J Plant Res (2006) 119:385–395
Digital Object Identifier (DOI) 10.1007/s10265-006-0286-y

REGULAR PAPER

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Evaluation of morphological and molecular variation in *Plantago asiatica* var. *densiuscula*, with special reference to the systematic treatment of *Plantago asiatica* var. *yakusimensis*

Received: February 17, 2006 / Accepted: March 30, 2006 / Published online: June 14, 2006

Abstract Morphological and molecular variations in *Plantago asiatica* L. var. *densiuscula* Pilg. were analyzed to evaluate the genetic basis for recognizing the dwarf variety *P. asiatica* var. *yakusimensis* (Masam.) Ohwi. Considerable variation in the leaf size of *P. asiatica* var. *densiuscula* was observed, and no morphological discontinuities were found between the dwarf types of *P. asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis*. Morphological analysis of plants grown under standardized conditions revealed that both environmental plasticity and genetic differentiation contributed to the dwarfisms. Molecular phylogenetic analysis of rDNA internal transcribed spacer (ITS) regions and the *SUC1* locus encoding a sucrose transporter revealed that *P. asiatica* var. *yakusimensis* was genetically unique although the differentiation level was low. From the above results, we concluded that *P. asiatica* var. *yakusimensis* should be reduced to a form of *P. asiatica* var. *densiuscula.* Furthermore, the geographic distribution of the *SUC1* genotype suggested multiple origins of dwarves, and possible hypotheses for the origins of dwarves are discussed.

Key words Dwarfism · Plasticity · Genetic differentiation · Leaf size · *Plantago asiatica* var. *densiuscula* · *Plantago asiatica* var. *yakusimensis*

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Introduction

Plantago asiatica L. var. *densiuscula* Pilg. (Plantaginaceae) is widely distributed in Japan, including the Ryukyu Islands, as well as in Taiwan and China, and many variations in morphology and size have been reported (e.g., Pilger 1922). In particular, two types of dwarfism are recognized in Japanese populations. One of the dwarves is *P. asiatica* L. var. *yakusimensis* (Masam.) Ohwi, which is endemic to Yakushima Island, Kagoshima Prefecture, Japan (Masamune 1930; Ohwi 1953) where many dwarf plants have been found (e.g., Tsukaya 2002, 2005; Yokoyama et al. 2003). The other dwarf is the "minima" type, which is known from various shrines and temples in Japan (Nakayama et al. 1996). The presence of this extreme dwarf type in the Miidera temple, Shiga Prefecture, has been known since the Edo period (1603–1867; Iwasaki 1828). Other dwarf forms have also been reported from various localities in Japan, e.g., Miyajima (Itsukushima) Island, Hiroshima Prefecture, and have sometimes been treated as *P. asiatica* var. *yakusimensis* (Seki 1975). Because many environmental factors can affect plant size in *P. asiatica* (Sunohara and Ikeda 2003), the validity of using leaf size in taxonomic treatments of these varieties is unclear. Previously, Nakayama et al. (1996) showed that four minima types collected from the Kyoto area exhibited stable dwarfism under cultivation. However, our preliminary observations showed that some dwarves lost a part of their dwarfism when cultivated. Moreover, variations in ploidy level ($2n = 24, 36$) that may result in size variations are known in *P. asiatica* var. *densiuscula* (Ishikawa 1916; Iwatsubo et al. 2000). In addition to the above characters, Nakayama et al. (1996) reported that the seed number of the minima type was slightly more than that of the standard type (six in the minima and five in the standard type) while the number of seeds per capsule of *P. asiatica* var. *yakusimensis* was reported to be less than that of *P. asiatica* var. *densiuscula* (Yamazaki 1981). Despite these factors, this taxon has not been recently reexamined.

Thus, in the present study, we analyzed the extent and plasticity of leaf size of *P. asiatica* in Japan to determine if

dwarves and non-dwarves are distinguishable based on leaf size. Moreover, number of seeds per capsule, ploidy levels, nucleotide sequences of rDNA internal transcribed spacer (ITS) regions, and the plasma membrane sucrose–proton symporter1 (*SUC1*) locus were determined to analyze the degree of genetic differentiation among populations of *P. asiatica* in Japan.

Materials and methods

Plant materials and growing conditions

Table 1 lists the 39 samples used for morphological characterization or molecular analysis or both. The sampling locations for *P. asiatica* var. *densiuscula* were selected to cover a wide range of locations in Japan and included some localities in Taiwan and Korea for comparison. In particular, temples and shrines were selected for sampling locations for the analysis of the minima type. The minima type is reported to be a genetically fixed dwarf found in well-managed areas of temples and shrines (Nakayama et al. 1996). In this study, we defined the minima type as dwarves with leaves less than 3-cm long growing in temples and shrines. *P. asiatica* var. *yakusimensis* was collected from two populations on Yakushima Island (permission no. Kyushu 473, Japanese Ministry of the Environment).

For morphological analysis of cultivated plants, all plants were grown under the same conditions. Seeds were germinated in well-moisturized plastic containers, and after germination, seedlings were transferred to rockwool moistened with 0.5 g l^{-1} of HYPONeX (HYPONeX, Osaka, Japan) and grown for 1 month at 22°C under white fluorescent light (30 µmol m^{-2} s^{-1}) with a long-day photoperiod (16 h light, 9 h dark). The young plants were then transplanted to plastic pots filled with vermiculite and grown at least until flowering at 22°C under continuous white fluorescent light (30–50 µmol m^{-2} s^{-1}).

Leaf measurements

For all leaf measurements, plants were selected that had at least one inflorescence to ensure maturity, and three mature leaves were measured per individual. For collected specimens, we measured the dimensions of leaf blades from 88 individuals of *P. asiatica* var. *densiuscula* from 71 localities and nine individuals of *P. asiatica* var. *yakusimensis* from five localities from the herbarium of the University of Tokyo (TI). In addition, 22 individuals collected from 12 localities in Japan were also measured after being dried in the same manner as the herbarium specimens. Only dwarf individuals collected from Yakushima Island were annotated as *P. asiatica* var. *yakusimensis*, and dwarves collected from temples and shrines were treated as the minima type.

For measurements of leaf-blade length before and after cultivation, we chose five standard types (Table 1, samples 18, 24, 25, 34, and 35), eight minima types collected from temples and shrines (Table 1, samples 4–7, 9, and 11–13), a dwarf from an area in which the density of deer populations is extremely high (Table 1, sample 3), two dwarves from islands with both shrines and high deer densities (Table 1, samples 14 and 15), and two *P. asiatica* var. *yakusimensis* from Yakushima Island, which is also deer habitat (Table 1, samples 1 and 2). For leaf-length measurements of plants grown in their native habitat, at least one fresh or dried individual was analyzed per locality. Drying of leaves resulted in less than 0.3 ± 0.1 cm differences in leaf length (average ± standard deviation, $n = 9$). Leaf sizes under cultivated conditions were determined by measuring leaves of progenies of representative individuals from each locality. Each strain was self-pollinated one to three times.

