Micropropagation and genetic relationships among three species of *Pogonia* (Orchidaceae)

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CONTENTS

Introduction

The genus *Pogonia* is a terrestrial orchid which is taxonomically placed in the Epidendreae, the Orchidoideae. They are commonly distributed throughout the temperate areas in the Northern Hemisphere. *Pogonia japonica* Reichenb.f., *P. minor* (Makino) Makino and *P. yunnanensis* Finet are distributed in East Asia (Chen 2009), while *P. ophioglossoides* (L.) Jussieu is distributed in the southeastern part of North America (Brown and Folsom 2003). The disjunctive distribution of congeneric orchids between East Asia and Eastern North America have been reported (Chen 1983). *Pogonia* is one of the genera which have disjunctive distribution.

Pogonia japonica and *P. ophioglossoides* grow commonly in bogs in coastal plain and lowland (Ohwi 1954, Radford *et al.* 1968) while *P. minor* grows rather in highland grass fields and bogs (Kitamura *et al*. 1964) and furthermore, *P. yunnanensis* grows in alpine grass fields and abies forest in Yunnan and Tibet (Chen 2008; 2009).

Since they are threatened or locally extinct due to degradation and decrease of their habitats by land reclamation and overharvest, it is very necessary to conserve them by *in vitro* germination and micropropagation. Many temperate terrestrial orchid species have proven to be difficult to propagate by axenic methods (Arditii 1967). To promote axenic germination of such a terrestrial orchid species, various researchers have been studied. For instance, medium, composition, photoperiod and temperature for culture give influences, various culture conditions have been investigated for the axenic germination (Ichihashi 1979; Harvais 1982; Oliva and Arditti 1984; Miyoshi 1988; Takahashi and Tsutsui 1992). Also it was reported that axenic germination was promoted by use of immature seeds (Withner 1974; St-Arnaud *et al*. 1992; Takahashi and Tsutsui 1992). Moreover, symbiotic germination *in vitro* of terrestrial orchids has been studied

(Clements *et al.* 1986; Clements 1987; Rasmussen 1992; Tomita 1995, 2001; Yagame and Yamato 2009).

The axenic germination of seeds of *P. japonica* was very high on Knudson C (KC) (Knudson 1946) while that of *P. minor* was not seen in spite of same genes (Sawa *et al*. 1979). Seeds of *P. ophioglossoides* germinate on KC medium with the addition of peptone and Curtis (1936) medium (Stoutamire 1964; Arditti 1982). However, *in vitro* germination of *P. yunnanensis* has not yet been reported.

Various orchids have been cultured *in vitro* on KC, Vacin and Went (VW) (Vacin and Went 1949), Murashige and Skoog (MS) (Murashige and Skoog 1962), Hyponex (Kano 1965) and Gamborg (B5) (Gamborg *et al.* 1968) media with some modifications which have specific effects on dedifferentiation, regeneration, micropropagation, organ formation, plant growth and so on. Growth of orchids *in vitro* can be regulated by and correlated with nitrogen ratio in total ion concentration and nitrogen-form ratio (NH_4-N:NO_3-N) (Uesato 1973, 1974; Ichihashi 1987; Mukoyama 1987; Shimasaki and Uemoto 1990).

Micropropagation of orchids from protocorm-like bodies (PLBs) produced from shoot tips, flower stalks, leaf tips and root tips (Rotor 1949; Morel 1960; Churchill *et al.* 1971, Tanaka *et al.* 1976; Sanchez 1988; Yam and Weatherrhead 1991; Kerbauy 1993; Vij 1993; Vij *et al.* 1994) and tissue-cultured shoot primodia (Na and Kondo 1995; Tanaka *et al.* 1997) can be regulated with auxins and cytokinins in combination.

Among the members of the orchidaceous plants, *Pogonia* has one of the largest chromosomes and there is favorable to work with chromosome studies (Tanaka 1968). Tanaka (1971) states that relic and primitive-origined *Pogonia* species were commonly characterized by the large chromosomes with no particular heterochromatin.

Pogonia japonica showed the chromosome number of 2n=20 (Tanaka 1962a; 1965; 1971; 1974), while *P. minor* showed the chromosome number of 2n=18 (Mutsuura and Nakahira 1960; Tanaka 1962a; 1965; 1971). *Pogonia ophioglossoides* also showed the chromosome number of 2n=18 (Baldwin and Speese 1957). However, *P. ophioglossoides* was reported to have 1-3 B-chromosomes (Oryu and Kondo 2001; Takahashi and Kondo 2004). The karyotype of *P. japonica* consisted of eight pairs of median-centromeric chromosomes, one pair of terminal-centromeric chromosomes and one pair of terminal-centromeric chromosomes containing nucleous organizing regions (NOR's) (Tanaka 1962a, b). In contrast, the karyotype of *P. minor* and *P. ophioglossoides* consisted of nine pairs of median-centromeric chromosomes (Tanaka 1962a, b; Baldwin and Speese 1957). It was speculated that the third pair of median-centromeric chromosomes in *P. minor* might make a fission at the centromeric region to produce four small, terminal-centromeric chromosomes containing NOR's in *P. japonica* (Tanaka 1962a, b). Thus, *P. japonica* could be a pseudoploid and a hyperploid of *P. minor* (Tanaka 1962a, b). *Pogonia japonica* and *P. minor* might be originated from *P. ophioglossoides* according to the molecular study by Cameron and Chase (1999).

Pogonia japonica and *P. minor* sometimes show mixoploids (Mizuno and Yamasaki 1952; 1963); *P. japonica* had 2n=19 to 23 while a plant of *P. minor* had 2n=18 to 21. Moreover, *P. japonica* displayed a case of a natural haploid plant (2n=9) (Kondo 1970).

The karyotype of *P. yunnanensis* has not reported until now.

Pogonia japonica, *P. minor*, *P. yunnanensis* and *P. ophioglossoides* usually do not show any interspecific hybrid in the wild state. *Pogonia japonica*, *P. minor* and *P. ophioglossoides* cultivated at Hiroshima University and Mukaishima orchid center were used for genetic and breeding studies (Table 1; Fig. 1).

A few decades some plants of *Pogonia* smaller than average *P. japonica* with unusual flowers were found; firstly at mountainous districts in Akita Prefecture (Takahashi 1987) and were tentatively called ‘Miyamatokisou.’ It might be due to a kind of “Edge effect.” In contrast, a plant grown in Higashi-Hiroshima City seemed to have different flower morphology from *P. japonica* as well as *P. minor* and showed somewhat similar to ‘Miyamatokisou’.

Polymerase chain reaction (PCR)-based molecular technique as RAPD (randomly amplified polymorphic DNA) technique is useful to generate DNA markers for the detection of polymorphism even genetic distance is close as interspecific and intergeneric relation. RAPD has been widely adopted here the simplicity of the assay and its immediate applicability to a wide range of respective species.

To clarify their identification, their molecular information were obtained by RAPD technique.

Table 1. General imformation of three species of *Pogonia*

Plant name	Distribution	Previous counts of chromosome numbers(2n)	Preference
Pogonia			
japonica Reichb.f.	East Asia (the Japanese Archipelago, Kunashiri to Shikotan Islands, Kuriles Archipelago, Far East Russian Continent, Chinese Continent, Korean Peninsula)	20	Tanaka, 1962a, 1965, 1971, 1974
		19-23	Mizuno and Yamasaki, 1952, 1963
		9	Kondo 1970
minor (Makino) Makino	East Asia (from Hokkaido to Kyushu of Japanese Archipelago, Korean Peninsula and Taiwan Island)	18	Mutsuura and Nakahira, 1960 ; Tanaka, 1962a, 1965, 1971
		18-21	Mizuno and Yamasaki, 1952, 1963
ophioglossoides (L.) Jussieu	Southeast Nouth America (From Newfoundland to Florida)	18	Baldwin and Speese, 1957

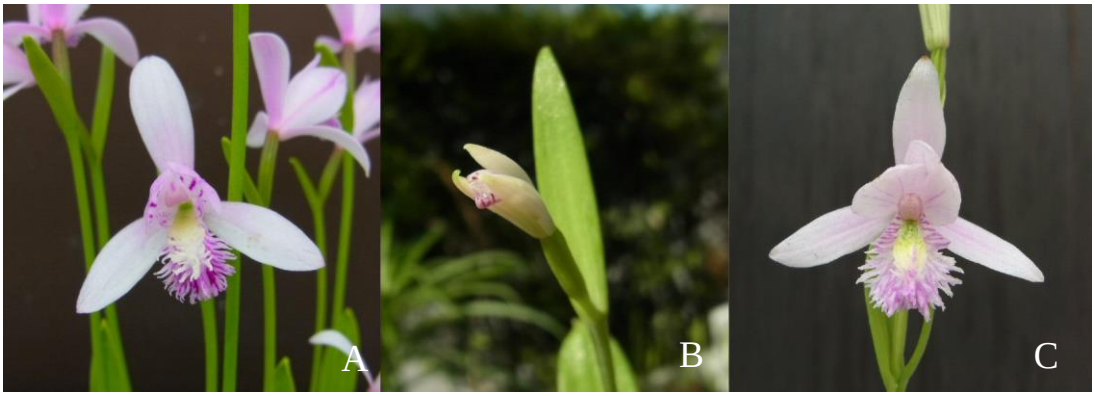

Fig. 1. Plants of wild species of *Pogonia* studied. A: *Pogonia japonica*, B : *P. minor*, C : *P. ophioglossoides.*

Chapter 1 Seed germination of *Pogonia*

Materials and methods

Plant materials

Plants of *Pogonia japonica* were collected on campus of Hiroshima University, Higashi-Hiroshima City, alt. ca 200 m while those of *P. minor* were collected in Ikenodan High-Moore, Mt. Hiba, alt. 1,279.5 m. Plants of *P. ophioglossoides* were collected in North Carolina, U.S.A. and were sent and presented by K. M. Cameron, New York Botanical Garden. They were cultivated in Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Higashi-Hiroshima City and Mukaishima Orchid Center, Onomichi City for continuous cultivation and research.

Axenic culture

In axenic germination of *P. japonica* and *P. minor*, effects of seed aging and culture medium types on axenic germination of the species were investigated. Fruit capsules or seeds were harvested at intervals of 15 days since the 30th days state after the hand pollination until the day when the capsules were matured, dried and dehisced. Aseptic seeds of *P. japonica* and *P. minor* were sowed on modified KC (mKC) medium [1,000mg/l $Ca(NO_3)_2 \cdot 4H_2O$, 250mg/l KH_2PO_4, 250mg/l $MgSO_4 \cdot 7H_2O$, 500mg/l $(NH_4)_2SO_4$, 40mg/l Fe-EDTA, 7.5mg/l $MnSO_4 \cdot 4H_2O$ and 20mg/l Saccharose] and modified Hyponex (Kano 1965) medium supplemented with no hormone. mKC medium adjusted pH 5.0 and 5.8 were used. Non-dehisced, immature capsules harvested were surface-sterilized with 1.2% (v/v) sodium hypochlorite solution for ten min. and were, then, dissected longitudinally and their seeds were scooped for sowing. Mature seeds

obtained from the dried, dehisced capsules were surface-sterilized with 1.2% (v/v) sodium hypochlorite solution and a few drops of Tween 20 for ten min. and rinsed three times with distilled, sterile water, and then, sowed on medium. They were cultured under continuous light at 22±2℃. Germination rate of seeds in *P. japonica* and *P. minor* were counted at the 90th days stage after sowing.

Also germination of immature seeds at 45th days stage after the hand pollination and mature seeds surface-sterilized in *P. japonica* and *P. minor* were observed at 175th days stages after sowing.

Non-dehisced mature capsules of *P. ophioglossoides* were harvested at about 90 days after self-pollination by hand. Then, mature seeds in the capsules were immersed in water with detergent for one night and were surface-sterilized with 1.2% (v/v) sodium hypochlorite solution for ten min and rinsed three times with distilled sterile water, and then sowed on mKC medium.

Several treatments such as hormone application, dark condition and low temperature were used to promote axenic seed germination of *P. minor* by using immature seeds at 45th days stage after pollination. Additionally non-dehisced mature capsules of *P. minor* at 90th days stage after pollination were harvested and their mature seeds in the capsules after sterilization by 1.2% sodium hypochlorite solution were sown on mKC solid medium.

Symbiotic culture

Spiranthes sinensis and *Cymbidium goeringii* from Mukaishima, Hiroshima Prefecture, *Habenaria radiata* from Himeji City, *Pogonia japonica* collected around a bog in Mt. Hiba, *P. japonica* cultivated in Mukaishima Orchid Center and *P. minor*

collected around a bog in Mt. Hiba were used to isolate a symbiotic fungus for this experiment for seed germination in *P. japonica* (Table 2). Their fungi were not identified (Table 2).

A piece of fungus infected root and rhizome of an adult plant was washed gently under running water to remove the soil and mosses. Particles on the piece were removed by a brush with soft bristles. Small segments of roots and rhizomes, 5-7 mm long, were cut from the tissue that showed yellow colored of the tissue. Those segments were soaked into 70% ethanol for a few seconds and were washed by sterilized water. The segments were sectioned, less than 1 mm thick, longitudinally under the binocular. These sections were examined under the binocular to check for the presence of pelotons. If the pelotons were observed, the epidermis was removed from the sections. The cleaned section of cortical cells with pelotons was transferred on the fungal isolating medium (FIM). FIM (Clements 1979) consisted of 300mg/l $NaNO_3$, 200mg/l KH_2PO_4, 100mg/l $MgSO_4 \cdot 7H_2O$, 100mg/l KCl, 100mg/l yeast extract, 50mg/l antibiotic streptomycin sulphate, 5g/l saccharose and 10g/l agar at pH 4.5-5.0. If the fungal hyphae were observed on the medium, agar cubes with the hyphae were cut out and were transplanted to 1/5 potato dextrose agar medium (Nissui Pharmaceutical Co., LTD) for propagation of the fungi.

Mature seeds at about 90th days stage after self-pollination were used for symbiotic seed culture. The method for seed sterilization was same to above. The seeds were sown on Oat-powdered agar (OPA) (Tomita 1995) and were used 12 fungal species for symbiotic germination of *P. japonica* (six fungal species isolated and transferred from M. Tomita, Hirosaki University and six fungal isolated as above) (Table 2). Also, mKC medium was used for the non-symbiotic germination. For the symbiotic germination investigation, a small fungal inoculation (5 mm^2) was added to the upper side of the slope

in the ⌀30 mm test tube. Cultures were maintained in a 16-hour light and 8-hour dark condition at 22±2℃. Seeds which the protocorm showed swelling to break the seed coat and changed to green in color were defined as germinated. Germination and protocorm development were settled and assessed on a scale 0-6 as follows: 0) No germination; 1) Embryos swollen and greening; 2) Protocorms with rhizoids; 3) Shoot formation; 4) Differentiation of a leaf more than 5 mm long; 5) Formation of rhizomes from a shoot; 6) dead.

