Plant Biotechnology 25, 341–346 (2008)

Original Paper

In vitro proliferation and genetic diversity of *Cypripedium macranthos* var. *rebunense*

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Received February 12, 2008; accepted March 27, 2008 (Edited by S. Ogita)

Abstract *In vitro* culture from seeds to full maturity of *Cypripedium macranthos* var. *rebunense*, one of the most endangered species in Japan, was studied. Immature seeds from wild plants germinated efficiently in Tsutsumi-Tomita medium and 1/3 strength Murashige-Skoog medium both supplemented with 1% sucrose. Formed protocorms were transferred onto the same Murashige-Skoog medium developed into seedlings, which were subjected to a cold-treatment. The seedlings were further successively cultured in artificial soils (Florialite and then Cryptomoss) under artificially controlled photoperiod and temperature conditions. After 7 years, fully matured plants successfully flowered. The genetic diversity of the wild colony of this orchid in Rebun Island was examined. Leaf samples randomly collected were subjected to random amplified polymorphic DNA analysis. Initially examined 31 samples were classified into independent 5 groups, indicating that the colony consists of genetically different individuals. This suggested that plants in the habitat are heterogenous as to the genetic structure. Introduction of artificially propagated plants with a similar genetic background to this habitat would match the genetic diversity of the existing population. The results of this study may be helpful in planning the rescue of other endangered plant species.

Key words: *Cypripedium macranthos* var. *rebunense*, endangered plant species, genetic diversity, protocorm growth, random amplified polymorphic DNA analysis.

Cypripedium macranthos var. *rebunense* (Orchidaceae) is found only in Rebun Island, which is situated off the north coast of Hokkaido, Japan in lat. 45°30′14″N. and long. 141°4′16″E. This plant once grew widely throughout this island. However, due to its specific flower feature, it was subjected to intensive and thoughtless over-collecting, resulting in declining of its population. Subsequently, in 1990, *C. macranthos* var. *rebunense* was designated as one of the most endangered plant species in Japan.

Natural proliferation of *C. macranthos* var. *rebunense* is very slow. Each seed capsule of this plant contains 15,000 and 20,000 seeds (unpublished observation). According to our series of field surveys on this variety in the protected area in Rebun Island, only few seeds survive to the seedling stage. Plants grow to maturity and flower at the four leaves stage when the height is usually more than 20 cm. The record of our unpublished observation indicates that it takes 7–13 years for wild plants to bear flowers after germination.

The artificial propagation of *Cypripedium* species from seeds is not easy. Many reports on this subject are found in reviews published in books and journals (Arditti 1967; Arditti and Ernst 1984, 1993; Miyoshi 1997). Both immature and mature dry seeds have been used for *in vitro* germination of *C. macranthos* (Tomita and Tomita 1997a; Miyoshi and Mii 1998), and *C. macranthos* var. *rebunense* (Shimura and Koda 2004, 2005). The Rebun Cultivation Center of Alpine Plants, an official workstation of Rebun-town, has been successful in seedling propagation from immature seeds of this variety (T. Iino, personal communication). A case of plant regeneration from immature seed-derived callus *of C. macranthos* var. *taiwanianum* has also been reported (Tomita and Tomita 1997b). Cold treatment (Tomita and Tomita 1997a), inoculation with a symbiotic fungus from the root of *C. macranthos* var. *rebunense* after cold treatment (Shimura and Koda 2005), and treatment with

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Abbreviations: RAPD, random amplified polymorphic DNA.
This article can be found at http://www.jspcmb.jp/

$Ca(ClO)_2$ followed by cold treatment (Miyoshi and Mii 1998) significantly increased germination. Since naturally grown *C. macranthos* var. *rebunense* is commonly associated with symbiotic fungi which inhabit in their roots, symbiotic culture with such fungi was proposed to be necessary for *ex situ* propagation (Shimura and Koda 2005). Despite of intensive efforts, however, cultivation of *in vitro* germinated seedlings of *C. macranthos* and *C. macranthos* var. *rebunense* to mature plants with flowering ability has not so far been scientifically reported.

In the present study, we attempted to establish a simple and efficient method to culture *in vitro* this orchid from seeds to maturity for the purpose of molecular analyses of its developmental processes and also supplying plant materials to be used for *in situ* reintroduction into the natural habitat of Rebun Island. We also examined the genetic diversity of this orchid in the wild colony of Rebun Island, because introduction of *ex situ* propagated plants may affect the genetic structure of the existing population.