Chromosome observations and determination of ploidy levels

We counted chromosome numbers for 24 individuals of *P. asiatica*, including one individual of *P. asiatica* var. *yakusimensis*. Root tips were pretreated in 2 mM 8-hydroxyquinoline solution for 1 h at 20°C and then transferred from the solution and stored at 4°C for 15 h. Root tips were then fixed in Newcomer's fluid (6:3:1:1:1 isopropanol, propionic acid, petroleum ether, acetone, 1,4-dioxane) and macerated in 1 N HCl at 20°C for 1 h, 60°C for 10 min, and 20°C for 0.5 h. Finally, the root tips were stained with 2% lactopropionic orcein and squashed for cytological observation. Because all examined individuals were found to be $2n = 24$ (tetraploid), we employed flow cytometry to determine ploidy levels for the remaining individuals using the plants already identified to be tetraploid as external standards. A fresh fragment (approximately 2 × 2 cm) of mature leaf was chopped with a razor blade in 500 µl buffer (10 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 0.1% Triton X-100; 10 µl ml^{-1} RNase; 40 mg ml^{-1} polyvinylpyrrolidone, PVP; 10 µl ml^{-1} ß-mercaptoethanol) on ice. The buffer, containing isolated nuclei, was filtered through a CellTrics 30-µm mesh filter (Partec, Münster, Germany) and stored at 37°C for 30 min. The isolated nuclei were stained with 50 µl propidium iodide solution for a few hours and then analyzed using flow cytometry (COULTER EPICS XL-MCL; Beckman Coulter, Fullerton, CA, USA). The ploidy level of each individual was determined by comparing the peak position of the fluorescence histogram with those of the external tetraploid standards.

Isolation of genomic DNA, sequencing, and phylogenetic analysis

Total genomic DNA was extracted from fresh or dried plant material using a DNeasy plant mini kit (Qiagen Japan, Tokyo). The ITS regions were amplified after incubation at 94°C for 2 min, with 30 cycles of incubation at 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min, and with a final extension for 7 min with AB101 and AB102 primers (Douzery et al. 1999).

Table 1. Samples used in this study

Species (type) sample name	Ploidy level[b], No. of Ch[c]	ITS, *SUC1* genotype	No. of seeds, Av (SD, *N*)	Voucher (herbarium)	Locality
P. asiatica var. *yakusimensis*					
1. Mt. Miyanoura	4x, 2*n* = 24	12, VIII	3.7 (1.2, 15)	H. Tsukaya (TI)	Japan, Kagoshima, Yakushima Island, Mt. Miyanoura
2. Mt. Kuromi	4x	12, VIII	4.0 (0.7, 21)	T. Kozuka (TI)	Japan, Kagoshima, Yakushima Island, Mt. Kuromi
P. asiatica var. *densiuscula* (dwarf from areas with high deer density)					
3. Nara-koen[a]	4x, 2*n* = 24	8, VII	4.5 (1.2, 26)	H. Tsukaya (TI)	Japan, Nara, Nara-koen park
Minima type					
4. Hachiman-jinjya shrine	4x, 2*n* = 24	1, II	4.4 (1.0, 31)	T. Kozuka (TI)	Japan, Gifu, Shirakawa Hachiman-jinjya shrine
5. Mii-dera temple[a]	4x	1, I	6.5 (1.6, 12)	N. Ishikawa (TI)	Japan, Shiga, Mii-dera temple
6. Nanzen-ji temple[a]	4x, 2*n* = 24	1, III	6.2 (1.2, 12)	N. Ishikawa (TI)	Japan, Kyoto, Nanzen-ji temple
7. Ise-jingu shrine	4x, 2*n* = 24	1, I	5.3 (1.0, 30)	N. Ishikawa (TI)	Japan, Mie, Ise-jingu shrine
8. Ohyamazumi-jinjya shrine[a]	4x	7, III	6.2 (1.0, 28)	N. Ishikawa (TI)	Japan, Ehime, Ohmi-shima Island, Ohyamazumi-jinjya shrine
9. Isono-jinjya shrine[a]	4x, 2*n* = 24	5, III	5.0 (1.1, 23)	H. Tsukaya (TI)	Japan, Ehime, Isono-jinjya shrine
10. Kushida-jinjya shrine	4x, 2*n* = 24	1, III	5.7 (0.5, 30)	N. Ishikawa (TI)	Japan, Fukuoka, Kushida-jinjya shrine
11. Dazaifu shrine	4x, 2*n* = 24	1, III	5.3 (1.5, 33)	N. Ishikawa (TI)	Japan, Fukuoka, Dazaifu-tenmangu shrine
12. Amamiohshima[a]	4x	1, III	4.9 (0.7, 29)	H. Tsukaya (TI)	Japan, Kagoshima, Amamiohshima Island, a shrine on Mt. Yuwan
13. Tanegashima[a]	4x, 2*n* = 24	6, III	5.6 (0.6, 18)	H. Tsukaya (TI)	Japan, Kagoshima, Tanega-shima Island, family cemetery for the Lord of Tanega-shima since 1468
Dwarves from islands with both a shrine and high deer density					
14. Kinkazan Island	4x, 2*n* = 24	1, I	5.3 (1.0, 31)	N. Ishikawa (TI)	Japan, Miyagi, Kinkazan Island
15. Miyajima Island	4x, 2*n* = 24	1, IV	4.8 (0.9, 25)	H. Tsukaya (TI)	Japan, Hiroshima, Miyajima Island
Standard types					
16. Hokkaido-jingu shrine	4x, 2*n* = 24	1, I	4.0 (0.9, 11)	N. Ishikawa (TI)	Japan, Hokkaido, Hokkaido-jingu shrine
17. Akita	4x, 2*n* = 24	2, I	3.4 (0.8, 15)	H. Tsukaya (TI)	Japan, Akita, Hiedahachimangu shrine
18. Hikariyama	4x, 2*n* = 24	1, I	3.7 (0.7, 35)	N. Ishikawa (TI)	Japan, Miyagi, Mt. Hikariyama
19. Hongo	4x, 2*n* = 24	1, I	5.7 (1.0, 22)	H. Tsukaya (TI)	Japan, Tokyo, Hongo campus of the University of Tokyo
20. Kamakura[a]	4x	3, I	5.2 (1.4, 29)	H. Tsukaya (TI)	Japan, Kanagawa, Kamakura, Zaimokuza
21. Tateyamaeki	4x, 2*n* = 24	4, ND	4.6 (0.7, 33)	T. Kozuka (TI)	Japan, Toyama, roadside near the Tateyama cable railway station
22. Ontake	4x	10, I	4.4 (0.7, 17)	H. Tsukaya (TI)	Japan, Nagano, Mt. Ontake
23. Kitayatsugatake	4x	9, ND	4 (0.7, 24)	N. Ishikawa (TI)	Japan, Nagano, near ropeway station on Mt. Yokodake, alt. 2,200 m
24. Bijyodaira	4x, 2*n* = 24	9, ND	5.3 (1.0, 28)	T. Kozuka (TI)	Japan, Toyama, near from Bijyodaira cable railway station, alt. 977 m
25. Okazaki[a]	4x, 2*n* = 24	8, V	5.1 (0.9, 23)	N. Ishikawa (TI)	Japan, Aichi, Okazaki, campus of the National Institute of Basic Biology
26. Maruyama	4x, 2*n* = 24	8, VII	5.8 (1.3, 24)	N. Ishikawa (TI)	Japan, Aichi, Mt. Maruyama
27. Sakatejima[a]	4x	8, VII	5.9 (1.0, 25)	H. Tsukaya (TI)	Japan, Mie, Sakatejima Island
28. Mii-dera temple (standard)	4x	1, ND	5.7 (0.6, 20)	N. Ishikawa (TI)	Japan, Shiga, Mii-dera temple
29. Awajishima	4x, 2*n* = 24	1, III	5.6 (0.6, 26)	N. Ishikawa (TI)	Japan, Hyogo, Awajishima Island, Higashiura, roadside
30. Daisen	4x, 2*n* = 24	1, ND	5.1 (0.4, 39)	H. Tsukaya (TI)	Japan, Tottori, Mt. Daisen, Amida-do temple, alt. 800 m
31. Hiruzen	4x, 2*n* = 24	1, III	4.7 (0.9, 30)	E. Mano (TI)	Japan, Okayama, Hiruzen, spring of Shiogama
32. Karasugasen	4x	1, ND	4.7 (0.6, 22)	H. Tsukaya (TI)	Japan, Tottori, Mt. Karasugasen, alt. 1,270 m
33. Hakozaki	4x	1, III	6.0 (0.8, 15)	N. Ishikawa (TI)	Japan, Fukuoka, Hakozaki campus of Kyushu University
34. Dazaifu shrine (standard)[a]	4x, 2*n* = 24	1, III	5.3 (1.0, 29)	N. Ishikawa (TI)	Japan, Fukuoka, Dazaifu, Dazaifu-tenmangu shrine, plum orchard
35. Oushushoumae[a]	4x	6, III	5.0 (0.6, 22)	T. Kozuka (TI)	Japan, Kagoshima, Sakurajima Island, Roadside of Oushushoumae junction
36. Sakurajimaguchi	4x, 2*n* = 24	11, III	5.4 (0.8, 16)	T. Kozuka (TI)	Japan, Kagoshima, Sakurajima Island, parking area
37. TW106	4x	1, VII	5.5 (0.8, 21)	H. Tsukaya (TI)	Taiwan, Keelung, Tawulun Fort
38. TW115	4x	1, VII	ND	H. Tsukaya (TI)	Taiwan, Ilan
39. Mt. Halla	4x	1, VI	4.1 (0.7, 24)	H. Ikeda (TI)	Korea, Cheju Island, Mt. Halla, alt. 1,700 m