Results

Axenic culture

Capsules of *P. japonica* and *P. minor* were matured, dried and dehisced approximately 105 days after hand-pollination at a year investigated. Embryos in the seeds of both species observed were not formed at the 30th day stage after hand-pollination but formed at the 45th day stage after hand-pollination in both species. The rate of embryo formation was more than 80% in at the 45th day stage after hand-pollination in both species.

Immature seeds in non-dehisced capsules of the 30th day stage after hand-pollination did not germinate at all on mKC and Hyponex media in both species. Immature seeds of *P. japonica* at the 45th day stage showed the best seed-germination rate of 76.9% on mKC medium (Table 3). Germination rates of the seeds at and after the 60-day-old stage in the non-dehisced capsules of *P. japonica* on mKC medium were between 40-70% and decreased in compared with those at the 45th day stage (Table 3).

On Hyponex medium, germination rate of *P. japonica* at the 45-day-old stage was 51.7%. In contract, a few seed germinations of *P. japonica* were observed on Hyponex

medium in their non-dehisced capsules except for that at the 45th day stage (Table 3). Germination rates of more immature seeds at the 45 day stage were the best in both media in *P. japonica* as for seeds in non-dehisced capsules.

Germination rates of immature seeds in the non-dehisced capsule of *P. minor* harvested were low in % on mKC medium, although embryos were formed in their seeds (Table 3). The highest germination rate of seeds on mKC medium in non-dehisced capsule of *P. minor* was only 1.4% using seeds at the 75th day stage (Table 3). Seeds in non-dehisced capsules of *P. minor* sowed on Hyponex medium did not show any germination (Table 3). Thus, axenic seed germination of *P. minor* was difficult and not correlated with seed maturation.

The surface-sterilized seeds from the matured and dehisced capsules obtained from the two species showed high germination rates on mKC medium in spite of more aged seeds. Germination rate of *P. japonica* was 83.3% cultured on mKC medium adjusted pH 5.8, as high as that of the seeds at the 45-days-stage (Table 3). However, germination rate of surface-sterilized mature seeds of *P. japonica* on Hyponex medium was 11.2% at the 90th days stage after sowing and was higher than that of immature seeds at 45th days after hand pollination. The seed-germination rate after sowing the surface-sterilized seeds of *P. minor* was 10.2%, the highest rate among the all data studied in *P. minor* (Table 3).

At 175th days stage after sowing the germination rate of surface-sterilized mature seeds of *P. japonica* on Hyponex medium reached up to around 75% (Fig. 2). In *P. japonica,* seedling growth after germination on mKC medium was better than that on Hyponex medium (Fig. 2). In *P. minor,* germination rate on mKC medium using surface-sterilized seeds reached up to more than 70% at 175 days stage after sowed, while that on Hyponex medium showed less than 1% (Fig. 2). Germination rate on mKC medium using

immature seeds at 45th days stage after the hand pollination in *P. minor* was around 1% even at 175 days after sowing.

Axenic germination of *P. ophioglossoides* was well, when mature seeds in non-dehisced capsules were surface-sterilized and sowed on mKC medium. The germination rate was 89.7%.

Several treatments were experimented to promote axenic seed germination of *P. minor* (Table 4). If seeds of *P. minor* were cultured under 4℃ for a month and then, shifted to 24℃, their germination rates got slightly high and was 8.5% (Table. 4). Treatments of hormone applications and condition of darkness did not show any significance of effects for promotion of axenic germination of *P. minor* in this research (Table 4).

Symbiotic culture

At 30 days after sowing, most of embryos of *P. japonica* examined swelled and changed to green color on non-symbiotic and symbiotic OPA media (Figs. 3 and 4). Germination rates of the seeds of *P. japonica* used under symbiotic condition were not different from those of the non-symbiotic OPA medium and were more than 85% (Figs. 3 and 4). The germination rate on mKC was 41.5% (Fig. 4). Compared with non-symbiotic OPA medium, inoculation of fungus isolated from *Cymbidium goeringii* collected in Mukaishima (No. MCG), one isolated from *Spiranthes sinensis* collected in Mukaishima (No. MSS), one isolated from *Habenaria radiata* collected in Himeji City (No. HHR) and one from *P. japonica* cultivated in Mukaishima (No. HoPJ) on OPA medium stimulated seedling growth (Figs. 3 and 4). On OPA media with their inoculation, protocorm with rhizoid and leaf differentiation were observed (Fig. 4). Especially

inoculation of No. MSS gave the best growth for the seedlings (Figs. 3 and 4). In No. MSS, formation rate of the seedlings with leaf was 82.5% (Fig. 4).

Sixty days after sowing, seedlings with more than 5 mm leaf were observed on OPA media with the inoculation of No. HHR, No. MCG and No. MSS (Figs. 5 and 6). The growth of the seeds inoculated with No. MCG and No. MSS were more remarkable. Percentages of differentiation with leaf more than 5 mm long on OPA media with No. MCG and No. MSS were 69.2% and 76.5%, respectively, while those of differentiation with more than 5 mm leaf on OPA media with other fungi were 0.0-2.4% (Figs. 5 and 6). Some seedlings were died on OPA media with No. 614 and No. 618 (Fig. 6). The germination rate on mKC were increased up to 66.0% (Fig. 6). Plant growth on OPA media with the inoculation of No. HHR, No. MCG, No. MSS and No. HoPJ were better than that on mKC.

Up to the 90th day after sowing, the germination rates on non-symbiotic and symbiotic OPA media reached more than 98% (Figs. 7 and 8) and the germination rate on mKC was 86.4% (Fig. 8). Compared with non-symbiotic OPA medium, inoculation of No. 624, No. MCG, No. HHR, No. HoPJ, No. HPM and No. MSS caused good growth (Figs. 7 and 8). Some of plants were differentiated to rhizomes on OPA media with MSS and with MCG. On symbiotic OPA media with No. 618, No. 706 and No. 824 seedlings were remarkably died (Fig. 8). In growth of *P. japonica* after the germination with fungus isolated from No. HPJ and No. HPM, growth with No. HPM was better than that with No. MPJ (Figs. 7 and 8). Growth pattern on mKC after axenic germination was similar to that on OPA medium with No. 624.

Discussion

In this study, axenic germination patterns of *P. japonica* and *P. minor* were different massively from each other. It was easy for *P. japonica* to germinate axenically on mKC medium but was difficult for *P. minor*. Sawa *et al.* (1979) reported that seeds of *P. minor* did not germinate on both Knudson C and Hyponex media. However, in this study, if immature seeds of *P. minor* were sowed on mKC medium and cultured by low temperature (4℃) for one month and treatment of surface-sterilization for mature seeds by 1.2% sodium hypochlorite solution, relative high germination rates were shown.

In this study, treatment of seed-surface sterilization was promoted in spite of mature seeds in *P. japonica* and *P. minor*. It was suggested that seed-surface sterilization could also be a promotion method for axenic germination of *Pogonia* seeds.

Corked element called Suberin, might be an inhibitor for germination and was accumulated into aging of seed coats (Harvais 1980). Suberin could be removed by sodium hypochlorite solution which sterilized the surface of seeds as well and then, permeability for water into seed could be promoted (Harvais 1980). Thus, the seeds treated by sodium hypochlorite solution showed high germination rate. Generally, seed germination of wild terrestrial orchids grown in the temperate climate had some difficulties because those seeds would stayed in cold winter and got stopped water flow permeating into them.

There is a report that endogenous abscisic acid (ABA) content in mature seeds of *Dactylorhiza maculata* was 14 times as much as that in immature ones (Guy van der Kinderen 1987). He described that ABA was a plant hormone which promoted dormancy and that mature seeds of orchids gave in dormancy by accumulation of ABA. Therefore, germination of mature seeds of orchids would be inhibited by ABA. After

seeds of *Dactylorhiza maculata* were surface-sterilized for two hours, there was practically no ABA to be detected (van Waes 1984).

In this study, the germination rate of immature seeds of *P. japonica* at the 45th days stage showed the best in using non-surface-sterilized seeds. Since immature seeds of *P. japonica* did not include certain inhibitors *e.g*. suberin and ABA, high germination rate would be shown. As germination inhibitors in seeds of *P. japonica* and *P. minor* were removed to by seed surface-sterilization, their germination rates would set well.

Treatment of low temperature for axenic seed germination of *P. minor* promoted germination rate than non-treatment in this study. Generally speaking, low temperature is one of a factor to break dormancy. The cold treatment seems to be either to decrease the level of endogeneous ABA or to increase the content of endogenous cytokinin, both of which can stimulate germination (Rasmussen 1995). There are some reports that orchid germination was promoted by low temperature (Ballard 1987; Ichihashi 1989).

Ecological effect of cold requirement in terrestrial orchid seeds is to prevent them from germinating immediately after dispersal. If orchid seeds germinate immediately after dispersal, seedlings would be died by cold temperature in winter. In this study, endogeneous ABA might decrease in seeds of *P. minor* by encountering cold. The increase in germination percentage observed by Pritchard (1984) in *Orchis morio* after repeated freezing and thawing could also be the result of seed coat rupture. Seeds coats of *P. minor* might be broken and suberin into seeds might be broken by freezing from encountering cold. Therefore, seeds of *P. minor* could germinate more after faced to low temperature in winter in nature.

Growth of *P. japonica* on mKC medium was synchronously occurred, while that on symbiosis OPA media was varied widely relatively. It was expected that the aspect was

differential ability of seedlings to maintain effective relationship to fungi (Alexander and Hadley 1983).

According to the result that germination rate was more than 98% on non-symbiotic OPA medium, it was suggested that seed germination of *P. japonica* can be started without symbiotic fungi and that seedling growth after germination was promoted by infected fungi. Species of *Dactylorhiza* and *Orchis* can germinate on water or water agar media without nutrients and they can survive for some days to some weeks without feeding (Rasmmussen 1995). That term means that orchid seeds survive by using nutrient themselves and wait for infection by symbiotic fungi (Vermeulen 1947).

Table 2. Orchid mycorrizal fungi used in the experiment

Isolate No.	Fungal Group	Origin of Isolate		remarks
		Host Orchid	Habitat	
614	Binucleate *Rhizoctonia*	*Dactylorhiza aristata* (Fisch.) Soo	Sapporo, Hokkaido	transferred from Dr. M. Tomita
618	*Rhizoctonia repens*	*Gymnadenia camtschatica*	Shakotan, Hokkaido	transferred from Dr. M. Tomita
624	*Rhizoctonia repens*	*Spiranthes sinensis* (Pers.) Ames var. *amoena* (M. v. Bieb) Hara	Futyuu, Toyama	transferred from Dr. M. Tomita
706	Binucleate *Rhizoctonia*	*Gymnadenia camtschatica* (Cham.) Miyabe et Kudo	Bikuni, Hokkaido	transferred from Dr. M. Tomita
864	*Rhizoctonia repens*	*Spiranthes sinensis* (Pers.) Ames var. *amoena* (M. v. Bieb) Hara	Bibai, Hokkaido	transferred from Dr. M. Tomita
9720	Binucleate *Rhizoctonia*	*Goodyera schiechtendaliana* Rechb. F.	Gosyogawara, Aomori	transferred from Dr. M. Tomita
MCG	(Unknown)	*Cymbidium goeringii*	Mukaishima, Hiroshima	
HHR	(Unknown)	*Habenaria radiata*	Himeji, Hyogo	
HoPJ	(Unknown)	*Pogonia japonica*	(Horticultural species)	
HPJ	(Unknown)	*Pogonia japonica*	Mt. Hiba, Hiroshima	
HPM	(Unknown)	*Pogonia minor*	Mt. Hiba, Hiroshima	
MSS	(Unknown)	*Spiranthes sinensis*	Mukaishima, Hiroshima	

Table 3. Differences in seed germination in *Pogonia japonica* and *P. minor* directly related in differences in seed maturation in days after hand pollination

Days after hand pollination (Days)	Medium	Germination rate % (no. of germinated seeds / no. of seeds sown)	
		P. japonica	*P. minor*
30	mKC 5.0	- (0/231)	- (0/309)
	mKC 5.8	- (0/389)	- (0/437)
	Hyponex	- (0/275)	- (0/168)
45	mKC 5.0	76.9 (560/728)	0.9 (11/1,231)
	mKC 5.8	86.2 (1,056/1,225)	0.6 (9/1,387)
	Hyponex	51.7 (736/1,424)	- (0/316)
60	mKC 5.0	56.6 (423/748)	- (0/148)
	mKC 5.8	57.1 (400/701)	- (0/133)
	Hyponex	0.0 (0/781)	- (0/344)
75	mKC 5.0	60.0 (404/673)	1.4 (3/210)
	mKC 5.8	60.0 (585/975)	0.3 (2/680)
	Hyponex	0.0 (0/781)	- (0/257)
90	mKC 5.0	42.1 (399/948)	0.7 (3/424)
	mKC 5.8	69.1 (797/1,154)	0.7 (3/410)
	Hyponex	2.0 (16/803)	- (0/257)
105(m)	mKC 5.0	80.6 (663/823)	10.0 (27/269)
	mKC 5.8	83.3 (620/744)	10.2 (19/186)
	Hyponex	11.2 (26/232)	- (0/189)

mKC = modified Knudson C

"m" means mature seeds

mKC 5.0 and mKC 5.8 designate pH 5.0 and 5.8, respectively

All media do not contain any hormone

Table 4. Seed germination in *Pogonia minor* in various culture condition

Culture condition	Seed germination % (no. of germinated seeds/no. of seeds sown)
Knudson C supplemented with:	
Hormone-free	0.6 (9/1,387)
1mg/l kinetin	0.6 (8/1,338)
1mg/l GA	0 (0/790)
1mg/l ABA to later hormone-free	0 (0/120)
1mg/l ABA to later 1mg/l kinetin	0 (0/170)
Culture treatment with:	
low temprature (4℃) to later 24℃	8.5 (86/1,017)
dark condition	0 (0/394)
surface-sterilization	19.4 (73/377)

