hatus.		Cultivated in Koishikawa Botanical Garden; *Tsutsumi s. n.*	AB623180	AB623166
V. boninense Nakai		Cultivated in Koishikawa Botanical Garden; *Tsutsumi s. n.*	AB623184	AB623168
V. bracteatum Thunb.		Yakushima, Kagoshima, Japan; *S. Tagane & Y. Tsujita* (TNS763429)	—	AB623177
V. ciliatum Thunb.		Cultivated in Tsukuba Botanical Garden; *Tsutsumi ARA1*	AB623188	AB623172
V. emarginatum Hayata	A	Kaohsiung Co., Taiwan; *Tsutsumi et al.*(TNS736868)	AB623181	AB623166
	B	Kaohsiung Co., Taiwan; *Tsutsumi et al.* (TNS736871)	AB623182	AB623166
V. hirtum Thunb.	A	Shinshiro, Aichi, Japan; *Tsutsumi Ho2-20100514*	AB623187	AB623171
	F	Fukushima, Japan; *Tsutsumi Fuku172-20100509*	AB623185	AB623169
V. oldhamii Miq.	K	Hamamatsu, Shizuoka, Japan; *Tsutsumi 175-19-N-1*	AB623189	AB623174
	M	Hamamatsu, Shizuoka, Japan; *Tsutsumi M20o-20100513*	AB623189	AB623173
V. sieboldii Miq.	K	Hamamatsu, Shizuoka, Japan *Tsutsumi 175ws-20100513*	AB623191	AB623175
	M	Hamamatsu, Shizuoka, Japan; *Tsutsumi M20s-20100513*	AB623190	AB623176
V. smallii A. Gray var. *glabrum* Koidz.		Hamamatsu, Shizuoka, Japan; *Tsutsumi 175sm-20100513*	AB623186	AB623170
V. wrightii A. Gray		Amami-Oshima, Kagoshima, Japan; *Ebihara et al.* (TNS764246)	AB623192	AB623178
V. yakushimense Makino		Yakushima, Kagoshima, Japan; *S. Tagane & Y. Tsujita* (TNS763260)	AB623183	AB623167

PCR products were purified with ExoSAP-IT (USB corporation, Cleveland, OH) following the manufacturer's instruction. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The sequences obtained were assembled using Seqman II (Dnastar, Madison, WI). The assembled sequences were aligned by Clustal X program (Thompson *et al.,* 1997) and then aligned manually. In addition to the sequences analyzed, registered sequences in GenBank used in Powell and Kron (2002) were added for phylogenetic analyses.

Phylogenetic analyses were performed by MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The bases that could not been identified were treated as unknown (N). Gaps were treated as missing data. MrModeltest 2.0 (Nylander, 2004) was used to determine the nucleotide substitution model for Bayesian analysis. Bayesian searches were conducted by mcmc with four chains over four million and two million generations for the ITS dataset and the *matK* dataset, respectively, sampling every 100 generations. Trees obtained before stationary generations were discarded as burn-in trees and the rest of trees were used to calculate posterior probabilities. *Leucothoe fontanesiana* (Steudel) Sleumer*, Andromeda polifolia* L.*,* and *Zenobia pulverulenta* (Bartram ex Willd.) Pollard were used as outgroups based on the results of Powell and Kron (2002).

Table 2. Differences of sequences in ITS and *matK* regions between four endangered species and their closely related species

	ITS	*matK*
V. amamianum–V. emarginatum	0–1	0
V. boninense–V. wrightii	1	1
V. boninense–V. bracteatum	—	12
V. sieboldii–V. ciliatum	0–3	0–1
V. sieboldii–V. oldhamii	0–3	5–7
V. yakushimense–V. smallii	3	3
V. yakushimense–V. hirtum (F)	2	1

Results

Differences of sequences in nuclear ITS (ca. 650 bp) and chloroplast *matK* (ca. 1520 bp) between the four endangered species and their closely related species are shown in Table 2. *Vaccinium amamianum* had no or one substitution distinguishable from *V. emarginatum* samples from Taiwan in the ITS and *matK* regions. *Vaccinium boninense* was close to *V. wrightii* and differed from it in only 1 substitution in each of the ITS and *matK* regions. In contrast, *V. boninense* differed from *V. bracteatum* in 12 substitutions in *matK,* although the ITS region of *V. bracteatum* could not be analyzed well in this study. *Vaccini-*