Av Average number of seeds per capsule, *SD* standard deviation, *N* number of capsules examined, *ND* not determined, *TI* University of Tokyo
[a]Sample used for determination of leaf size in a native habitat (Fig. 1)
[b]Ploidy level determined by flow cytometry
[c]Chromosome (*Ch*) number determined by microscopy

To design polymerase chain reaction (PCR) primers for *SUC1* analysis, sequence information of the entire genomic sequence of *SUC1* (4.7 kb) in *P. major* L. (Gahrtz et al. 1996), which is very closely related to *P. asiatica* (e.g., Rønsted et al. 2002) was used. In general, more polymorphisms are found at noncoding regions than at coding regions. Therefore, for sequencing analysis of the 1.4-kb region of the *SUC1* locus, a 2.2-kb region consisting of 0.1 kb of the 5′ untranslated region and 2.1 kb of the 5′ upstream region was amplified. Among 39 strains examined, *SUC1* sequences from six strains could not be obtained because of smeared PCR amplification or difficulty in determining the sequence. Thus, we used the remaining 33 strains for *SUC1* analysis. PCR reactions were performed after incubation at 94°C for 2 min, with 30 cycles of incubation at 94°C for 15 s, 58°C for 30 s, and 68°C for 3 min, with a final extension for 7 min with SUC1-F1 (5′-TGCATCGTGCGCGATTAATG-3′) and SUC1-R1 (5′-TTCAATTCCTGACAATTCAC 3′) primers.

All PCR reactions were performed in a 50-µl volume using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). Amplified products were purified using a QIAquick PCR purification kit (Qiagen Japan) according to the manufacturer's procedures. Cycle sequencing reactions were performed with approximately 80–100 ng purified product and PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan). Primers used for sequencing reactions of the *SUC1* fragment were SUC1-F1, SUC1-F2 (5′-GTCATGTTTATTATAGGATG-3′), SUC-F3 (5′-CCCATGTTCATTCTTAGT-3′), SUC1-R1, and SUC1-R2 (5′-ATTTGATCTTTTCATCAATA-3′).

Sequences were aligned using the CLUSTALX program (Thompson et al. 1997), and results were manually modified to minimize the numbers of insertions and deletions (indels). Phylogenetic relationships of samples used in this study were then analyzed with the maximum-parsimony (MP) method. For the analysis, we used the PAUP* 4.0b program (Swofford 1998). All characters were weighted equally. All indels were coded as missing data. MP analysis was conducted through a branch-and-bound search. Bootstrap analysis (Felsenstein 1985) with 1,000 replications was performed using the same program. We also applied the median-joining (MJ) network method (Bandelt et al. 1999) to determine the relationships of ITS haplotypes and *SUC1* genotypes. The computer program Network ver. 3.1 (Röhl 1999; available at http://www.fluxus-engineering.com) was used for the analyses, with $\varepsilon = 0$. All mutation events were weighted equally in the MP analysis.

Results

Leaf-size variation in herbarium specimens

As shown in Fig. 1, great variation in leaf size (average and standard deviation for an individual: 1.2 ± 0.1–12.6 ± 0.3 cm in length, 0.7 ± 0–8.8 ± 1.6 cm in width; white circles in Fig. 1) was observed in *P. asiatica* var. *densiuscula*. This size range almost encompassed the range of variation seen in *P. asiatica* var. *yakusimensis* (1.0 ± 0–4.8 ± 0.1 cm in length, 0.6 ± 0–2.9 ± 0.2 cm in width; black circles in Fig. 1) and the minima type (0.5 ± 0.1–2.6 ± 0.1 cm in length, 0.4 ± 0.1–2.5 ± 0.1 cm in width; gray squares in Fig. 1). These results indicate that *P. asiatica* var. *yakusimensis* and the minima type cannot be distinguished from *P. asiatica* var. *densiuscula* based only on leaf size.