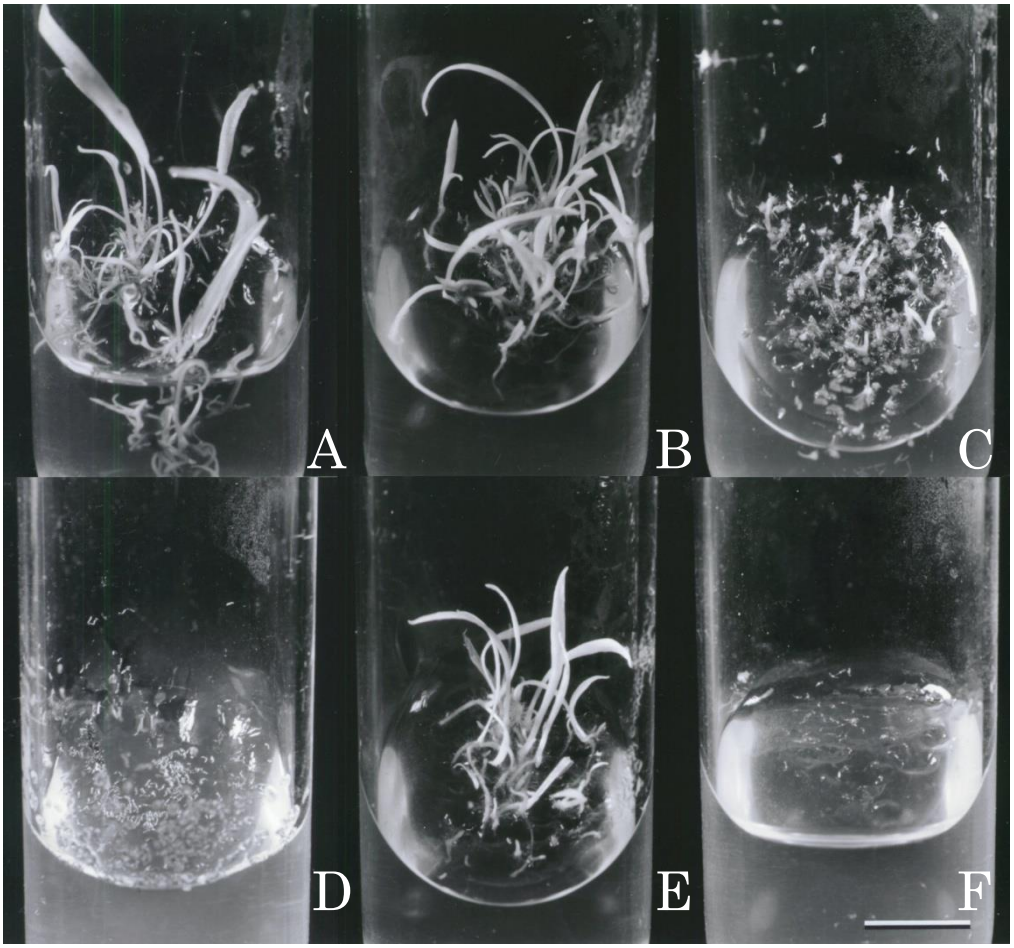

Fig. 2. Seed germination and development in two species of *Pogonia* at 175 days stage after sowed. A-C. *P. japonica*. D-F. *P. minor*. A and D. Immature seeds were sowed on KC gellan-gum medium, B and F. Mature seeds were sowed on KC gellan-gum medium. C and F. Mature seeds were sowed on Hyponex gellan-gum medium. Bar = 10mm.

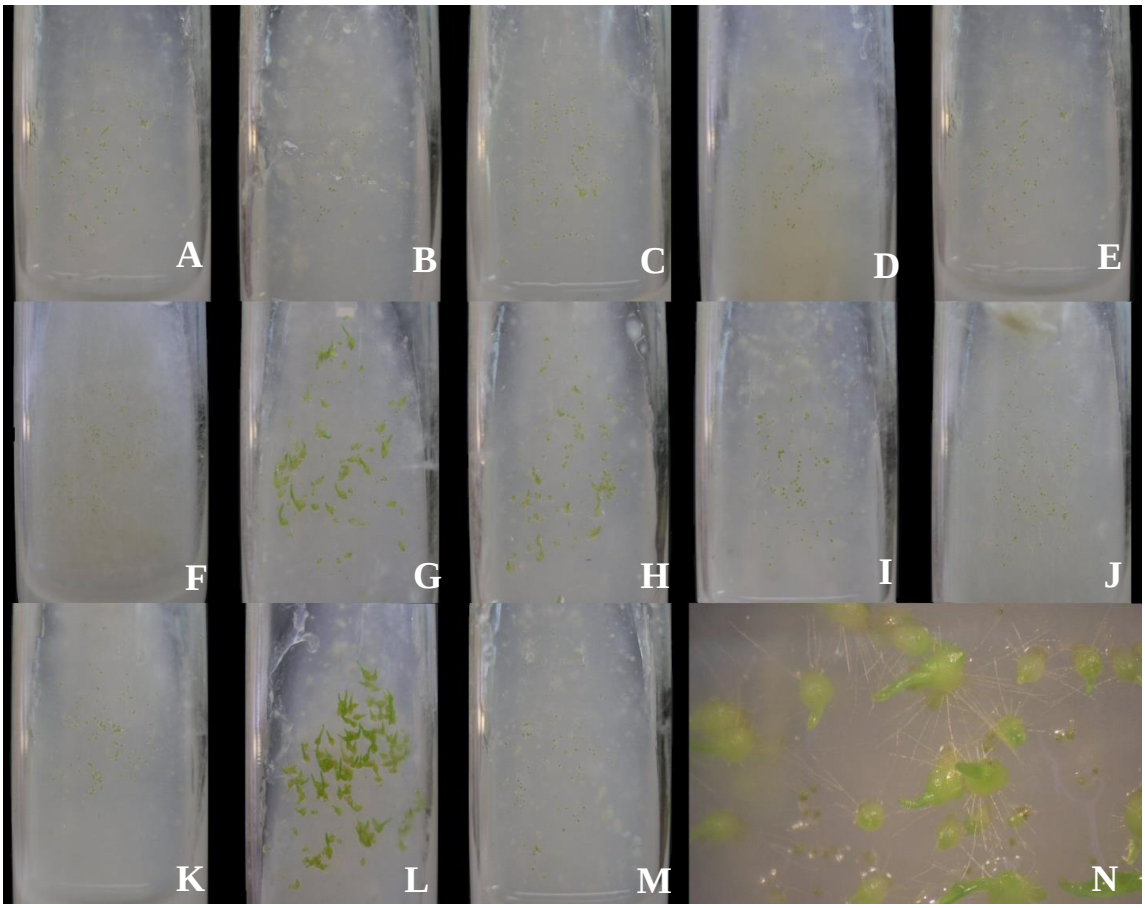

Fig. 3. Germination and development of *Pogonia japonica* seeds as affected by orchid endophytes under non-symbiotic and symbiotic conditions 30 days after sowing. A: OPA with isolated No. 614, B: OPA with isolated No. 618, C: OPA with isolated No. 624, D: OPA with isolated No. 706, E: OPA with isolated No. 864, F: OPA with isolated No. 9720, G: OPA with isolated No. MCG, H: OPA with isolated No. HHR, I: OPA with isolated No. HoPJ, J: OPA with isolated No. HPJ, K: OPA with isolated No. HPM, L: OPA with isolated No. MSS, M: OPA without fungus, N: Ptotocorms on OPA with isolated No. MSS.

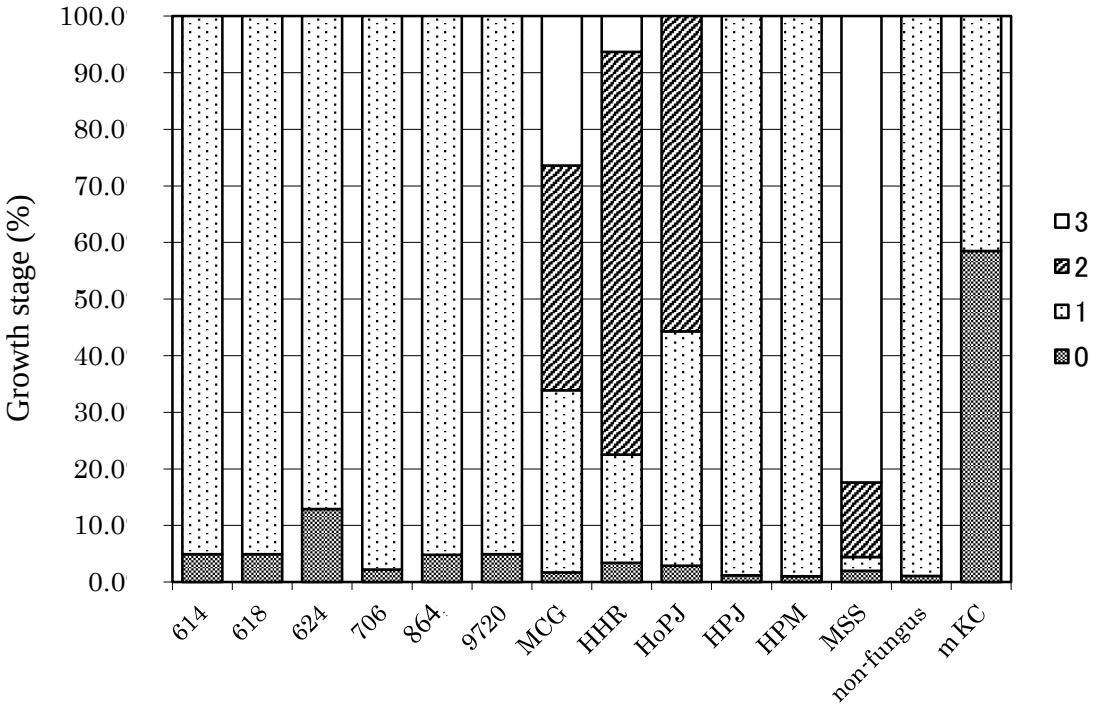

Fig. 4. Germination and development of *Pogonia japonica* seeds as affected by orchid endophytes under non-symbiotic and symbiotic conditions 30 days after sowing. Germination and protocorm development was assessed as follows: 0: Non-germination, 1: Embryos swollen and greening, 2: Protocorms with rhizoids, 3: Leaf differentiation.

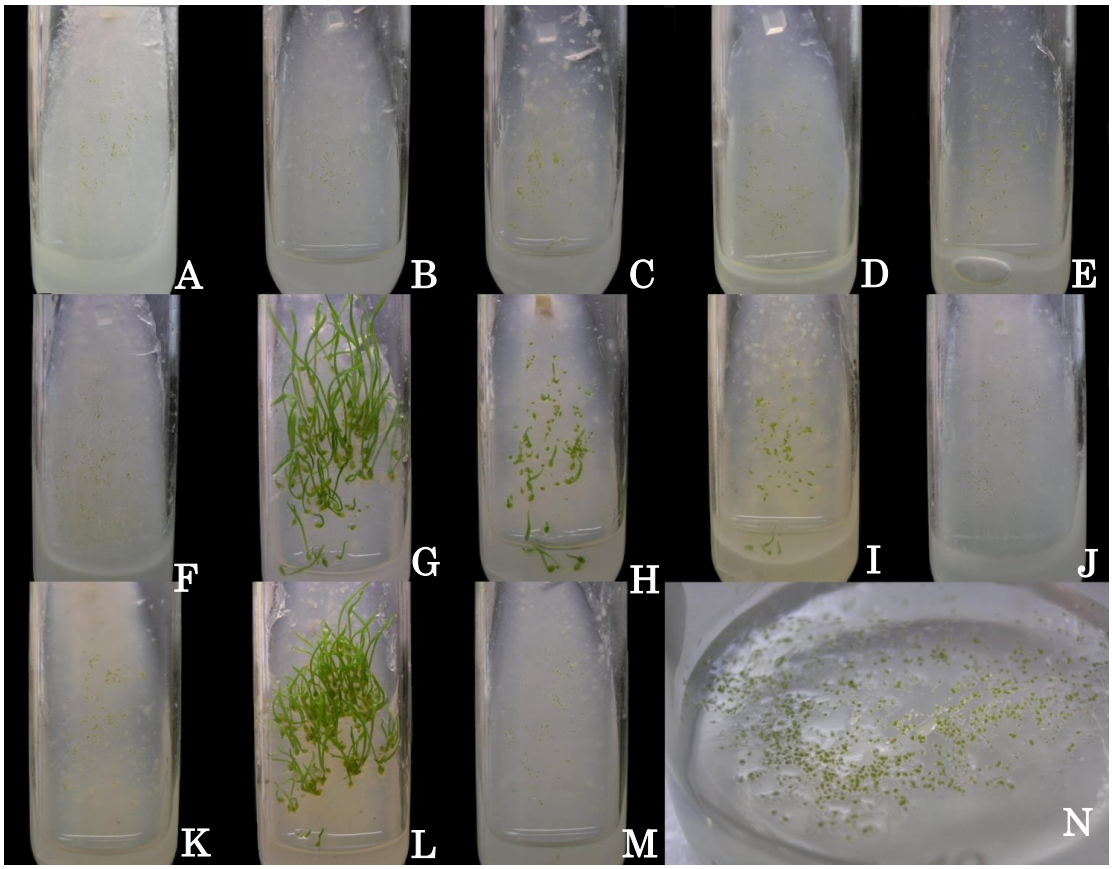

Fig. 5. Germination and development of *Pogonia japonica* seeds as affected by orchid endophytes under non-symbiotic and symbiotic conditions 60 days after sowing. A: OPA with isolated No. 614, B: OPA with isolated No. 618, C: OPA with isolated No. 624, D: OPA with isolated No. 706, E: OPA with isolated No. 864, F: OPA with isolated No. 9720, G: OPA with isolated No. MCG, H: OPA with isolated No. HHR, I: OPA with isolated No. HoPJ, J: OPA with isolated No. HPJ, K: OPA with isolated No. HPM, L: OPA with isolated No. MSS, M: OPA without fungus, N: mKC.