Materials and methods

Plant materials

Capsules containing immature seeds of *Cypripedium macranthos* var. *rebunense* were sampled from wild type plants grown in the habitat colony in Rebun Island 8 weeks after the time of flowering. They were kept at below 10°C under a moist condition during transportation, and were subjected to procedures for *in vitro* germination within 2 days after sampling.

In vitro cultivation

Collected capsules were washed thoroughly with tap and distilled water, and sterilized first in 70% ethanol for 1 min and then in 2% sodium hypochlorite solution for 8 min. After rinsing 5 times in sterilized distilled water, the capsules were dissected in a Petri dish, and immature seeds were scraped out. Approximately 100 seeds per plate were transferred to each one of the four types of culture media and kept at 20°C: Medium I, half strength Norstog (N) medium (Norstog 1973); Medium II, medium I supplemented with 100 mg L^{-1} KNO_3 and 60 mg L^{-1} NH_4NO_3; Medium III, full strength T medium (Tsutsumi and Tomita 1990); Medium IV, 1/3 strength MS medium (Murashige and Skoog 1962). All media tested contained 1% sucrose and 1% agar, and adjusted to pH 5.8 before autoclaving at 121°C for 20 min. Rupture of the testa after embryo swelling was regarded as germination. Percentage germination was estimated after 2 to 3 months of culture by counting numbers of germinated seeds among total numbers of sown seeds in at least 5 plates.

After 3 months of initial culture in the four types of culture media, all germinated seeds were collected and transferred to 1/3 strength MS medium with 1% sucrose and 1% agar for further growth. At the beginning of root and shoot development, they were subjected to a cold treatment at 4°C in the dark for 1 month, and then were back to a growth cabinet under a 16h/8h light/dark photoperiod at 20°C. Plantlets with well-developed roots were transferred onto Florialite (Nisshinbo Co. Ltd., Tokyo) supplemented with 1/2 strength MS liquid medium containing 1% sucrose. Plantlets were grown aseptically at 20 °C for 6 months, and then subjected to a 4°C cold treatment in the dark for 3 months. After cold treatment, plantlets were again transferred to a growth cabinet under a 16h/8h light/dark photoperiod at 22°C for shoot growth.

Finally, plantlets were transferred to Cryptomoss (Imaichi Wood Materials Development Cooperative Association, Tochigi, Japan) in pots and grown in a controlled greenhouse at 22°C under a 16h/8h light/dark photoperiod condition. After 6 months, shoots became withered and plantlets were subjected to a cold treatment at 4°C for up to 2 months before new shoots (buds) emerged. After this stage, potted plants were repeatedly subjected to the culture cycle with a 5-month growth and a 1-month cold treatment (2 cycles a year) for 7 years, when plants matured and formed the first flower.

DNA extraction and RAPD analysis

Leaf samples were obtained from naturally growing mature plants in the conserved area in Rebun Island, Japan, and also from cultivated plants, of which seeds were initially collected in the same conserved area, and grown in the Rebun Cultivation Center of Alpine Plants. Cut leaf samples were kept under a desiccated condition with silica powder at 4°C until use. DNA was extracted by the cetyl-trimethyl ammonium bromide method (Murray and Thompson 1980). DNA concentration was measured using a spectrophotometer, and after being confirmed to be at high molecular weight by 1.0% agarose gel electrophoresis, DNA samples were used for RAPD analysis as previously described (Yamamoto et al. 2006). Initially, 20 independent primers (Operon Technologies, Alameda, CA, USA) were screened for specific amplification, and 5 were finally selected: OPM01, 5′-GTTGGTGGCT-3′; OPM06, 5′-CTGGGCAACT-3′; OPN04, 5′-GACCGACCCA-3′; OPM05, 5′-GGGAACGTGT-3′; and OPN01, 5′-CTCACGTTGG-3′. PCR was performed in a volume of 10 μl containing 20 ng total DNA, 10 pmol primer, 250 μM dNTP and 0.5 U DNA polyme rase (ExTaq, Takara, Kyoto, Japan) under a condition of 45 cycles of 94°C for 0.5 min, 36°C for 1 min and 72°C for 2 min, with a final termination at 72°C for 6 min. Amplified products were fractionated by 1% agarose gel electrophoresis, visualized by staining with ethidium bromide and photographed.