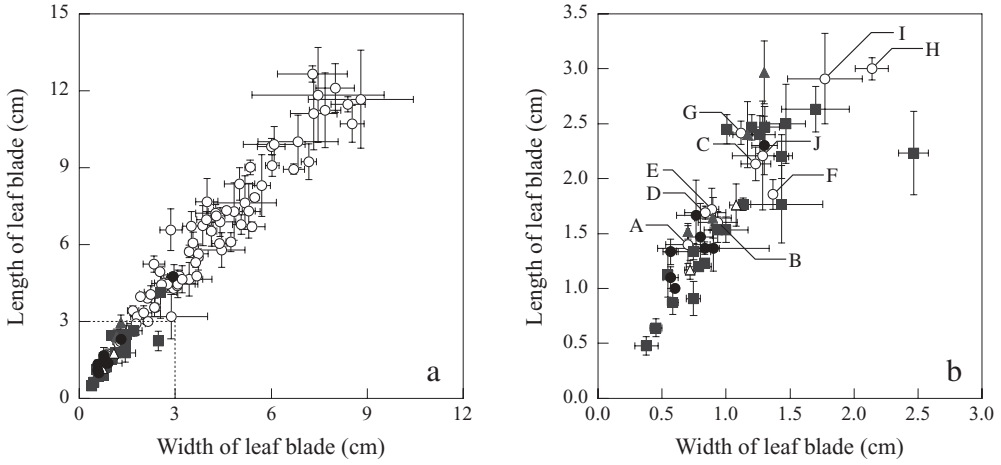

Fig. 1. Variations in leaf size in individuals of *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis*. **a** Scatter plot of leaf dimensions. Individuals defined as dwarves, having leaf blades less than 3 cm in length and width, are enclosed by the *dotted line*. **b** Magnification of the area enclosed by the *dotted line* in **a**. Note that the size of leaves of *P. asiatica* var. *yakusimensis* falls within the range of leaf-size variation in individuals of *P. asiatica* var. *densiuscula*. Dwarves of *P. asiatica* var. *densiuscula* were categorized into those collected from temples and shrines (minima type: *gray squares*), areas with high deer densities (*white triangles*), islands with both deer and a shrine (*gray triangles*), and other locations (*white circles*). Specimens of *P. asiatica* var. *yakusimensis* are represented by *black circles*. Data are presented as means ± SD. Individuals shown as a *white circle* with a *letter* were collected from the following localities: *A* Mt. Kanadate, Saga; *B* and *C* the area around the tennis court on the Komaba campus of the University of Tokyo; *D* and *E* Isoyama, Tochigi; *F* Mt. Ichifusa, Kumamoto; *G* a pasture at Kamikita-gun, Aomori; *H* a serpentinite site on Mt. Apoi, Hokkaido; *I* and *J* Kamakura City, Kanagawa

Dwarf individuals, i.e., plants with small leaves (less than 3 cm in length) were collected not only from temples and shrines but also from deer habitat (e.g., Nara-koen Park, Nara Prefecture; white triangle in Fig. 1b), pasture (e.g., Kamikita-gun, Aomori Prefecture; white circle-G in Fig. 1b), and a serpentinite site (e.g., Mt. Apoi, Hokkaido; white circle-H in Fig. 1b). Leaves of *P. asiatica* var. *yakusimensis* were not small relative to leaves of the above-mentioned dwarves. The individual with the smallest leaves was collected from the Mii-dera temple (Shiga Prefecture; 0.5 ± 0.1 cm in length, 0.4 ± 0.1 cm in width).

We also noticed variation in the density of leaf hair, as *P. asiatica* var. *yakusimensis* has often been characterized by a high density of leaf hair (Masamune 1930; Yamazaki 1981). However, not only individuals of *P. asiatica* var. *yakusimensis* but also individuals of standard *P. asiatica* var. *densiuscula* were found to be highly pubescent (data not shown), as previously noted by Yamamoto (1939). Moreover, the density of leaf hairs was not always high in *P. asiatica* var. *yakusimensis*. Based on these morphological results, *P. asiatica* var. *yakusimensis* appeared to warrant treatment as a dwarf type of *P. asiatica* var. *densiuscula*.

Stabilities of dwarfism under cultivation

The above analyses revealed high variability in leaf size of *P. asiatica* var. *densiuscula*. To determine whether these variations were the result of environmental plasticity, the stability of leaf length was investigated for 18 selected strains, including dwarves and standard types grown under standard culture conditions. As a result, they were classified into three groups based on the difference in leaf-blade length before and after cultivation (Fig. 3). The strains in the first group had leaf blades shorter than 3 cm both in their native habitat and after cultivation and were considered to be genetically fixed extreme dwarves [Fig. 3, group A: plants from Mii-dera temple, Shiga Prefecture (Fig. 2); Miyajima Island, Hiroshima Prefecture; and Nanzen-ji temple, Kyoto]. Members of the second group were another type of dwarf; the leaf blades were shorter than 3 cm in their native habitats but were longer than 3 cm when cultivated [Fig. 3, group B: e.g., plants from Isono-jinjya shrine, Ehime Prefecture, and *P. asiatica* var. *yakusimensis* (Fig. 2)]. In both group A and B dwarves, leaf-blade length more or less increased with cultivation (Fig. 3), suggesting that the dwarfisms were attributable, to some extent, to environmental plasticity of leaf size and dependent on environmental factors. At the same time, both types of dwarves retained a certain degree of dwarfism, even after cultivation. This fact suggests that the dwarfism in these plants was in part attributable to genetic characteristics. The third group of plants was composed of standard types that had leaf blades longer than 4 cm in both their native habitat and in cultivation (Fig. 3, group C: e.g., plants from Okazaki, Aichi Prefecture (Fig. 2)].

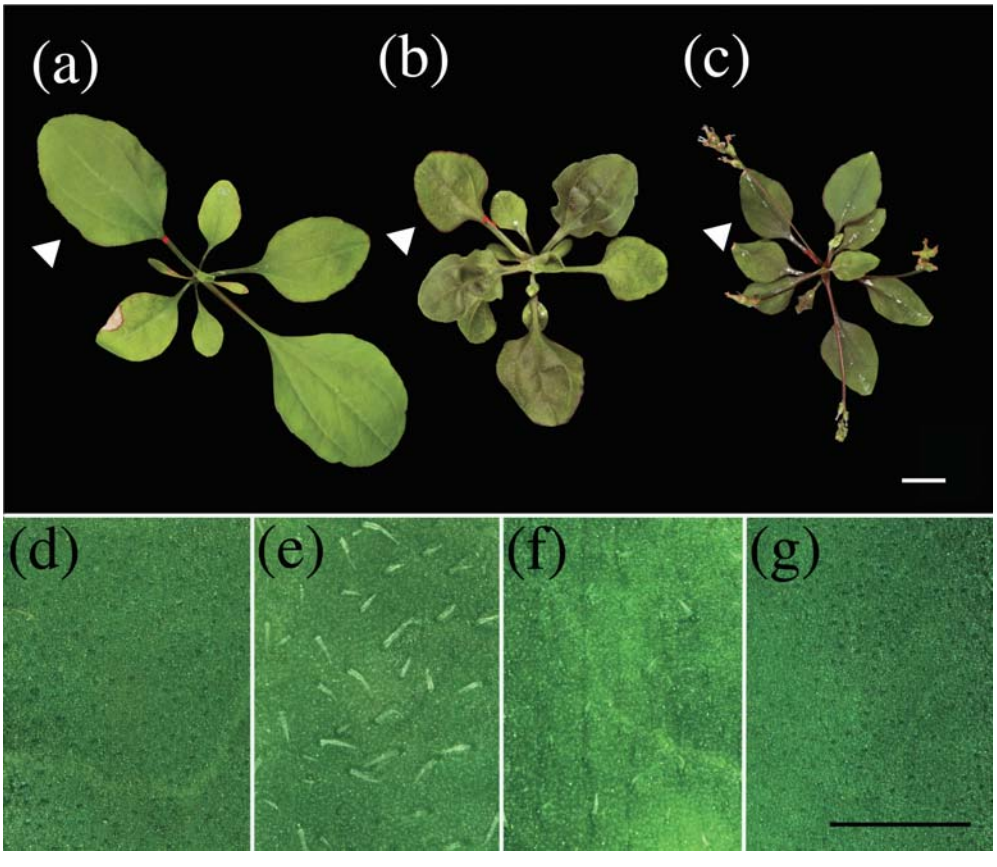

Fig. 2. Morphology of the standard and minima types of *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis* under cultivation. Typical examples of the gross morphology at 70 days after sowing (**a–c** *bar* 1 cm) and magnified views of leaf surface (**d–g** *bar* 2 mm) are shown. **a, d** Standard type of *P. asiatica* var. *densiuscula* (Okazaki). **b, e, f** *P. asiatica* var. *yakusimensis* (Mt. Miyanoura, Yakushima). Note variation in density of hairs on leaf surface among leaves of *P. asiatica* var. *yakusimensis* under cultivation (**e, f**). **c, g** A minima type of *P. asiatica* var. *densiuscula* (Mii-dera temple). *Arrowhead* indicates the fifth leaf of each individual

Leaf-hair density of *P. asiatica* var. *yakusimensis* was not stable under cultivation (Fig. 2e, f). The hair did not disappear completely, but hair density alone was not enough to distinguish *P. asiatica* var. *yakusimensis* from other dwarf types of *P. asiatica* var. *densiuscula* (Fig. 2), further suggesting that *P. asiatica* var. *yakusimensis* cannot be characterized by leaf-hair density.