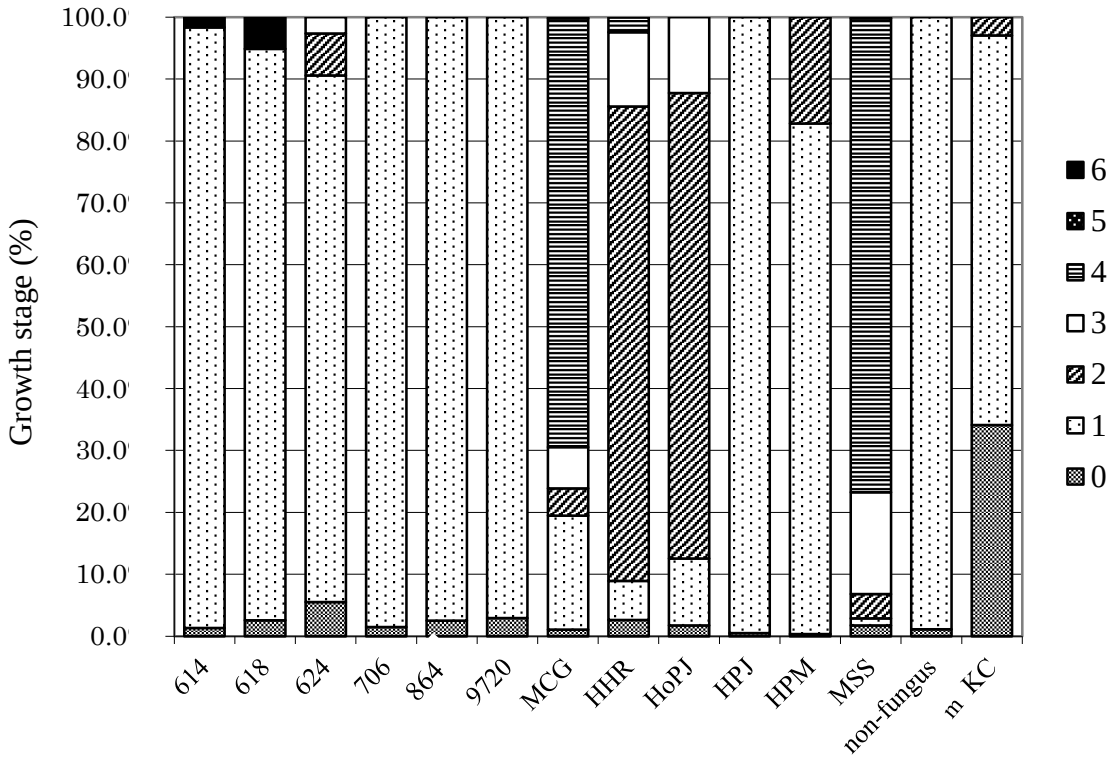

Fig. 6. Germination and development of *Pogonia japonica* seeds as affected by orchid endophytes under non-symbiotic and symbiotic conditions 60 days after sowing. Germination and protocorm development was assessed as follows: 0: Non-germination, 1: Embryos swollen and greening, 2: Protocorms with rhizoids, 3: Leaf differentiation, 4: Leaf (more than 5mm) differentiation, 5: Rhizome differentiation from plants, 6: Dead.

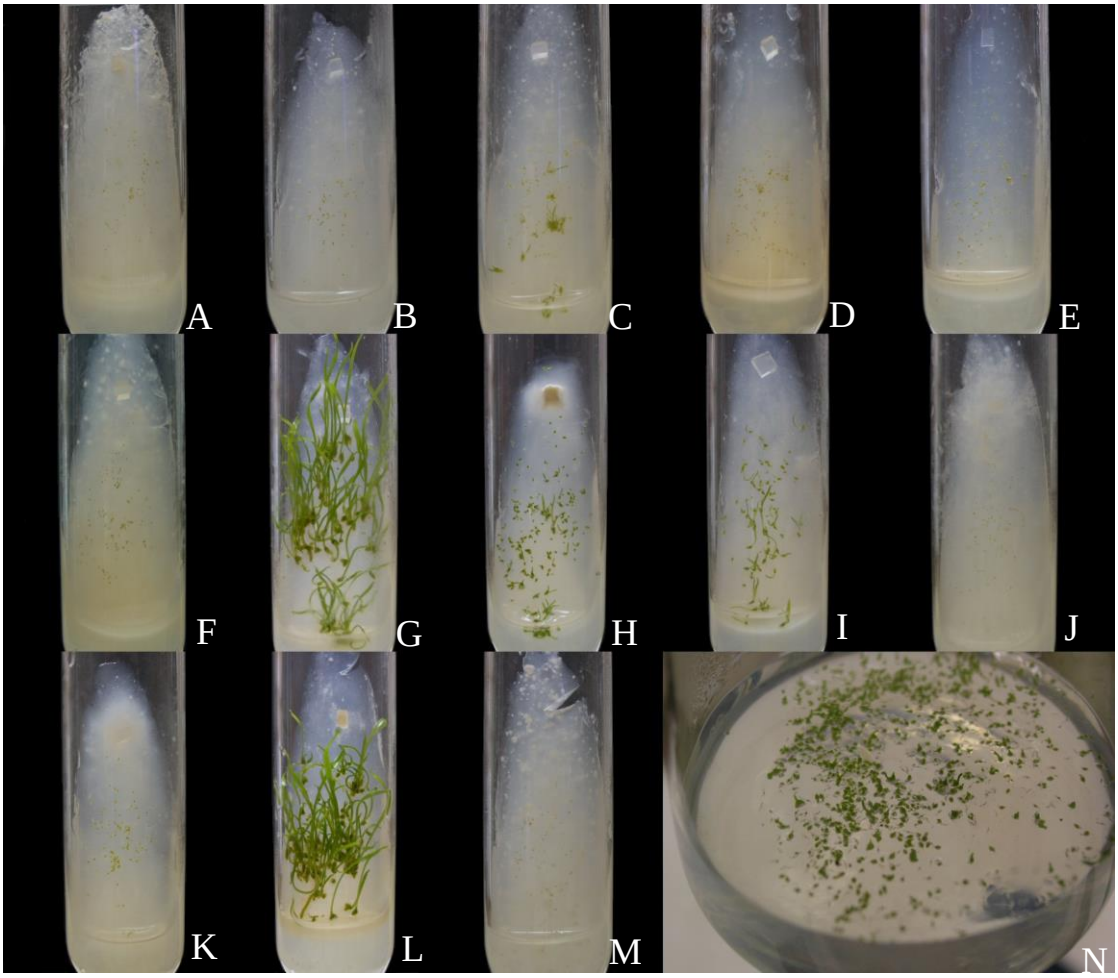

Fig. 7. Germination and development of *Pogonia japonica* seeds as affected by orchid endophytes under non-symbiotic and symbiotic conditions 90 days after sowing. A: OPA with isolated No. 614, B: OPA with isolated No. 618, C: OPA with isolated No. 624, D: OPA with isolated No. 706, E: OPA with isolated No. 864, F: OPA with isolated No. 9720, G: OPA with isolated No.MCG, H: OPA with isolated No. HHR, I: OPA with isolated No. HoPJ, J: OPA with isolated No. HPJ, K: OPA with isolated No. HPM, L: OPA with isolated No. MSS, M: OPA without fungus, N: mKC.

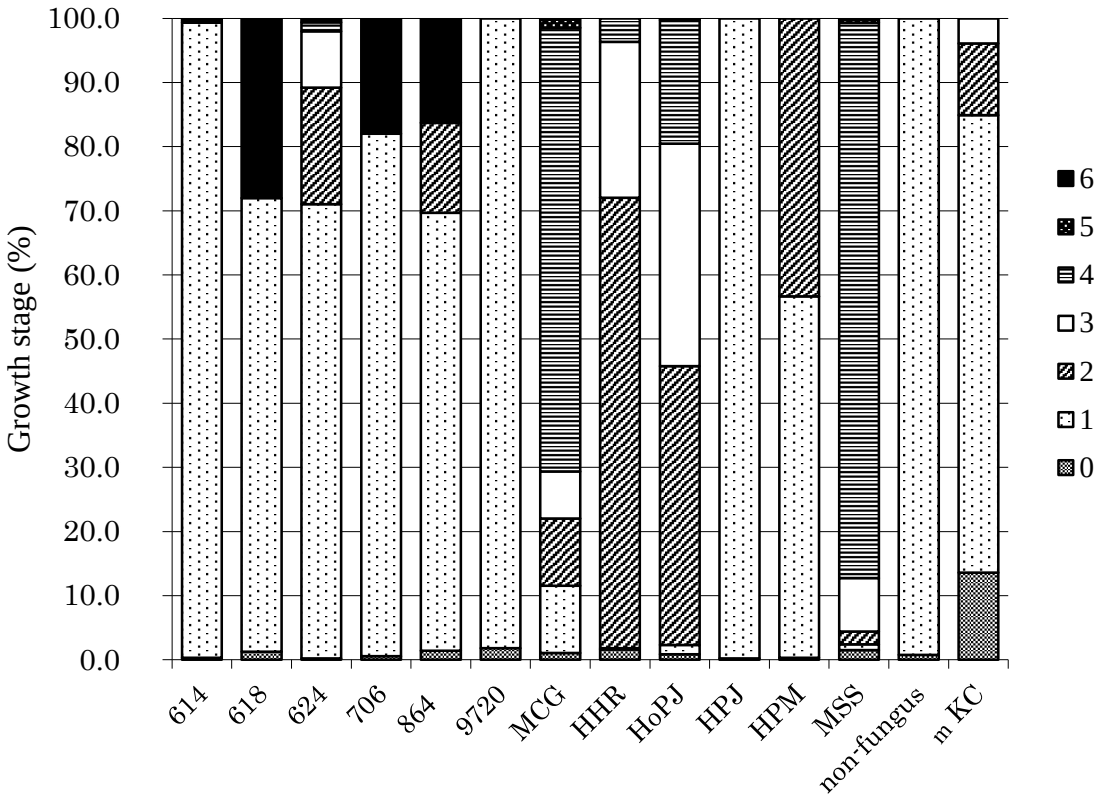

Fig. 8. Germination and development of *Pogonia japonica* seeds as affected by orchid endophytes under non-symbiotic and symbiotic conditions 90 days after sowing. Germination and protocorm development was assessed as follows: 0: Non-germination, 1: Embryos swollen and greening, 2: Protocorms with rhizoids, 3: Leaf differentiation, 4: Leaf (more than 5mm) differentiation, 5: Rhizome differentiation from plants, 6: Died.

Chapter 2 Plant growth

Materials and methods

Rhizome tips 2-3 mm long of ca. 100-day-old juvenile plants of *P. japonica* and *P. ophioglossoides in vitro* were harvested and used as explants.

They were cultured on media such as B5, Hyponex, mKC in which the $FeSO_4 \cdot 7H_2O$ was replaced with Fe-EDTA (40 mg/l), VW and MS media solidified with 0.3% Gelrite without plant hormones at pH 5.8. Some rhizome tips were used to investigate the effects of concentration of MS basal medium. Rhizome tips were planted on MS, 1/2MS and 1/4MS media. They were placed under 1,000 lux continuous light illumination at 24 ± 1℃.

Some rhizome tips of *P. japonica* were utilized to investigate the effects for growth of adventitious buds, leaves and rhizomes on nitrogen form ratio (NH_4-N:NO_3-N) in the MS basal medium. 1,650 mg/l NH_4NO_3 and 1,900 mg/l KNO_3 as inorganic nitrogen source in MS basal medium were replaced with either 825 mg/l $(NH_4)_2SO_4$ (NH_4-N:NO_3 = 1:0), 1,650mg/l NH_4NO_3 (NH_4-N:NO_3-N = 1:1) or 1,900 mg/l KNO_3 (NH_4-N:NO_3-N 0:1) with 1,000 lux continuous illumination at 24 ±1℃.

To observe the plant growth under symbiotic condition, rhizome tips from plants of *P. japonica* and *P. ophioglossoides* grown on 1/2 MS medium without any plant hormones were transplanted on OPA medium with or without some fungi. Twelve types of fungi were used for investigation of symbiotic germination in this study (Table 2). They were cultured under 22℃±2 with 1000 lux for two months.

Results

Adventitious shoot buds were formed at the rhizome tip of the explants in *P. japonica* at seven days after planting on five different gelrite-solidified media with no growth regulators (*e.g.*, Fig. 9A and B), and they soon developed into leaves and rhizomes (*e.g.*, Fig. 9C and D). After 60 days, they grew up to form a juvenile plant per explant (Fig. 9E). In basal five media, plant growth was well on MS and VW media but was inferior on B5, mKC and Hyponex media. The largest leaves were averaged 43.73 mm long on MS medium, and the smallest leaves were averaged 24.13 mm, (1/2 times smaller the largest leaves) on B5 medium (Table 5; Fig. 10). Then, in MS basal medium, more the concentration was low, more plant growth got slow (Table 5; Fig. 10). The smallest leaves were averaged 11.20 mm, (1/4 times smaller the largest leaves) on 1/4MS medium (Table 5; Fig. 10). The leaves of *P. japonica* cultured on MS were green, those on B5 were pale green, and those on modified mKC, modified Hyponex and VW media were yellowish green or chlorotic (Table 5; Fig. 10). Rhizome growth in plants cultured on mKC medium was not well. When rhizome tips were planted on diluted MS media, 1/2MS and 1/4MS, the rhizome growth of differentiated plants became well (Table 5).

At the 60 days stage after from rhizomes of *P. ophioglossoides* were transplanted, the longest leaves got average 19.87 mm on VW while the shortest leaves got average 8.87 mm on B5 (Table 6; Fig. 11). Leaf color pattern of *P. ophioglossoides* was similar to that of *P. japonica*. Plant growth of *P. japonica* was better than that of *P. ophioglossoides* on five basal media. Plant growth of *P. ophioglossoides* on 1/2MS and 1/4MS media were inferior to that on MS basal medium (Table 6; Fig. 11).

Main difference of MS and B5 media had different content ratio of NH_4-N:NO_3-N: MS had 1:2, while B5 had 1:15. Inhibition of leaf growth in the cultures on B5 medium

could be correlated with high concentration of NO_3-N in the medium. The additional experiment in nutritional change as the nitrogen form ratio in MS basal medium indicated that different ratios could cause organ formation and development of the cultures. The medium containing only NO_3-N (NH_4-N:NO_3-N = 0:1) inhibited leaf growth and induced chlorosis in *P. japonica*, whereas those containing NH_4-N (NH_4-N:NO_3-N = 1:1 and 1:0) did not (Table 7; Fig. 12).

Rhizome tips of *P. japonica* and *P. ophioglossoides* were planted on non-symbiotic and symbiotic OPA media. Adventitious shoot buds were formed at the rhizome tip of the explants in the both species on all media. After 60 days, they grew on to form one juvenile plant per one explant. In both species, plant growths were well on OPA media inoculated No. MCG and No. MSS (Table 8 and 9; Fig. 13 and 14). Plant growths of *P. japonica* were better than those of *P. ophioglossoides* on OPA media inoculated No. 706, No. 864, No. HHR and No. HoPJ and non-symbiosis (Table 8 and 9; Fig. 13 and 14). On OPA media inoculated No. 624, No. MCG and No. MSS, plant growths of *P. ophioglossoides* were better than those of *P. japonica* (Tables 8 and 9; Figs. 13 and 14). Leaves and rhizomes of *P. japonica* were not differentiated on symbiotic OPA media inoculated No. 614, No. 9720, No. HPJ and No. HPM. Those of *P. ophioglossoides* were not differentiated on symbiotic OPA media inoculated No. 614, No. 706, No. 864, No. 9720, No. HPJ (Tables 8 and 9; Figs. 13 and 14).