Statistical analysis

The amplified PCR products were visually scored for the presence (1) or absence (0) to produce a binary matrix. All statistical analyses, including Jaccard's similarity coefficient, Pseudo-F (psF) statistics, and cluster analysis, were performed with the SAS 9.1 software (SAS Institute 1998) based on the following model;

$$D_{KL} = \frac{1}{N_K N_L} \sum_{i \in C_K} \sum_{i \in C_L} d(Xi, Xj)$$

Where D_{KL} represents the distance between cluster C_K and C_L, N_K is the number of observations in C_K, N_L is the number of observation in C_L, Xi is the ith observation, Xj is the jth observation, $d(Xi, Xj)$ is the distance between observations Xi

and *Xj*, C_K is the *k*th cluster, and C_L is the *l*th cluster (Elliot 2000).

Results and discussion

In vitro proliferation

Immature seeds isolated from capsules of *Cypripedium macranthos* var. *rebunense* after 7–8 weeks of pollination were yellow brown. They were cultured on four types of media, and swelling and germination were examined. On medium I, 23.5% of embryos germinated after 3 months of culture. In contrast, on medium II, embryo swelling was suppressed and no germination was observed (Figure 1A). On medium III, 73% embryos swelled after 2 months, and 37% showed the final rupture of testa after 3 months. On medium IV, swelling was fast, and up to 56% of samples were found to germinate after 3 months (Figure 1B–C). These observations suggested that media III (T-medium) and IV (1/3 MS medium) were suitable for *in vitro* germination. Accordingly, germinated embryos grown in these 4 media were collected after 3 months and transferred onto medium IV to further induce protocorms. Root and shoot primordia began to protrude from spherical embryos after 1 month (Figure 1D).

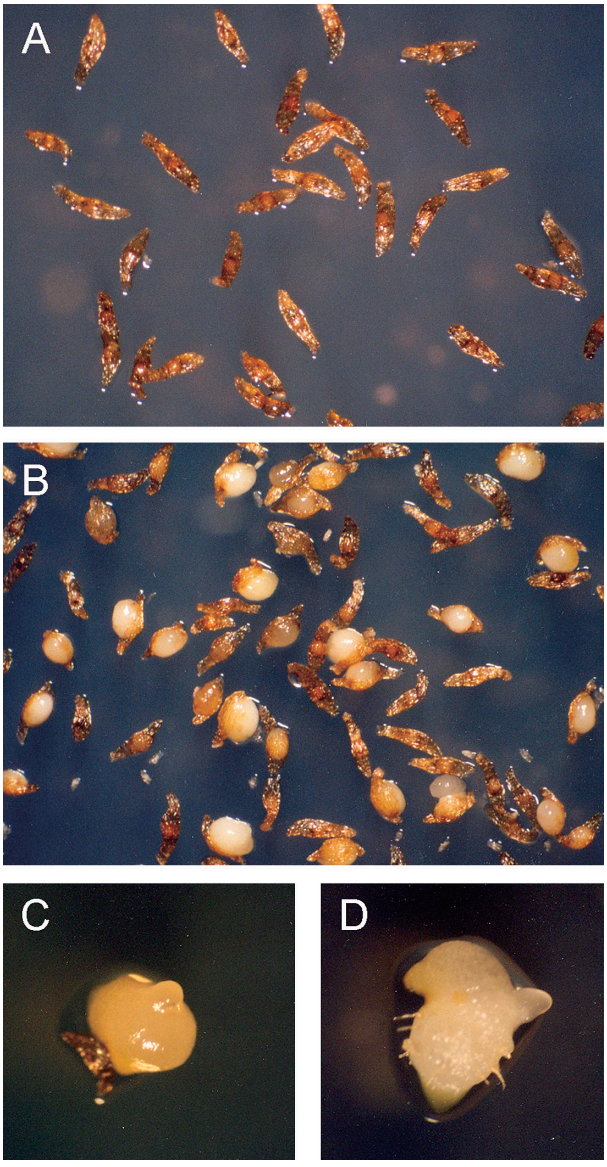

Figure 1. Germination and protocorm formation. Immature seeds were spread on agar plate containing medium II (1/2 N plus 200 mg L^{-1} KNO_3 and 120 mg L^{-1} NH_4NO_3) (A) and medium IV (1/3 MS medium) (B, C), and incubated at 20°C for 3 months. On medium IV, 45% of seeds successfully germinated (B). Further incubation for one month resulted in protocorm development (D).