Number of seeds per capsule

As mentioned in the Introduction, the number of seeds per capsule has been treated as a key character in genus *Plantago*, in particular, for distinguishing *P. asiatica* from a closely related species *P. major* L. (Pilger 1922; Yamazaki 1981; Matsuo 1989), and we reexamined this character in detail. As a result, great variation was found in the number of seeds per capsule among standard types having leaves more than 4 cm in length, ranging from 3.4 ± 0.8 to 6.0 ± 0.8, even under the same culture conditions (Table 1). Similar variations were recognized among dwarves of *P. asiatica* var. *densiuscula*, with the number of seeds per capsule ranging from 4.4 ± 0.1 to 6.5 ± 1.6 (Table 1). On the other hand, we confirmed that *P. asiatica* var. *yakusimensis* had 3.7 ± 1.2 or 4.0 ± 0.7 seeds per capsule, as reported previously (Yamazaki 1981; Table 1). Taken together, we did not recognize a clear correlation between number of seeds per capsule and leaf size.

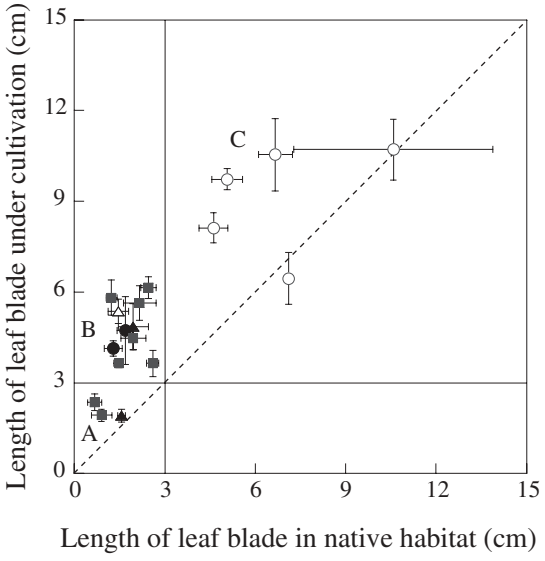

Fig. 3. Comparison between leaf-blade length in native habitats and under cultivation. Populations were separated into three groups, A, B, and C, based on the plasticity of the dwarfism (see text). The *plot* above the *diagonal dotted line* indicates that leaf blade was larger after cultivation. Abbreviations of symbols are the same as in Fig. 1. Data are presented as means ± SD

Variations in ploidy level

In plants, size variation among strains is often attributed to differences in ploidy levels (Stebbins 1950). Therefore, we determined the ploidy level of the 39 strains. As shown in Table 1, all examined strains of *P. asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis* were tetraploid with $2n = 24$, in accordance with earlier reports (Ishikawa 1916; Yamazaki 1981; Iwatsubo et al. 2000).

Sequence diversity at ITS locus

Sequence analysis of approximately 740 bp of the ITS locus was performed to determine if there was any relationship between genetic differentiation and size variations in Japanese *P. asiatica*. Among the 39 strains examined, 16 substitutions and one deletion were recognized. The variable positions are summarized in Table 2. Based on differences in those sites, 12 genotypes were recognized. Molecular phylogenetic analysis revealed two synapomorphic substitutions, one between genotypes 9 and 10 and the

Table 2. Differences found in sequences of the internal transcribed spacer (ITS) locus

Genotype	1	1	1	2	2	2	2	3	3	4	5	6	6	6	7
	6	6	9	3	4	8	9	4	7	6	0	4	7	8	0
	3	4	3	5	4	5	5	2	1	3	9	4	3	8	4
1	T	C	A	A	G	C	C	C	C	C	C	C	A	G	C
2	–	–	–	–	–	–	–	–	Y	–	Y	–	–	–	–
3	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–
4	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	M	–	–	–	–	–	–	–	–	–	R	–
6	–	–	–	–	–	T	–	–	–	–	–	T	–	–	–
7	–	–	–	–	–	–	Y	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	Y	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	Y	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	T	–	–	–	–	O
11	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	–
12	W	M	–	–	–	–	–	–	–	–	–	–	T	–	–

Sequences are numbered from the 5′ end to the 3′ end
– Same as genotype-1, *O* absence, *R* A or G, *Y* C or T, *M* C or A, *W* A or T

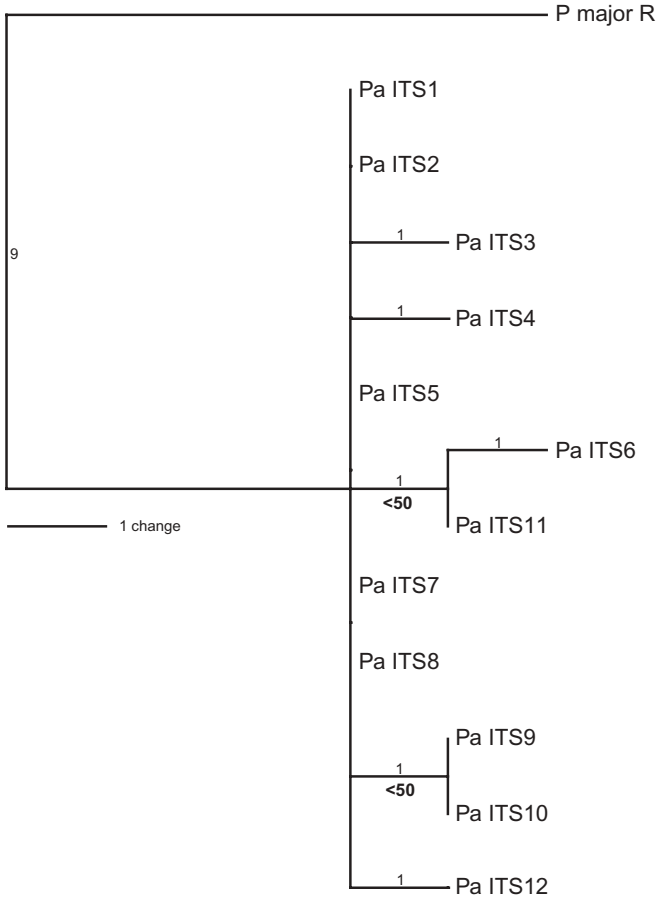

Fig. 4. One of four most parsimonious phylogenetic trees based on the ITS sequences of *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis.* The tree has 15 steps, and CI = 1.00. The phylogenetic relationship among 12 genotypes of *P. asiatica* are shown. Genotypes are represented by *numerals* from 1 to 12, as shown in Tables 1 and 2. Accession numbers for each genotype are listed in Table 4. *Numbers* above the branches indicate branch length. *Numbers* below the branches are bootstrap values