Discussion

Juvenile plants growing on Hyponex, B5, mKC and VW media did not have any leaf with dull green but have leaves with rather whitish green, chrolosis (Tables 5 and 6). In cultures of *Orchis laxiflora* Lam., leaves developed chlorosis if the MS basal medium lacked thiamine (or vitamin B_1) among the organic elements, while those of *Ophrys*

sphegodes Miller did not (Mead and Bulard 1975). Hyponex, mKC and VW media do not contain thiamine as nutrition. It was expected that chlorosis occurred on Hyponex, mKC and VW media because of lack of thiamine. When rhizomes of *P. japonica* were cultured on Hyponex, mKC and VW media containing 0.1mg/l thiamine for two months, juvenile plants with dull green were differentiated.

Chlorosis sometimes occurred in leaves of *P. japonica* and *P. ophioglossoides* cultures on B5 and modified MS which contained high NO_3-N ratio as the nitrogen source, even though their media contained thiamine. Chlorosis can be caused not only by lack of the organic elements but also by high NO_3-N ratio in the culture media. *P. japonica* grows with carnivorous plants in oligotrophic bogs with high acidity and low NO_3-N content (Idei and Kondo 1998). Mycorrhizal fungi symbiotic with orchids in this natural habitat may provide a source of organic nitrogen to make up a shortage of NO_3-N content. On the other hand, *P. japonica* may be a plant which can utilize ammonium. Shoots of *P. japonica* did not grow well on media such as B5 which contain high concentrations of NO_3-N. If this species absorbs NH_4-N, the uptake of other cations would be suppressed, and thus, the pH of the tissues would drop. On the contrary, if it absorbs NO_3-N, the base absorption would increase, tissue pH would also increase, and the solubility of Fe and the inorganic minor elements would decrease (Haynes and Goh 1978). Chlorophyll does not contain iron, but it is involved in its biosynthesis, and thus chlorosis might also be due to Fe deficiency.

It was reported that effective symbiotic fungi for orchid germination were different from those for growth of adult plants (Rasmussen 1995). However, in this study effective symbiotic fungus for seed germination and plant growth of *P. japonica* was same.

However, effective fungi for germination and plant growth were not ones isolated from *Pogonia* but *Cymbidium goeringii* and *Spiranthes sinensis* which did not exist at bogs.

Table 5. Quantitative characters in organ formation in rhizome tips of *Pogonia japonica* in five different media at different concentrations of MS media with no hormone

	Leaf length (mm)	No. of leaves	Rhizome length (mm)	No. of rhizomes	Leaf color
Hyponex	27.80 ± 22.049	1.07 ± 0.258	29.33 ± 5.876	2.67 ± 0.816	Yellowish gray - Pall yellow green
B5	24.13 ± 10.636	1.47 ± 0.516	22.60 ± 6.717	2.53 ± 0.516	Soft yellow green
mKC	26.80 ± 13.685	1.50 ± 0.527	9.40 ± 3.950	1.80 ± 0.632	Yellowish gray
VW	37.40 ± 16.677	1.27 ± 0.458	18.87 ± 8.305	2.27 ± 0.704	Yellowish gray
MS	43.73 ± 7.545	1.33 ± 0.488	19.53 ± 9.039	2.40 ± 0.632	Dull green
1/2MS	23.40 ± 19.449	1.00 ± 0.000	36.93 ± 9.838	2.07 ± 0.704	Dull green
1/4MS	11.20 ± 16.959	1.00 ± 0.000	45.33 ± 8.139	1.53 ± 0.743	Dull green

Mean ± Standard error

Table 6. Quantitative characters in organ formation in rhizome tips of *Pogonia ophioglossoides* in five different media at different concentrations of MS media with no hormone

Medium	Leaf length (mm)	No. of leaves	Rhizome length (mm)	No. of rhizome	Leaf color
Hyponex	11.93 ± 6.902	1.53 ± 0.516	27.80 ± 11.359	2.20 ± 0.941	Yellowish gray - Pall yellow green
B5	8.87 ± 6.653	1.07 ± 0.258	14.00 ± 9.079	1.80 ± 0.941	Soft yellow green
mKC	10.20 ± 6.805	1.20 ± 0.414	25.67 ± 9.447	1.43 ± 0.646	Yellowish gray
VW	19.87 ± 6.927	1.53 ± 0.516	33.07 ± 14.646	2.40 ± 1.121	Yellowish gray
MS	14.00 ± 6.794	1.77 ± 0.599	19.29 ± 12.579	2.23 ± 0.832	Dull green
1/2MS	1.93 ± 0.799	1.00 ± 0.000	50.60 ± 7.799	1.20 ± 0.414	Dull green
1/4MS	1.87 ± 0.743	1.00 ± 0.000	56.73 ± 7.096	1.13 ± 0.352	Dull green

Mean ± Standard error

Table 7. Effects of concentration changes and combinations in NH_4-N : NO_3-N ratio in MS basal medium on some quantiative characters of *Pogonia japonica*

Chemical	Nitrogen ratio (NH4-N:NO3-N)	Leaf length (mm)	Rhizome length (mm)
1650mg/l NH_4NO_3	1 : 1	31.8±8.52	13.7±3.28
825mg/l $(NH_4)_2SO_4$	1 : 0	33.9±8.24	20.1±4.11
1900mg/l KNO_3	0 : 1	2.5±1.12	15.1±2.09

mean ±standard error

Table 8. Quantitative characters in organ formation on rhizome tips of *Pogonia japonica* on OPA media with orchid endophytes and 1/2MS medium

	Leaf length (mm)			Rhizome length (mm)		
614	1.67	±	1.723	0.25	±	0.622
624	16.00	±	0.737	2.00	±	2.171
706	10.72	±	6.935	1.56	±	1.199
864	4.72	±	7.169	2.22	±	1.734
9720	1.50	±	0.850	0.80	±	1.549
MCG	40.64	±	17.861	13.21	±	9.048
HHR	18.82	±	14.236	6.71	±	4.043
HoPJ	16.92	±	12.724	7.31	±	4.939
HPJ	1.50	±	0.760	0.00	±	0.000
HPM	1.33	±	0.651	0.17	±	0.389
MSS	42.78	±	24.920	16.78	±	9.723
control	5.27	±	5.675	1.67	±	2.743
1/2MS	15.50	±	10.961	21.61	±	7.617

Mean ± Standard error

Table 9. Quantitative characters in organ formation on rhizome tips of *Pogonia ophioglossoides* on OPA media with orchid endophytes and 1/2MS medium

Fungus	Leaf length (mm)	Rhizome length (mm)
614	1.00 ± 0.000	0.00 ± 0.000
624	3.14 ± 5.201	2.43 ± 4.467
706	1.10 ± 0.316	0.00 ± 0.000
864	1.00 ± 0.000	0.00 ± 0.000
9720	1.17 ± 0.577	0.00 ± 0.000
MCG	55.11 ± 20.419	13.89 ± 6.790
HHR	6.67 ± 9.530	3.44 ± 3.276
HoPJ	2.78 ± 5.001	0.67 ± 1.572
HPJ	1.50 ± 0.707	1.00 ± 1.414
HPM	2.83 ± 4.218	1.08 ± 2.466
MSS	58.82 ± 21.892	13.18 ± 6.013
control	1.00 ± 0.000	0.00 ± 0.000
1/2MS	8.44 ± 8.444	25.17 ± 8.133

Mean ± Standard error

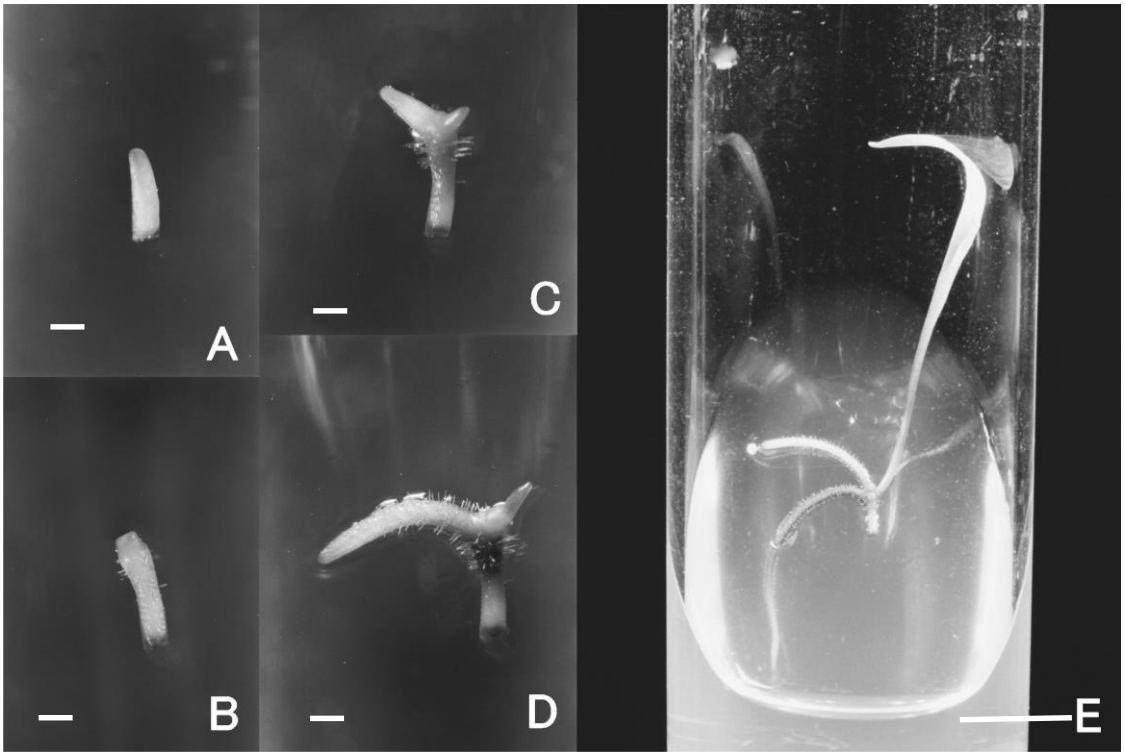

Fig. 9. Tissue-cultured rhizome-tips and their organ formation in *Pogonia japonica* on MS gellan-gum medium with no hormone. A. A rhizome tip at the beginning of the culture. B. Seven days culture-stage of the rhizome tip. C. 14 days culture-stage of the rhizome tip. D. 21 days culture-stage of the rhizome tip. E. 60 days culture-stage of the rhizome tip. Bar = 1mm for A-D. Bar =10mm for E. (Takahashi, C and Kondo, K. 1998)

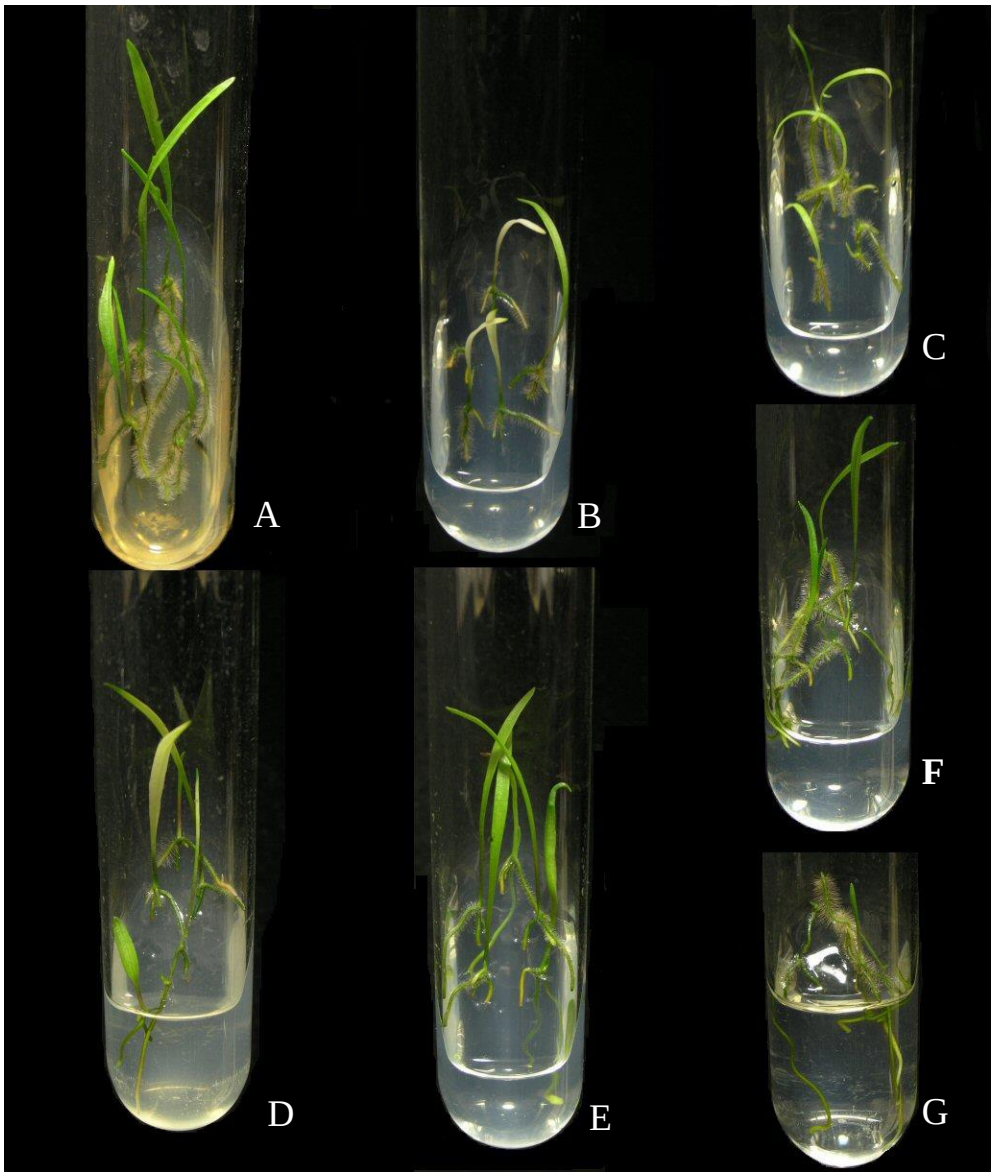

Fig. 10. Difference in organ development of *Pogonia japonica* rhizome-tip on five different media and two differentiation of MS media on with no hormone. A. Hyponex. B. mKC. C. B5. D. VW. E. MS. F. 1/2-diluted concentration of MS. G. 1/4-diluted concentration of MS.