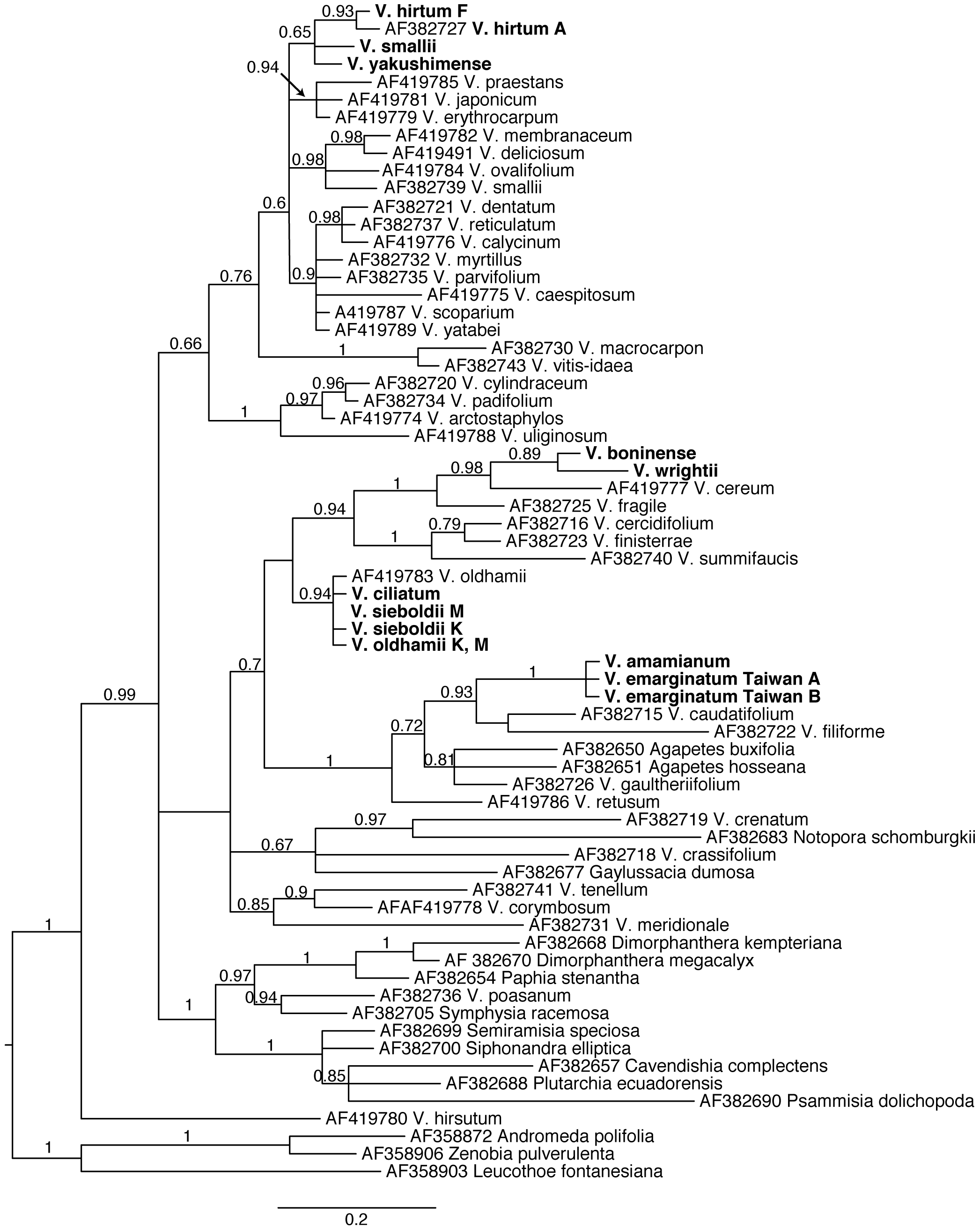

Fig. 1. Consensus tree by Bayesian analysis based on the nuclear ITS sequence dataset. Figures above branches indicate posterior probabilities (p>0.6) calculated by Bayesian analysis. Names shown by bold indicate samples analyzed in this study.

um sieboldii had no or a few substitutions distinguishable from *V. ciliatum,* and differed from *V. oldhamii* in 0–3 substitutions in ITS and 5 to 7 substitutions in *matK*. *Vaccinium yakushimense* differed from *V. smallii* or *V. hirtum* by a few substitutions in both the nuclear ITS and the chloroplast *matK* regions.