Table 3. ITS genotype of each sample

Genotype	Sample name
1	**4. Hachiman-jinjya shrine, 5. Mii-dera temple, 6. Nanzen-ji temple, 7. Ise-jingu shrine, 10. Kushida-jinjya shrine, 11. Dazaifu shrine, 12. Amamiohshima, 14. Kinkazan Island, 15. Miyajima Island, 16.** Hokkaido-jingu shrine, 18. Hikariyama, 19. Hongo, 28. Mii-dera temple (standard), 29. Awajishima, 30. Daisen, 31. Hiruzen, 32. Karasugasen, 33. Hakozaki, 34. Dazaifu shrine (standard), 37. TW106, 38. TW115, 39. Mt. Halla
2	17. Akita
3	20. Kamakura
4	21. Tateyamaeki
5	**9. Isono-jinjya shrine**
6	**13. Tanegashima,** 35. Oushushoumae
7	**8. Ohyagmazumi-jinjya shrine**
8	**3. Nara-koen,** 25. Okazaki, 26. Maruyama, 27. Sakatejima
9	23. Kitayatsugatake, 24. Bijyodaira
10	22. Ontake
11	36. Sakurajimaguchi
12	**1. Mt. Miyanoura, 2. Mt. Kuromi**

Dwarves are shown in bold face

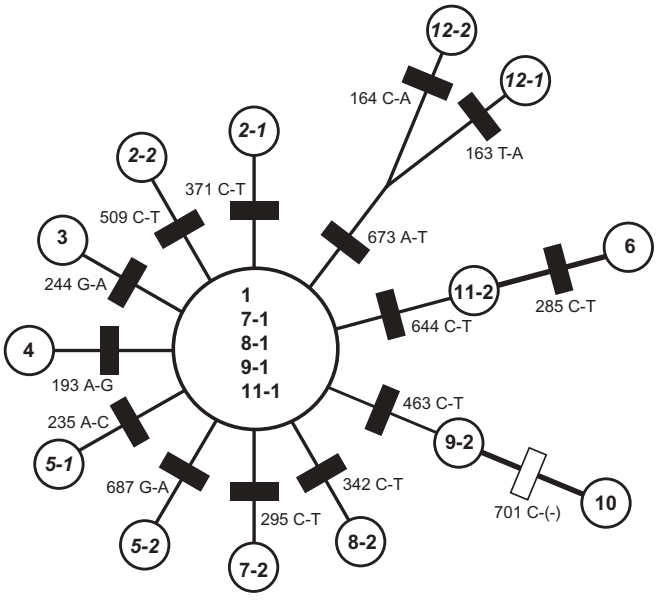

Fig. 5. Median-joining network of ITS sequences of *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis.* Names in *block letters* indicate haplotypes determined unambiguously, and those in *italics* indicate haplotypes inferred from the assumption that sequences with two or more ambiguous sites are combinations of two haplotypes. *Black bars* indicate substitutions and a *white bar* indicates an indel

other between genotypes 6 and 11 (Fig. 4). In both cases, however, the substitutions were heterogeneous in one of the genotypes (genotype 9 in the former case and genotype 11 in the latter), suggesting that these substitutions were polymorphic in a given population. Genotypes 5 and 7 were found only in dwarf types (Table 3). *P. asiatica* var. *yakusimensis* (genotype 12) was characterized by three substitutions, of which one was unique to the taxon but two were heterogeneous even within a single individual.

The above 12 genotypes were explained by the combination of 15 haplotypes of the ITS region. The relationship among these haplotypes is illustrated by the MJ network (Fig. 5). Eleven haplotypes were considered to have arisen from the acquisition of a single substitution on a common haplotype. Four other haplotypes accumulated additional mutations (substitutions in three haplotypes and deletion in one haplotype), and two of these were unique to *P. asiatica* var. *yakusimensis*.

From these results, *P. asiatica* var. *yakusimensis* was concluded to have specific genotypes from the combination of unique ITS haplotypes although the substitution level was not significant when compared with those observed among strains of *P. asiatica* var. *densiuscula*.

Table 4. Differences found in sequences of the *SUC1* locus

Genotype																	
		1	2	2	2	2	2	3	5	9	9	2	2	2	2	3	3
	1	7	1	8	8	8	8	1	9	1	8	0	6	6	7	7	9
	4	1	2	1	2	3	4	0	4	5	0	2	0	1	8	7	0
I	C	T	C	O	O	O	O	C	T	A	O	G	A	A	A	A	A
II	–	–	–	–	–	–	–	–	–	C	–	A	–	–	–	–	–
III	–	C	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IV	–	C	T	–	–	–	–	–	O	–	–	–	–	–	–	–	–
V	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
VI	T	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
VII	T	C	–	G	C	A	T	A	–	–	T	–	G	G	G	–	O
VIII	T	C	–	G	C	A	T	–	–	–	T	–	G	G	–	G	–

Sequences are numbered from the 5′ end to the 3′ end
– Same as genotype-I, *O* absence

Sequence diversity at the *SUC1* locus

Although the above analyses revealed genetic differentiation of *P. asiatica* var. *yakusimensis*, the relationship among dwarves and standard types remained elusive. Therefore, we conducted sequence analysis of the nuclear-encoded *SUC1* locus. Based on the sequence comparisons, eight genotypes were recognized (Table 4). Only a single MP tree of 43 steps, CI = 0.98, and RI = 0.91 was obtained by the analysis (Fig. 6). In this tree, a clade consisting of genotypes VII and VIII was sister to clades of all other genotypes. In the large clade including genotypes I–VI, two major genotypes, I and III, belonged to different monophyletic groups, in which the former was with genotype II and the latter was with genotype IV.

The relationship among the genotypes of *SUC1* is illustrated by the MJ network (Fig. 7). As inferred in the MP tree, a cluster consisting of genotypes VII and VIII was largely separated from the other genotypes by two substitutions and two indels. Genotypes VII and VIII were also separated from each other by three substitutions and one indel. In contrast, the other six genotypes were in a relatively tight cluster and were connected to each other by one to two substitutions or indels. As a result of the ITS analysis, *P. asiatica* var. *yakusimensis* has a specific genotype, VIII, of the *SUC1*, and this fact also supports the distinctness of the taxon. Dwarf phenotypes other than *P. asiatica* var. *yakusimensis* were found in genotypes I, II, III, IV, and VII, and each of genotypes II and IV was observed only in a single locality in the dwarf type (Table 5).

In addition to the above relationship among genotypes, we found an interesting distribution pattern of the *SUC1* genotypes in terms of geographic structure (Fig. 8). The two main genotypes, I and III, were allopatric. The former was distributed from Hokkaido to central Honshu, and the latter appeared in western Honshu, Shikoku, Kyushu, and in the northern Ryukyu archipelago. Genotype VII was observed in a small area of the Pacific Ocean side of central Honshu and was disjunctly distributed from the most closely related genotype, VIII, on Yakushima (Fig. 8).