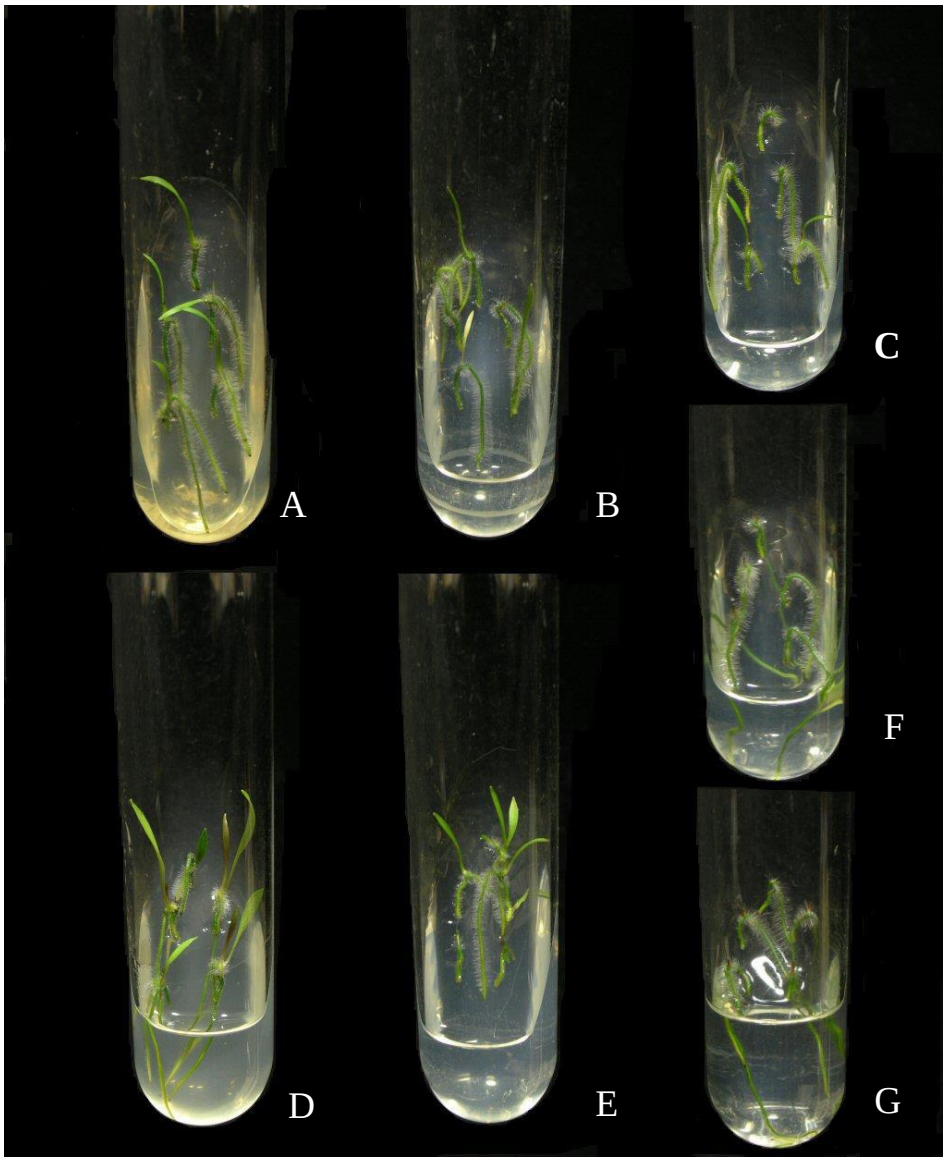

Fig. 11. Difference in organ development of *Pogonia ophioglossoides* rhizome-tip on five different media and two differentiation of MS media on with no hormone. A. Hyponex. B. mKC. C B5. D. VW. E. MS. F. 1/2-diluted concentration of MS. G. 1/4-diluted concentration of MS.

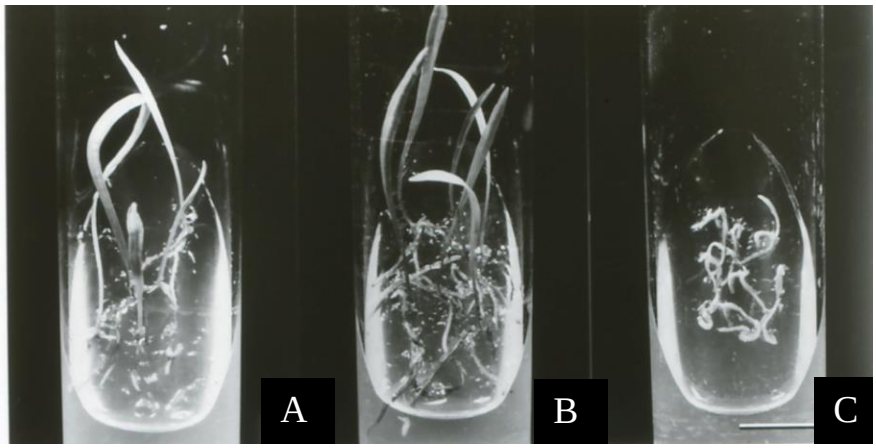

Fig. 12. Effects of combinations of nitrogen ratio (NH_4-N : NO_3-N) on organ formation on *Pogonia japonica* rhizome-tips. A. 1 : 1. B. 1 : 0. C. 0 : 1. Bar = 10mm.

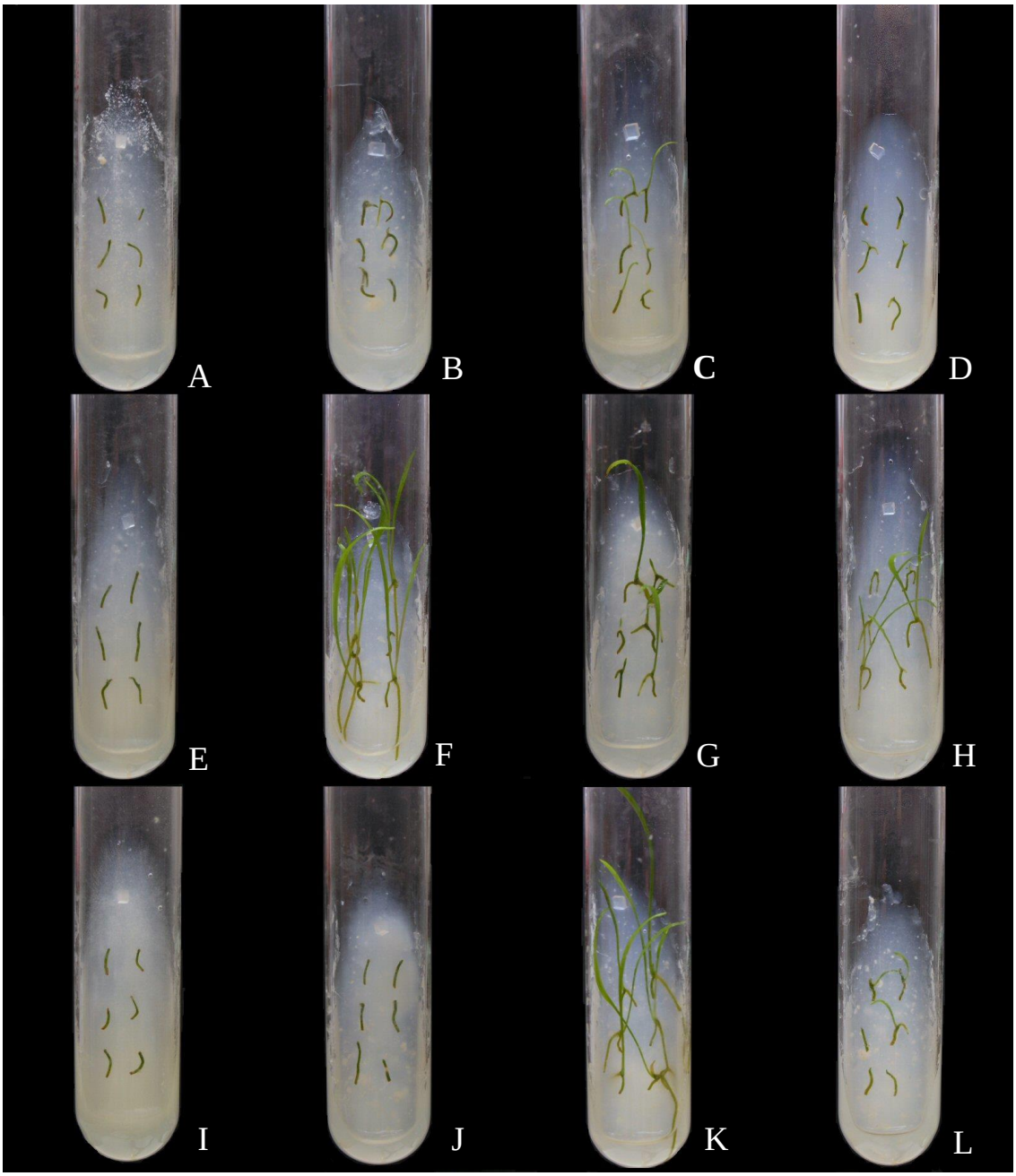

Fig. 13. Development of *Pogonia japonica* as affected by orchid endophytes under non-symbiotic and symbiotic conditions 60 days after planting. A: OPA with isolated No. 614, B: OPA with isolated No. 624, C: OPA with isolated No. 706, D: OPA with isolated No. 864, E: OPA with isolated No. 9720, F: OPA with isolated No. MCG, G: OPA with isolated No. HHR, H: OPA with isolated No. HoPJ, I: OPA with isolated No. HPJ, J: OPA with isolated No. HPM, K: OPA with isolated No. MSS, L: OPA.

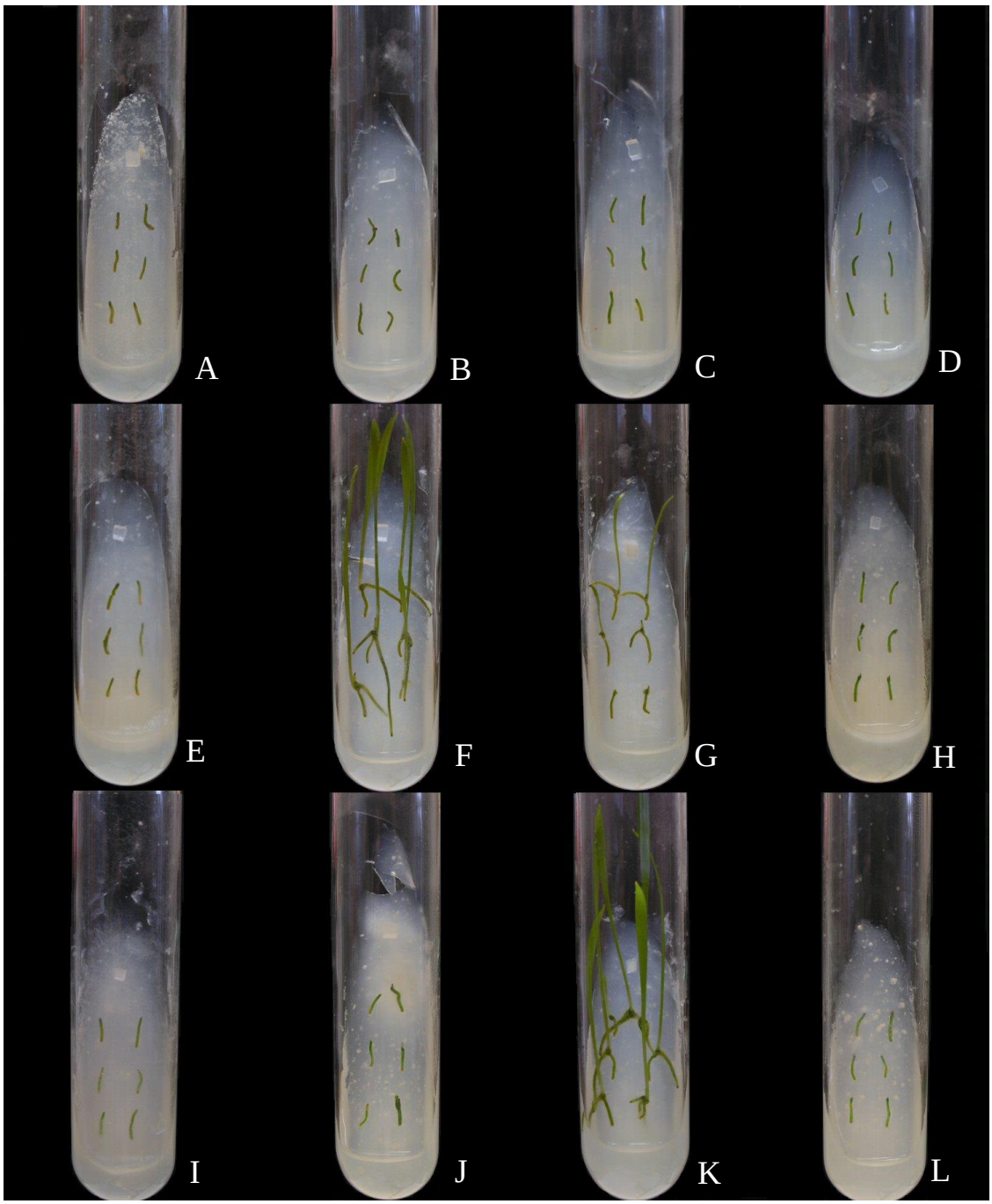

Fig. 14. Development of *Pogonia ophioglossoides* as affected by orchid endophytes under non-symbiotic and symbiotic conditions 60 days after planting. A: OPA with isolated No. 614, B: OPA with isolated No. 624, C: OPA with isolated No. 706, D: OPA with isolated No. 864, E: OPA with isolated No. 9720, F: OPA with isolated No. MCG, G: OPA with isolated No. HHR, H: OPA with isolated No. HoPJ, I: OPA with isolated No. HPJ, J: OPA with isolated No. HPM, K: OPA with isolated No. MSS, L: OPA.

Chapter 3 Micropropagation of *Pogonia japonica*

Materials and methods

For micropropagation, rhizome tips of ca. 100-day-old juvenile plants of *Pogonia japonica in vitro* were harvested and placed in B5 and MS liquid media supplemented with α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA), commonly at concentrations of 0.00, 0.02, 0.20 and 2.00 mg/l in combination and 3% sucrose at pH 5.8. The cultures were maintained in test tubes (30 mm diameter × 200 mm length) with 20 ml liquid medium and shaken at 2 rpm on a rotary culture equipment at 24±1℃ with 2,000-10,000 lux continuous illumination.