Flower induction

At this stage, embryos were treated with low temperature for 1 month, and then returned to standard culture condition (Figure 2A). During 3 months culture under this condition, shoot development was observed. The culture medium was renewed when it became brownish, usually once a month. Plantlets with well-developed roots were selected and transferred onto artificial soil and

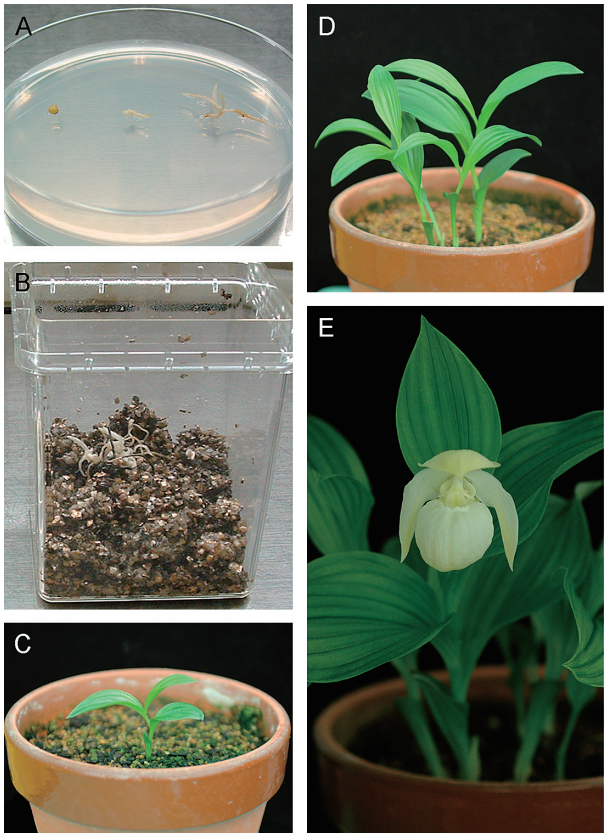

Figure 2. Growth to maturation. Germinated immature seeds with protocorms and roots were transferred onto agar plate containing 1/2 MS medium with 1% sucrose, and cold treated at 20°C for one month and then transferred to growth cabinet under a long day condition of a 16 h/8 h light/dark cycle at 20°C for further three months (A). When their size reached approximately 3 cm in length, they were transplanted on artificial soil and maintained for 6 months (B). Emergence of shoot from a cold treated plantlet for three months on soil (C) and incubated under natural day conditions in a green house (D). After 7 years, they grew to maturity and flowered normally (E).

cultured for 6 months (Figure 2B). At this stage, they were again treated with low temperature for 1 month, and returned to the standard growth condition for 5 months. When new green shoots emerged, plantlets were transferred to pots and grown in a green house (Figure 2C) under a cycle of 1 month cold and 5 months growth (Figure 2D). After 6 years culture, one out of 7 plants formed the first flower bud, although it failed to bloom. At the 7th year, however, the same plant opened a healthy flower of normal size (Figure 2E).

Genetic diversity

Genetic diversity among population was assessed by the random amplified polymorphic DNA (RAPD) analysis using DNA samples isolated from leaves of randomly selected wild plants growing in the protection area. Thirty one samples were obtained from six locations (Figure 3A, **a** through **f**) in the conserved area of 1.5 hectare and serially numbered. Location **a** contained plants #1–5, #21–26 and #31; location **b** contained #9 and #10; location **c** contained #6–8 and #11; location **d** contained #13–15; location **e** contained #16 and #17; and location **f** contained #18–20. In this habitat, plant size was variable, showing leaf numbers from one to five (Figure 3B). Plants with four or five leaves were regarded as mature plants as they flower (Figure 3B), and leaf pieces for DNA extraction were sampled mostly from these plants.