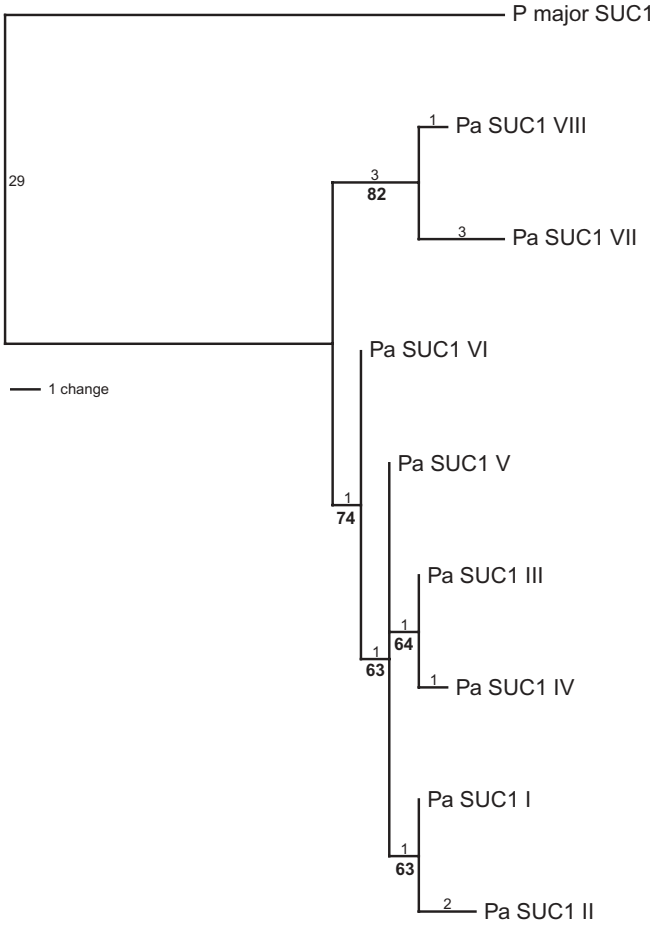

Fig. 6. Most parsimonious phylogenetic tree based on the *SUC1* sequences of *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis*. The phylogenetic relationships among eight genotypes of *P. asiatica* are shown. Genotypes are represented by *roman numerals* from I to VIII, as shown in Tables 1 and 3. Accession numbers for each genotype are listed in Table 4. *Numbers* above the branches indicate branch lengths. *Numbers* below the branches are bootstrap values

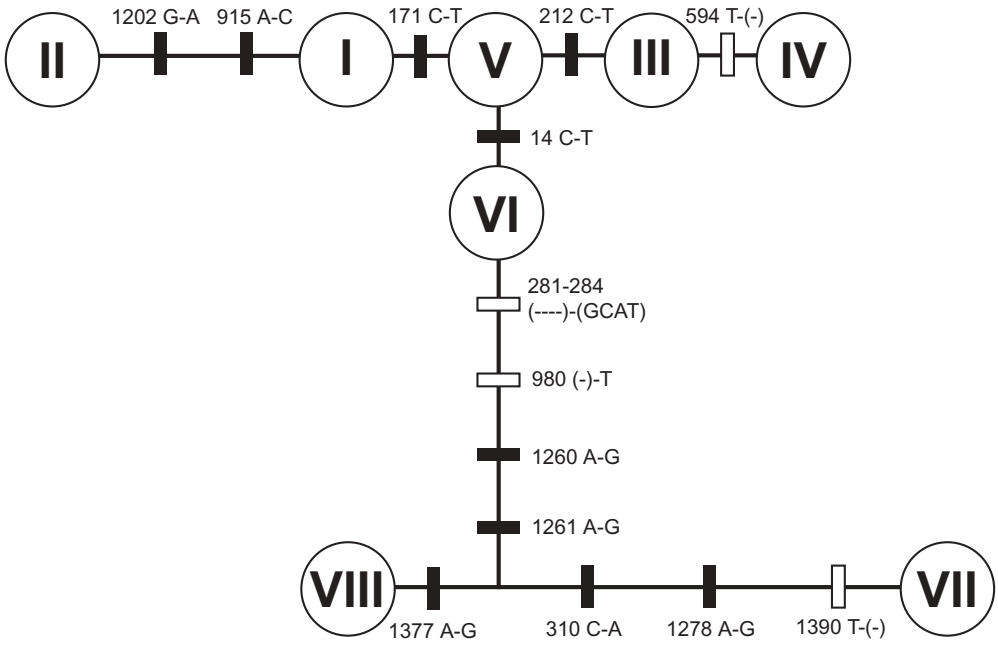

Fig. 7. Median joining network of *SUC1* sequences of *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis*. *Black bars* indicate the substitutions and *white bars* indicate indels

Table 5. *SUC1* genotype of each sample

Genotype	Sample name
I	**5. Mii-dera temple, 7. Ise-jingu shrine, 14. Kinkazan Island**, 16. Hokkaido-jingu shrine, 17. Akita, 18. Hikariyama, 19. Hongo, 20. Kamakura, 22. Ontake
II	**4. Hachiman-jinjya shrine**
III	**6. Nanzen-ji temple, 8. Ohyamazumi-jinjya shrine, 9. Isono-jinjya shrine, 10. Kushida-jinjya shrine, 11. Dazaifu shrine, 12. Amamiohshima, 13. Tanegashima,** 29. Awajishima, 31. Hiruzen, 33. Hakozaki, 34. Dazaifu shrine (standard), 35. Oushushoumae, 36. Sakurajimaguchi
IV	**15. Miyajima Island**
V	25. Okazaki
VI	39. Mt. Halla
VII	3. Nara-koen, 26. Maruyama, 27. Sakatejima, 37. TW106, 38. TW115
VIII	**1. Mt. Miyanoura, 2. Mt. Kuromi**
ND	21. Tateyamaeki, 23. Kitayatsugatake, 24. Bijyodaira, 28. Mii-dera temple (standard), 30. Daisen, 32. Karasugasen

Dwarves are shown in bold face
ND Not determined

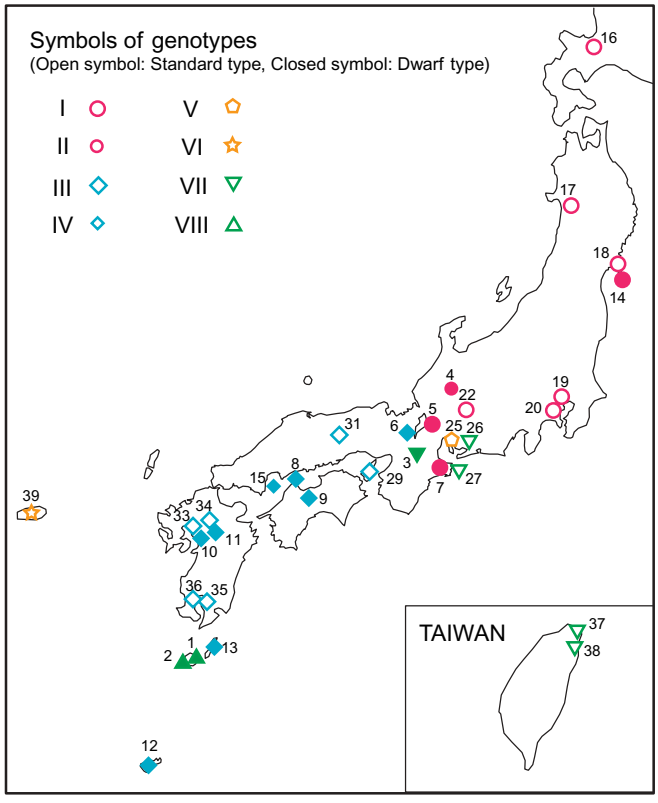

Fig. 8. Geographic distribution of dwarves and eight genotypes of *SUC1* in *Plantago asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis*. *Letters* indicate sample numbers shown in Table 1

Discussion

Morphological variation in *P. asiatica* var. *densiuscula*

Morphological analysis of herbarium specimens revealed that leaf sizes of *P. asiatica* var. *densiuscula* were highly variable. *P. asiatica* var. *yakusimensis* and the minima type could not be distinguished from *P. asiatica* var. *densiuscula* based on leaf size (Fig. 1). Considering the highly plastic nature of leaf morphology in *P. asiatica* as discussed below (Fig. 3), leaf size at the native habitat alone is not sufficient to distinguish these varieties.