For histological studies, cultures were fixed in 4% (v/v) formaldehyde for 24-36 hrs. at 0℃, dehydrated in a series of 2-methoxyethanol, 100% ethanol, n-propanol and n-butanol for 12-16 hrs at 0℃, and then infiltrated in a monomer mixture solution of 94.5% purified glycol methacrylate, 0.5% (v/v) 2'2-azobis, and 0.5% (v/v) polyethylene glycol 400 for 12 hrs at 0℃ three times. Then, they were transferred to polyethylene capsules, embedded in monomer mixture at 30℃ for a day, 40℃ for one day and then 60℃ for a day in an incubator to prepare hard blocks. The embedded samples were sectioned to a thickness of 8-10 μm by a sledge microtome. The sections were affixed to glass slides and stained with 0.05% (v/v) toluidine blue O.

Results

Two months after rhizome tips of *P. japonica* were placed either in B5 or MS liquid media by rotary culture equipment, they were differentiated into various culture types (Figs. 15 and 16): 1) Plantlets formed in the media without any growth regulators; 2) Rhizomes formed in the media supplemented with 0.02 mg/l NAA; 3) Abnormal

rhizomes formed in the media supplemented with NAA≧0.20mg/l ; 4) Cultures with numerous rhizome primordia, some of which produced several leaf buds at the apex called "Rhizome-derived Protocorm-like Bodies" (RPLBs), were formed in both media supplemented with BA≧0.02 mg/l and NAA≦0.02 mg/l (Figs. 17A-C); and 5) Small globular cultures with numerous "Abnormal shoot-tip aggregations" (ASTAs) formed only in MS supplemented with BA≧0.02 mg/l and NAA at concentration 0.2 ≦0.02 mg/l (Figs. 15 and 17D-F). The RPLBs in B5 and MS supplemented with BA≧0.02 mg/l proliferated and sometimes produced shoots, but the ASTAs did not.

A comparison was made between cross-sections of an RPLB which indicated differentiation into a rhizome tip, a regenerated shoot tip, an inner region, an established vascular bundle (Fig. 17C), and an ASTA (Fig. 17F), which indicated no organ differentiation. The RPLB produced numerous rhizome tips and was, thus, an excellent micropropagation line, whereas the ASTA was not.

If RPLBs and ASTAs were transplanted onto MS with no growth regulators, they regenerated multiple shoots (Fig. 18). The RPLBs produced shoot directly from the rhizome primodia (Fig. 18A), whereas the ASTAs thickened and then produced shoot primodia or possibly adventitious embryos (Fig. 18B). The plantlets regenerated from RPLBs had healthy, succulent leaves and several rhizomes (Fig. 18C) and thus, were better propagules (Table 10) than those from ASTAs which had delicate-looking, twisted, thin leaves and only a few rhizomes (Fig. 18D). Juvenile plants grown from RPLBs *in vitro* were successfully acclimatized *in vitro.*

Discussion

The RPLBs were characterized by the presence of numerous rhizome primodia on their surface, whereas the ASTAs were not clearly characterized by any specific organ. The five types of cultures histologically classified and described above were the same as those occurring in *Spiranthes sinensis* (Pers.) Ames (Sato *et al*. 1987; Tanaka *et al*. 1997). One of the five culture types morphologically quite closely resembled the ASTA was called "Fascicled dwarf-leaves on the globular tissue" and was characterized by a plateau-like dome of shoot tip in which the epidermal layer pushed up to develop into a new meristematic region (Sato *et al.* 1987). However, the cross-section of the ASTA in *P. japonica* did not correspond to this and thus appears to be another new type of culture. The RPLBs and the ASTA seemed to be affected by nitrogen form in the media used. The rate of formation of ASTAs is particularly in inverse proportion to the NO_3-N concentration.

A schematic diagram of a possible pathway for artificial multiplication of *P. japonica* is shown in Fig. 19.

Table 10. Regeneration of shoots and rhizomes from RPLB and ASTA of *Pogonia japonica* on MS Gelrite medium with on growth regulators

Culture	No. of shoot/culture[1]	No. of shoots which produced rhizomes/total no. of shoots (%)
RPLB[2]	10.0±1.86	121/190（63.7）
ASTA[3]	5.8±1.64	24/69（34.8）

[1] mean ±standard error

[2] rhizome-derived protocorm-like body

[3] abnormal shoot-tip aggragation

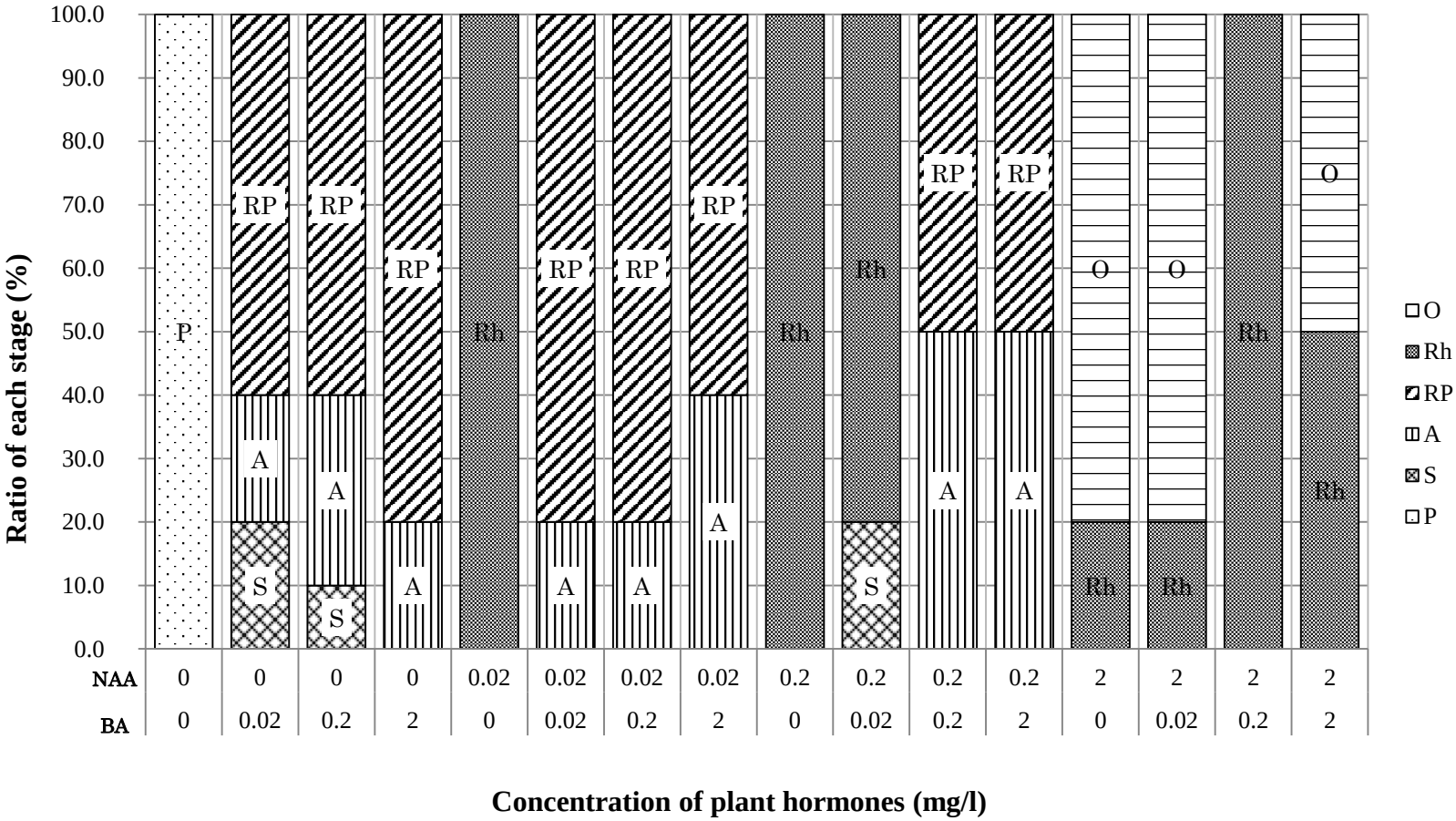

Fig. 15. Effects of volumes and combinations of NAA and BA in MS basal medium on organ formation in *Pogonia japonica* rhizome-tips. P: Plant. S: Shoot. A: ASTA. RP: RPLB. Rh: Rhizome. O: The other.

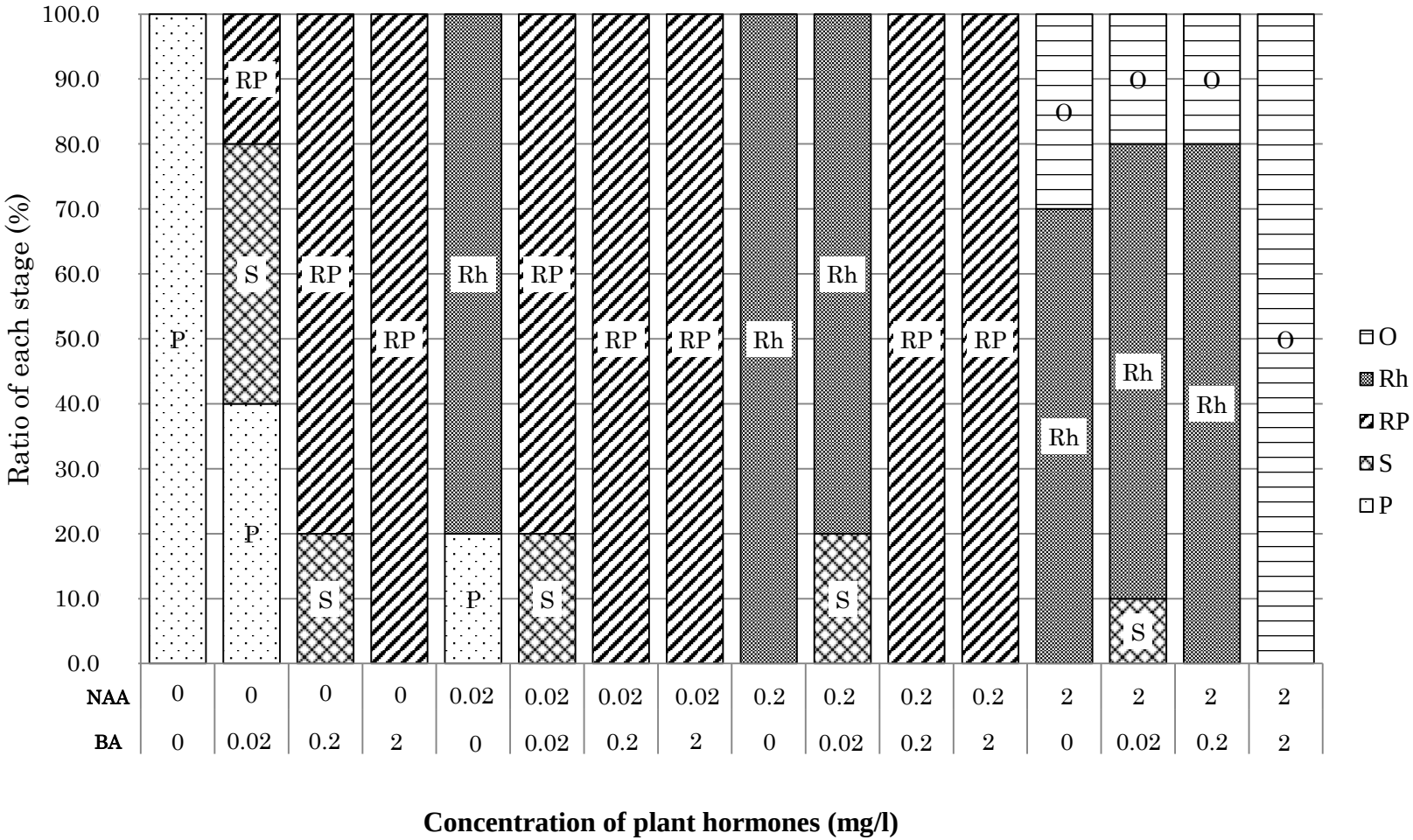

Fig. 16. Effects of volumes and combinations of NAA and BA in B5 basal medium on organ formation in *Pogonia japonica* rhizome-tips. P: Plant. S: Shoot. RP: RPLB. Rh: Rhizome. O: The other.