Screening initially with 20 RAPD primers, we found 5 to yield distinct and reproducible polymorphic patterns of amplified DNA fragments (Figure 4A). The primer-specific polymorphic fragments in kb were as followings: OPM01, 0.8 and 0.7; OPM06, 1.8, 1.0 and 0.6; OPN04, 1.5, 1.2, 1.0 and 0.8; OPM05, 1.3, 1.2, 0.8 and 0.3; OPN01, 0.7, 0.6, 0.4 and 0.3. The presence or absence of each fragment was pair-wisely compared in the population, and scored for cluster analysis (Figure 4B). Results showed samples to be classified into five groups. The numbers of scored plants are 10 each for Group A and D, 3 each for Group B and C, and 2 for Group E. Plants in each group were then classified according to their growing locations; Group A contained plants from locations **a**, **b**, **e** and **f** (see Figure 3A); Group B contained those from **a** and **f**; Group C contained those from a and **b**; Group D contained those from **a**, **c**, **d** and **f**; and Group E contained plants from **a**. Plants from Rebun Cultivation Center of Alpine Plants were also diverse, three belonging to Group A, and one each to Group D and E (Figure 4B). These observations suggested that plants in the habitat are genetically diverse, and that each genotype may distribute throughout the habitat without being restricted to particular location.

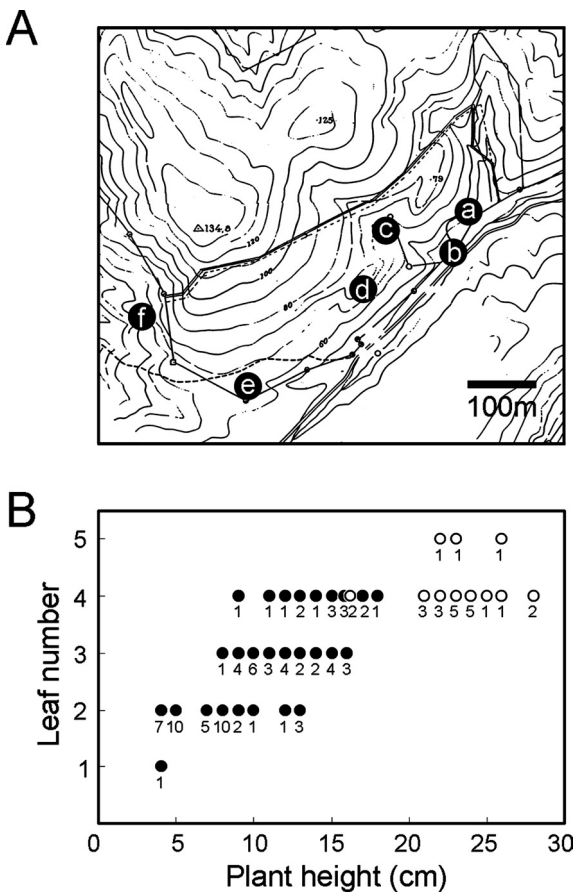

Figure 3. Natural habitat and properties. (A) Sampling spots. The protection area of *C. macranthos* var. *rebunense* is located in the northern part of Rebun Island, which is situated in lat. 45°30′14″N. and long. 141°4′16″E. The contour interval is 10 m. Pieces of leaves were collected from plants naturally growing in the indicated area. Each plant was numbered and categorized into 6 groups according to the location (see text). Correlation between the plant height and leaf number. Measurements were made on plants grown in an 1 m^2 quadrat set up in the preservation area of Rebun Island (June 10, 2000) Each numerical value beneath each symbol represents respective number of plants counted. Plants with and without flowers are indicated by open and closed circles, respectively.

Conclusions

The present work has demonstrated that *C. macranthos* var. *rebunense* can be grown artificially from immature seeds to flowering maturity without transplanting to the open air. The procedure is simple and no specialized techniques are required, though selection of appropriate culture conditions such as medium components, temperature, vernalization, watering, etc. are necessary. The *in vitro* cultured plants can be used as the experimental material for analyses on molecular mechanisms of development and flower induction of this orchid, which have so far scarcely been known. The results also indicate that it is possible to propagate this plant species *in vitro* on a large scale. A similar approach has been undertaken in the Rebun Cultivation Center of Alpine Plants. *In vitro* grown seedlings were transplanted