The molecular phylogenetic trees deduced from each of the nuclear ITS region and the *matK* sequences showed that all samples were nested in the clade Vaccinieae (Figs. 1 and 2). *Vaccinium amamianum,* along with *V. emarginatum,* formed a monophyletic clade with *V. caudatifolium* Hayata and *V. filiforme* (J.J. Smith) Sleumer and the clade was nested in the Agapetes clade shown in Powell and Kron (2002). *Vaccinium boninense* formed a clade with *V. wrightii* with high supports in both the ITS and *matK* trees. The clade was sister to *V. cereum* (L.f.) Forster, and then they were close to *V. fragile* Franch. in the ITS tree, while the clade formed a monophyletic clade with *V. bracteatum, V. sieboldii, V. ciliatum* and *V. oldhamii* in the *matK* tree with high posterior probabilities. *Vaccinium sieboldii, V. ciliatum* and *V. oldhamii* formed a monophyletic clade both in the ITS tree and the *matK* tree, although the phylogenetic position remained unclear in the ITS tree because of low supports of the clade. *Vaccinium yakushimense* merged in the clade with *V. smallii* and *V. hirtum.* The clade then was sister to the group of *V. japonicum* Miq. and *V. praestans* Lamb. in the *matK* tree, while the phylogenetic position of *V. yakushimense, V. hirtum* and *V. smallii* remained unsolved in the ITS tree.

Discussion

The present results revealed the phylogenetic positions of the four endangered species in *Vaccinium,* and suggested their closely related species, which were the same as the candidates morphologically estimated. The results also showed genetic divergences between the endangered species and their relatives.

Similarities in both the ITS and the *matK* sequences between *V. amamianum* and *V. emarginatum* supported the taxonomic treatment that *V. amamianum* is synonymous to *V. emarginatum* (Yamazaki, 1993), a common species in Taiwan (Li *et al.,* 1998). To clarify the taxonomic treatment and the genetic diversity of *V. amamianum* and *V. emarginatum*, further molecular and morphological analyses are needed using more samples. *Vaccinium amamianum* and *V. emarginatum* are not found in the Okinawa Prefecture, located between Taiwan and Amami-Oshima Island (Yamazaki, 1993). They were sister to Taiwanese *V. caudatifolium* Hayata and Bornean *V. filiforme* (J.J. Smith) Sleumer. The phylogenetic relationships and patterns of their distributions suggest that *V. amamianum* was likely derived from southeastern Asia and it survives only in Amami-Oshima Island in Japan. Thus, the endangered *V. amamianum* occurs at the isolated northern end of the species group and hardly differentiated from the neighboring *V. emarginatum.*

About 70% of the species on the Bonin Islands are related to Southeast Asian species, and considered to be recently derived (Toyoda, 1981; Shimizu, 2010). *Vaccinium boninense* is also morphologically and phylogenetically close to *V. wrightii,* a common species in Ryukyus and Taiwan. *Vaccinium cereum,* close to *V. boninense* and *V. wrightii* in the ITS tree and far from them in the *matK* tree (Figs. 1 and 2), is suspected to have a hybrid origin (Powell and Kron, 2002). The conflict of the phylogenetic position of the clade of *V. boninense* and *V. wrightii* between the ITS tree and the *matK* tree seems also likely to be caused by the introgression in the group.

Few difference was observed in the ITS and *matK* regions between *V. sieboldii* and *V. ciliatum,* while a detail analysis using SSR markers suggested their differentiation (Hirai *et al.,* 2010). Hirai *et al.* (2010) also showed a clear distinction between *V. sieboldii* and *V. oldhamii* in SSR markers. *Vaccinium sieboldii* occurs in quite restricted area in central Japan and is separate from *V. ciliatum* distributed fragmentally in western Japan. Thus, *V. sieboldii* and *V. ciliatum* differentiated recently in fragmental areas, and