Investigation of the stability of dwarfism in 13 lines of dwarves collected from temples and shrines, areas with high deer densities, and Yakushima Island identified three lines of genetically fixed extreme dwarves (Fig. 3, group A: plants from Mii-dera temple, Nanzen-ji temple, and Miyajima

Table 6. Accession numbers for sequences of the ITS and the *SUC1* locus

Locus	Species	Genotype	Accession no. (reference)
ITS	*Plantago major*		AY101861 (Rønsted et al. 2002)
	P. asiatica var. *densiuscula*	1	AB223151
		2	AB223152
		3	AB223153
		4	AB223154
		5	AB223155
		6	AB223156
		7	AB223157
		8	AB223158
		9	AB223159
		10	AB223160
		11	AB223161
	P. asiatica var. *yakusimensis*	12	AB223162
SUC1	*P. major*		AB246394
	P. asiatica var. *densiuscula*	I	AB246395
		II	AB246396
		III	AB246397
		IV	AB246398
		V	AB246399
		VI	AB246400
		VII	AB246405
	P. asiatica var. *yakusimensis*	VIII	AB246406

Island). Interestingly, Miyajima Island is the locality where genetically fixed dwarfism is found also for *Paederia foetida* f. *microphylla* (Tsukaya et al. 2006). The other dwarves, including *P. asiatica* var. *yakusimensis*, lost their extreme dwarfism through cultivation although they retained some degree of dwarfism (Fig. 3, group B). The observed dwarfisms can therefore be attributed to both plasticity to environmental factors and genetic background. The difference between groups A and B may reflect a different ratio of plasticity-dependence in dwarfism. Various genetic factors can affect dwarfism, as reported in several dwarf mutants of the model plant *Arabidopsis thaliana* (Tsukaya 2002). The difference in ploidy level is a plausible factor for the size variation in plants (Stebbins 1950). However, all individuals of *P. asiatica* examined in this study had the same ploidy level. Thus, other genetic factors may be responsible for the observed variations.

Molecular variation in *P. asiatica* var. *densiuscula*

Molecular analysis of the ITS region and the *SUC1* locus revealed genetic relationships among dwarves, standard types of *P. asiatica* var. *densiuscula*, and *P. asiatica* var. *yakusimensis*. There were no obvious patterns of distribution or phylogenetic relationships in ITS haplotypes of *P. asiatica* var. *densiuscula* because of a low genetic differentiation of the locus. Pairwise sequence divergence values of ITS between *P. asiatica* var. *densiuscula* (including *P. asiatica* var. *yakusimensis*) haplotypes were 0–0.0059. These values were lower compared with other studies of intraspecies variations in ITS sequences (e.g., *Mitchella undulata*: 0–0.014, Yokoyama et al. 2003; *Symphonia globulifera*: 0.0016–0.044, Dick et al. 2003; *Saxifraga oppositifolia*: 0–0.015, Holderegger and Abbott 2003; *Cichorium intybus*: 0–0.02, *C. spinosum*: 0–0.003, Gemeinholzer and Bachmann 2005; *Passiflora actinia*: 0–0.02, *P. elegans*: 0–0.005, Lorenz-Lemke et al. 2005). On the other hand, results of our analysis of the *SUC1* locus support the independent origin of dwarf strains because the distribution pattern of *SUC1* genotypes shows a clear geographic structure, suggesting that gene flow among genotypes has probably been prevented. Among the dwarf types, only *P. asiatica* var. *yakusimensis* has specific genotypes in both the ITS and the *SUC1* locus. From these results, we concluded that *P. asiatica* var. *yakusimensis* has a different divergence history from other dwarf types. However, the level of genetic differentiation is still comparable with other genotypes in *P. asiatica* var. *densiuscula*. From the results of our morphological and molecular analyses, we propose that *P. asiatica* var. *yakusimensis* should be reduced to a form as follows.

Taxonomic treatment

Plantago asiatica L. var. ***densiuscula*** Pilg. forma ***yakusimensis*** (Masam.) N. Ishikawa, J. Yokoyama, H. Ikeda, et H. Tsukaya, stat. et comb. nov.

BASIONYM: *P. yakusimensis* Masam. in Bot Mag Tokyo 44:220 (1930).

Type: Insula Yakusima monte Yaegadake ca. 1,600–1,900 m alt. (G. Masamune in 1927, holotype: TI (?), not seen). Two specimens [G. Masamune, 25 July 1927 (with fruits) & July 1927 (sterile)] are preserved at TI and are considered as syntypes (Yahara et al. 1987: 111).

SYNONYM: *P. asiatica* L. var. *yakusimensis* (Masam.) Ohwi in Bull Nat Sci Mus Tokyo (33):86 (1953).

The origins of *P. asiatica* var. *densiuscula* f. *yakusimensis* and other dwarves of *P. asiatica* var. *densiuscula* in Japan

Based on the results described above, we discuss the origins of dwarves in *P. asiatica* var. *densiuscula*. There are at least two possible evolutionary processes that led to dwarfism in *P. asiatica* var. *densiuscula.* One is that mutations responsible for dwarfism occurred in an ancestral population of Japanese individuals and were retained during the establishment of the geographic structure recognized in the *SUC1* genotypes. In this case, these mutations would have been shared by all populations and then selected at each habitat to generate dwarves. The other possibility is that mutations responsible for dwarfisms occurred independently at each habitat over a relatively short period of time after establishment of the geographic structure. In the former case, mutated genes should be shared at least in part among dwarves with different *SUC1* genotypes. In the latter case, however, mutations should differ among dwarves. To determine which scenario is correct, genetic analyses are under way. In addition to the similar dwarves with small leaves reported from Miyajima Island, Kinkazan Island, and Yakushima Island in other species (e.g., Tsukaya 2002; Yokoyama et al. 2003), dwarves of *P. asiatica* var. *densiuscula* can served as model plants for elucidating the mechanisms of convergent evolution of dwarves in Japan.

Acknowledgements We would like to thank the herbarium curator of the University of Tokyo who kindly permitted us to examine specimens of *P. asiatica* var. *densiuscula* and *P. asiatica* var. *yakusimensis.* We also thank Ms. C. Yamaguchi and Ms. M. Nagura for technical assistance, and Dr. T. Kozuka (Kyoto University) and Ms. E. Mano (Graduate University for Advanced Studies, Japan) for providing plant materials. The collection of samples in Taiwan, Miyajima, and Yakushima was permitted by Prof. Ching-I Peng (Academica Sinica, Taiwan), Drs. G. Toyohara and S. Mukai (Hiroshima University), and by the Ministry of the Environment, Japan, respectively. We also thank Profs. H. Okada (Osaka City University) and R. Imaichi (Japan Women's University) for their kind assistance with the fieldwork. This study was supported by a grant from the Sumitomo Foundation, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan, and a grant from the SOKENDAI group research project of the Graduate University for Advanced Studies.

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