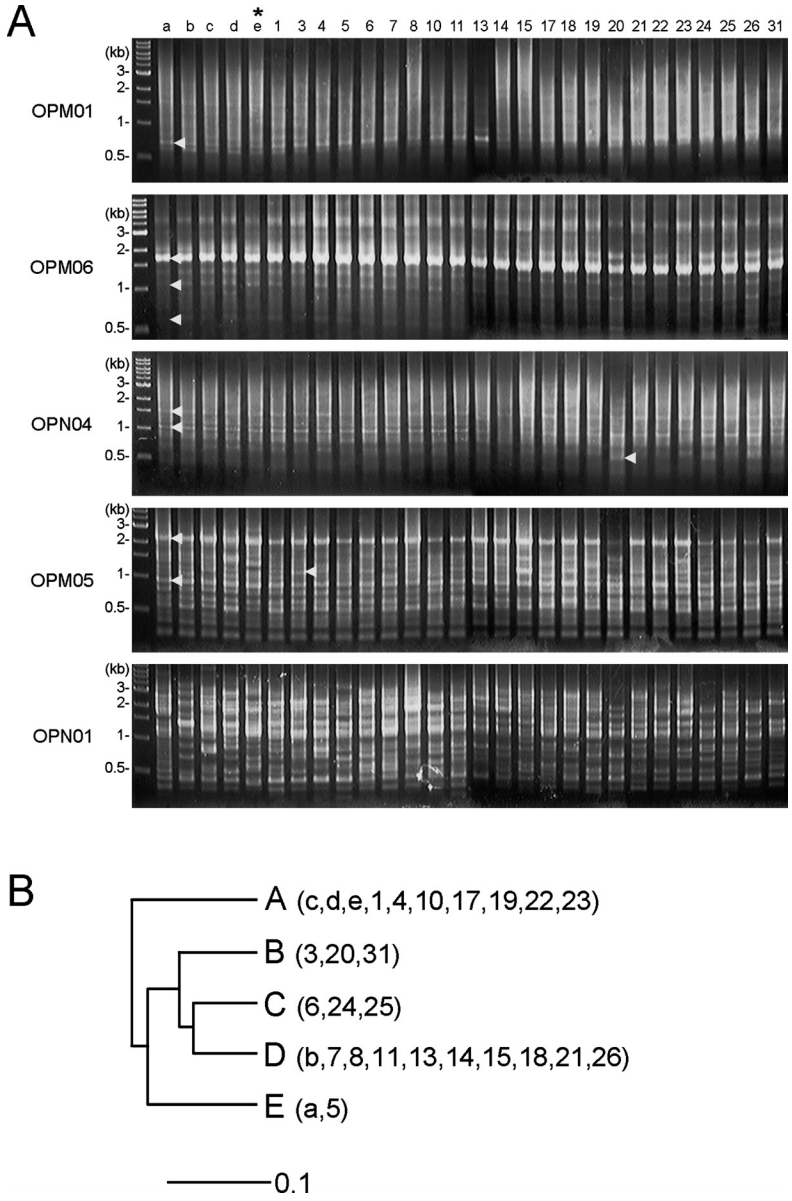

Figure 4. Random amplified polymorphic DNA analyses. (A) RAPD patterns. Total DNA isolated from indicated plants was subjected to PCR with the specific primers indicated at the left. Samples were from plants propagated from seeds at the Rebun Cultivation Center of Alpine Plants (a through d), from a plant propagated in this study (e) (see Figure 2E), and from wild stocks grown in the conserved area in Rebun Island (1 through 31). Amplified DNA samples were fractionated by agarose gel electrophoresis, and visualized after ethidium bromide staining. Some polymorphic fragments are indicated by open arrowheads (see text). (B) Average linkage cluster analysis. Tree diagram was produced from RAPD profiles. The scale is calculated by the SAS program as described in text. Individual plant shown in Figure 4A is indicated in parentheses.

to the open air, and many of them flowered (T. Iino, personal communication).

It is thus conceivable that these artificially propagated plants can be transplanted back to the natural habitat in Rebun Island. However, one critical problem concerning introduction of *ex situ* propagated plants is genetic disturbance. If the currently conserved colony is composed of a single, or at most few genotypes, introduction of the same genotype would accelerate reduction of genetic diversity. To address this argument, we examined genetic polymorphism among randomly selected samples by RAPD analysis. Although the number of analyzed samples was rather small due to difficulties in sampling of this strictly conserved plant, at least five independent groups were found to co-exist in the colony. This indicates that the wild colony in Rebun Island is consisted of genetically different individuals, and suggests that the introduction of artificially propagated plants with a similar genetic background originating from the same colony may not disturb the genetic structure of the existing wild colony.

Acknowledgements

The authors thank Mr. T. Iino (Rebun Cultivation Center of Alpine Plants, Rebun Town, Hokkaido, Japan) for providing us leaf samples for DNA extraction. This work was partly supported by grants from the Japan Society for the Promotion of Science and grants-in-aid from the Ministry of Education.

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