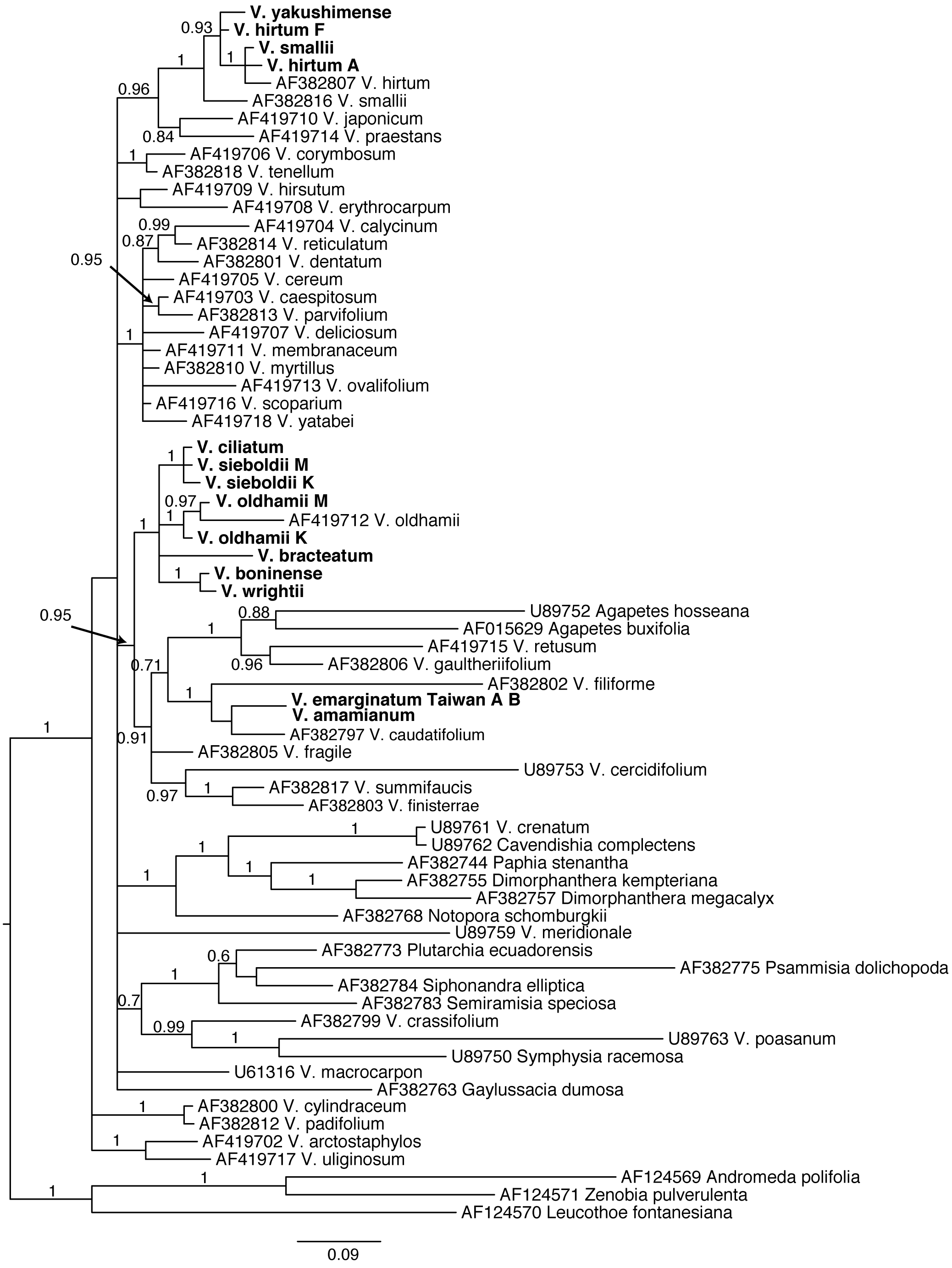

Fig. 2. Consensus tree by Bayesian analysis based on the chloroplast *matK* sequence dataset. Figures above branches indicate posterior probabilities (p>0.6) calculated by Bayesian analysis. Names shown by bold indicate samples analyzed in this study.

the former is threatened. In contrast, *V. oldhamii* is widely distributed in Japan, Korea and China, overlapping with *V. sieboldii* and *V. ciliatum* (Yamazaki, 1993; Hirai *et al.,* 2010).

Vaccinium yakushimense differed from *V. smallii* and *V. hirtum* in a few substitutions, although they are morphologically similar. *Vaccinium yakushimense* is distinct from those of *V. smallii* and *V. hirtum* in the distribution (Vander Kloet and Dickinson, 1992; Yamazaki, 1993); *V. yakushimense* occurs only at high altitudes of Yakushima Island, southern Kyusyu. *Vaccinium smallii* s.l. is distributed in the Kurile islands and Sakhalin, Russia, North and South Korea, and Honshu and Shikoku, Japan. *Vaccinium hirtum* s.l. is distributed in Hokkaido to northern Kyushu, Japan, and South Korea. Thus, *Vaccinium yakushimense* evolved as an epiphyte in the isolated southern end of the predominantly terrestrial group.

The four endangered species, *V. amamianum, V. boninense, V. sieboldii* and *V. yakushimense,* are endemic species to quite restricted distributions areas, disjunct from the most closely related species. Although the samples analyzed are insufficient, the small genetic divergences between the endangered species and their relatives infer that the endangered species are neo-endemic and threatened partly due to limited distribution areas, or a local variant in the periphery of the distribution region. Further comparison between the endangered plants and relatives is necessary to clarify the taxonomic status and diversification of endemic or local species that are apt to be threatened.

Acknowledgments

I thank K. Ando, A. Ebihara, Y. Hirayama, C.-I Huang, M. Kato, G. Kokubugata, T. Muramatsu, A. Okuda, C.-I Peng, S. Tagane, Y. Tsujita, H. Yamashita, C.-K. Yang, and Koishikawa Botanical Garden, The University of Tokyo, for providing the materials and/or help during the field trips. I also gratefully thank Y. Hirayama, N. Katayama, and A. Uehara for technical assistance and M. Kato for useful comments to the manuscript. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and Showa Seitoku Memorial Foundation.

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