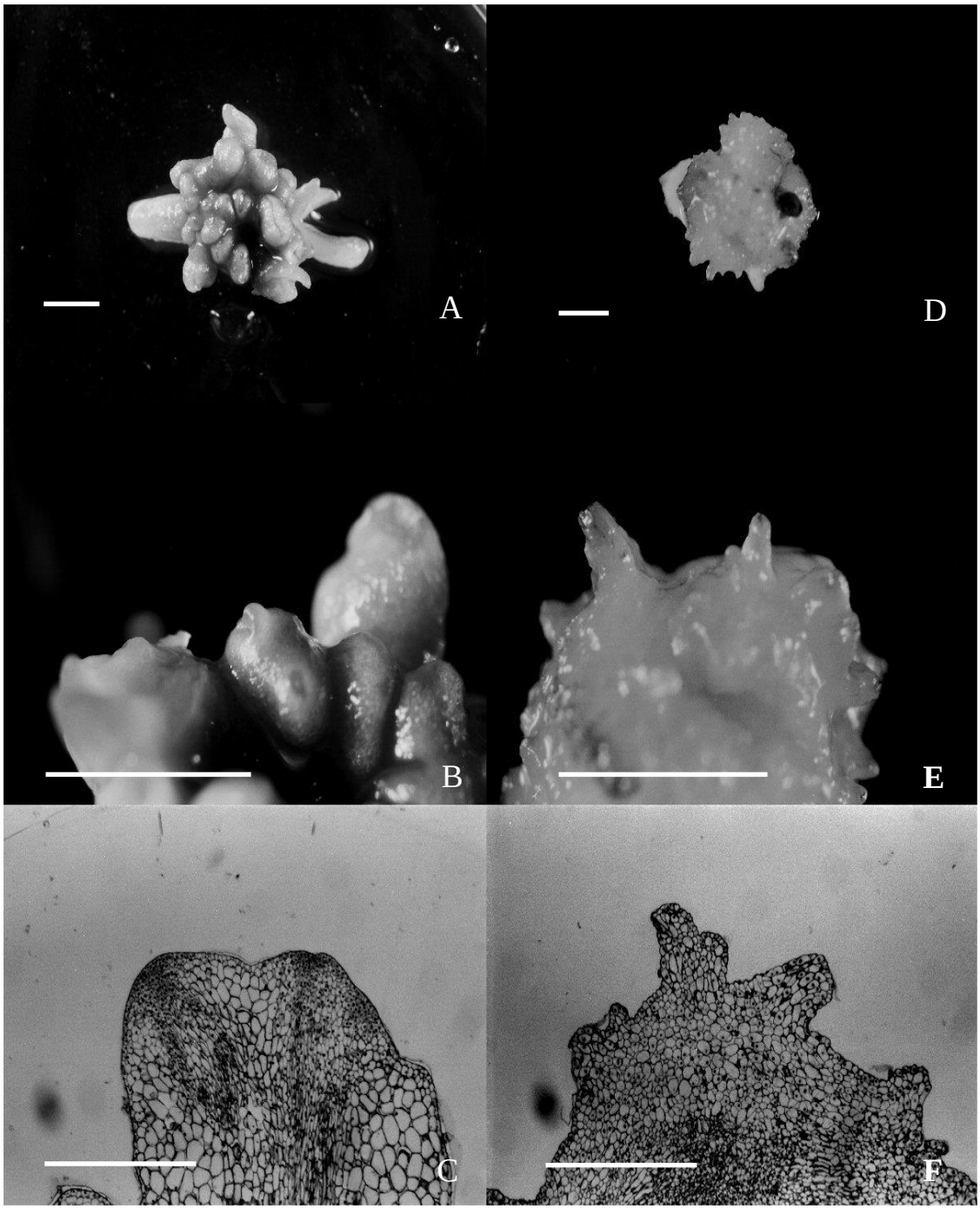

Fig. 18. Micropropagation cultures of *Pogonia japonica* induced from the rhizome tip in MS medium supplemented with 0.2mg/l NAA and 0.2mg/l BA, maintained by shaking at 2rpm on a rotary shaker with continuous illumination. A-C. RPLB. D-F. ASTA. A and D. Block. B and E. Close-up view of surface. C. Cross-section of a block of RPLB, which shows regenerated shoot and rhizome tips, inner region, and established vascular bundles. F. Cross section of block of ASTA, which shows no organ differentiation. Bars = 2mm for A, B, D and E and 1mm for C and F. (Takahashi, C. and Kondo, K. 1998)

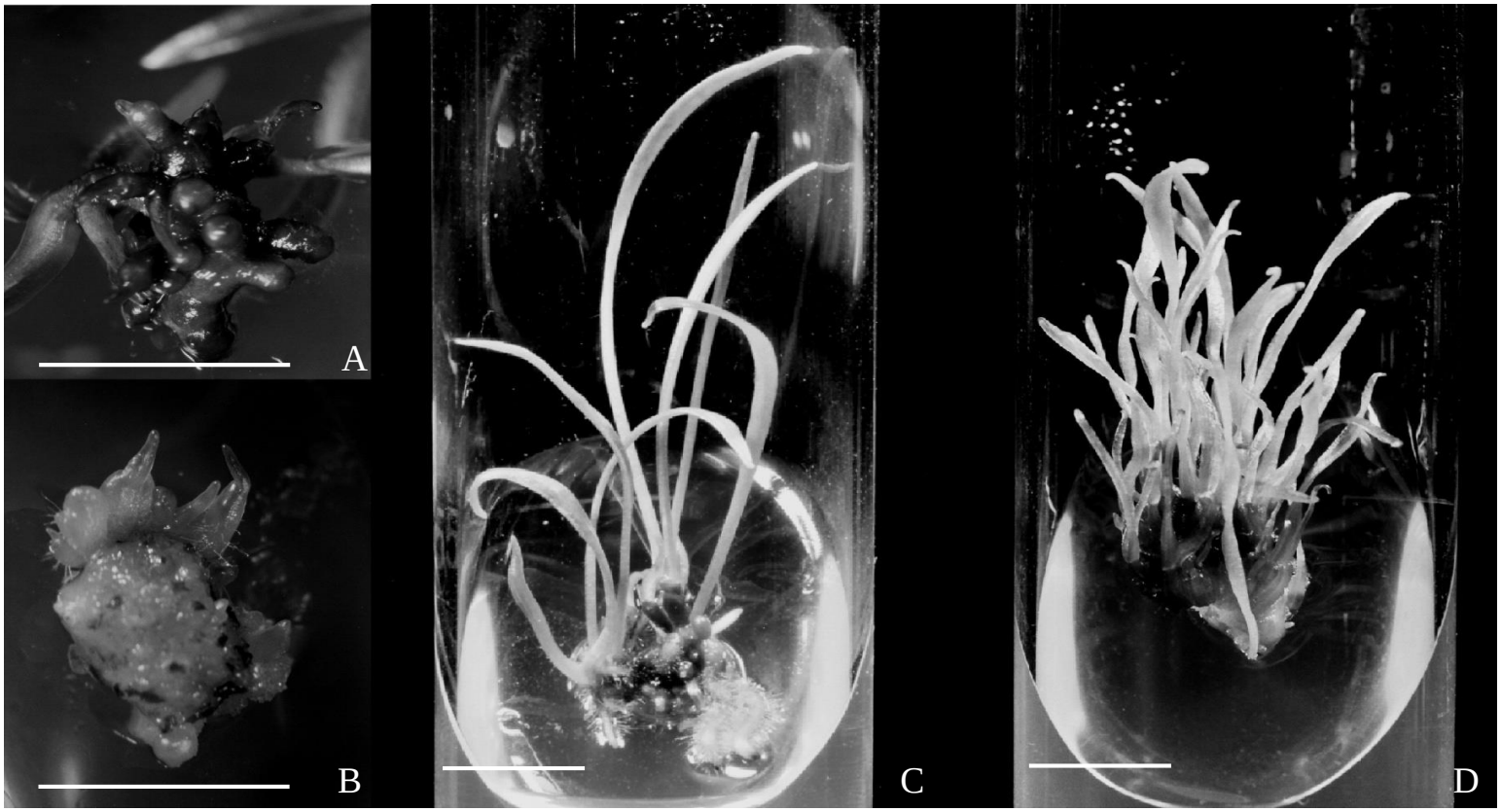

Fig. 18. Regeneration of numerous plantlets of *Pogonia japonica* arisen from RPLB (A and C) and ASTA (B and D) on MS hormone free gellan-gum medium. A and B. 30 days old regenerated-plantlets. C and D. 90 days old regenerated-plantlet. Bar = 10mm. (Takahashi, C. and Kondo, K. 1998)

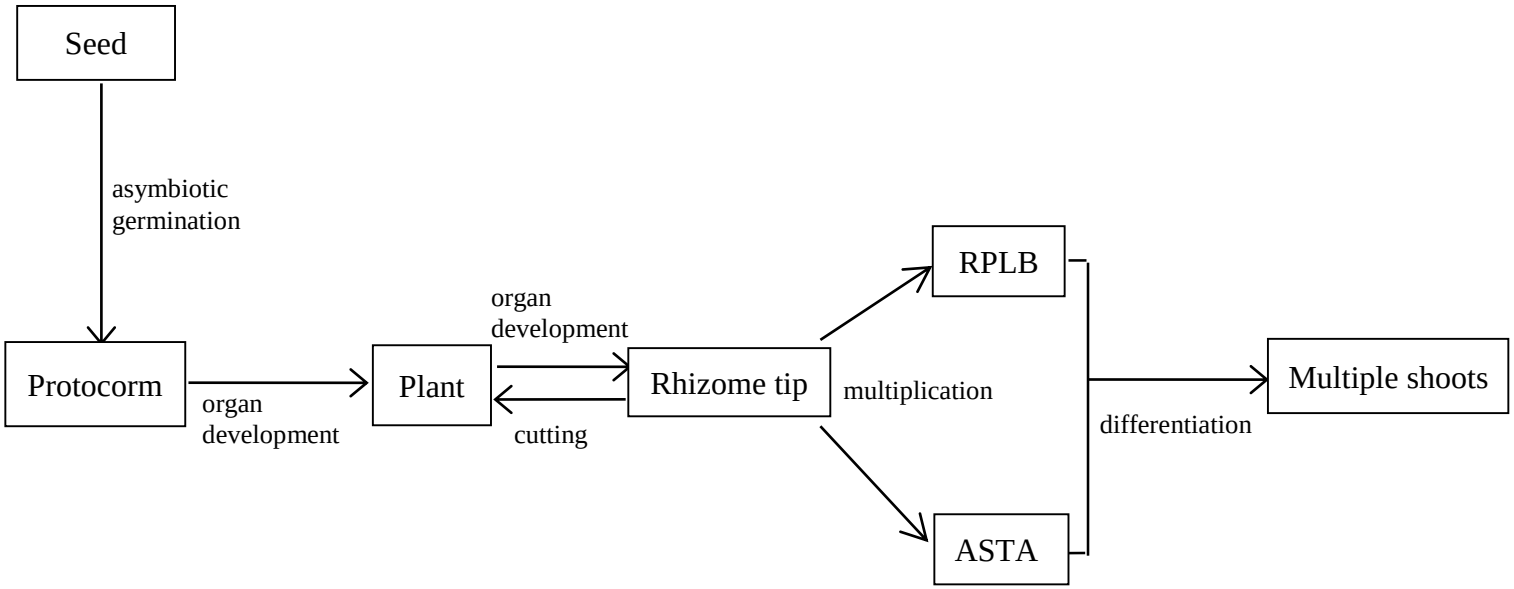

Fig. 19. Schematic diagram of possible pathway for artificial multiplication of *Pogonia japonica.*

Chapter 4 Cytogenetic Analysis

Materials and methods

Plant materials

Naturally growing *Pogonia japonica* were collected on campus of Hiroshima University, Higashi-Hiroshima City, alt. about 200m above the sea-level while those of *P. minor* were collected in Ikenodan High-Moore, Mt. Hiba, alt. 1,279.5m above the sea-level. In contrast, plants of *P. ophioglossoides* were collected in North Carolina, U.S.A. by K. Cameron, New York Botanical Garden. These three species of *Pogonia* were cultivated in Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University.

Currently, another type of *Pogonia* called ‘Miyamatokisou’ a little bit flower-shape different from the former two Japanese species of *P. japonica* and *P. minor* have spontaneously appeared (Fig. 20). Some capsules were produced on ‘Miyamatokisou’ grown in Mt. Hakusan, Gifu Prefecture were found and collected by Mr. Hiroshi Nakayama, Yokogawa electric corporation for the purpose of determining and clarify what ’Miyamatokisou’ was. How old the capsule obtained it took after pollination could not be clearly determined since it would be produced by natural-pollination and Mr. Nakayama who was not specialized to ‘Miyamatokisou’. Two capsules of so-called ‘Miyamatokisou’ were collected, sent to Takahashi and immediately fruit-surface-sterilized by 70% ethanol for a few seconds. Then, seeds inside the capsule were scooped and sowed on mKC medium for axenic germination.

Also another plant which had flower shape different from either *P. japonica* or *P. minor* but a little bit similar to 'Miyamatokisou' was found in a bog in Higashi-Hiroshima City (Fig. 21).

Some plants of *Pogonia* were collected in a floating marine wet meadows with boggy areas near by the sea shore of the Japan Sea at the north latitude of 42°46'38"N and the east longitude of 131°15'73"E, alt. 5 m above the sea level, near Village Ryaznovka, Khasan District, Primorye Territory, Russia. They were brought and cultivated in Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University. Later, they were transplanted to Mukaishima Orchid Center.

Pollination experiments

Reciprocal crosses among *P. japonica, P. minor* and *P. ophioglossoides* were made by hand pollination. Their 75 to 90-day-old, ripen fruits were harvested and their seeds were scooped and soaked in distilled water containing a few drops of wetting agent overnight. Then, those seeds were surface-sterilized in 1.2% sodium hypochlorite solution for ten minutes and rinsed in distilled sterilized water. The surface-sterilized seeds were sowed on mKC agar medium, pH 5.0 with no hormone and were cultured under continuous 1000 lux illumination at 24±1℃. In reciprocal hybrids between *P. japonica* and *P. minor,* non-surface-sterilized F_1 seeds taken from surface-sterilized capsules by 70% ethanol were sown on mKC medium.

Protocorms were transplanted on 1/2MS or MS medium to stimulate their growth.

Some of *in vitro* young plant of F_1 hybrids were acclimatized to pots planted with sphagnum moss and/or commercially so-called Akadama (red granular) soil and were cultivated.

Self-pollination in F_1 hybrids of six cross combinations were carried by hand-pollination. Approximately 90-day-old ripen fruits were harvested and surface-sterilized by 70% ethanol for ten seconds. Half amount of the seeds scooped from the surface-sterilized fruits was sowed on mKC medium. Other seeds were directly surface-sterilized by 1.2% sodium hypochlorite solution before they were sowed on mKC medium. Germination rate of seeds was counted at the 150th days stage after sowing.

Chromosome preparation

Rhizome tips of *Pogonia* ca 5 mm long of the plants *in vivo* studied were harvested. Also rhizome tips from growing on Hyponex or mKC solid media *in vitro* were cut ca 5mm and were used. They were pretreated in 0.002M 8-hydroxyquinoline at 20℃ for 3 h and then at 6℃ for 2 h. They were fixed in 3 : 1 mixture of ethanol and acetic acid at 6℃ for 1 h and were, then, stored in 70% ethanol for a week, before they were macerated in 1N hydrochloric acid at 60℃ for 1 min. They were stained by 1% aceto-orcein and squashed in 45% acetic acid.

Results

Pollination experiments

Rates of maturity and formation of embryos and seed germination were observed and measured in *P. japonica*, *P. minor*, *P. ophioglossoides* and their reciprocal F_1 hybrids (Table 11). Rates of embryo formation in F_1 hybrid seeds were rather same to those of parental seeds (Table 11). Rates of surface-sterilized seed germination in F_1 hybrids were quite similar to those of the parents and were higher than 80% on modified mKC medium (Table 11). However, germination rate of non-surface-sterilized seeds of *P. minor* x *P.*

japonica was average 1.7% and the rate was as low as that of non-sterilized seeds of *P. minor*, while that of *P. japonica* x *P. minor* was 56.1% and it was same to the non-sterilized seeds germination rate of *P. japonica* (Table 12). Thus, the germination patterns of reciprocal F_1 hybrids were similar to those of their female parents.

Protocorms of their reciprocal hybrids grew up and differentiated leaves and rhizomes on 1/2MS and MS agar medium.

Seedlings of F_1 hybrids which were grown *in vitro* were acclimated and were cultivated by sphagnum moss and/or Akadama soil. They grew well.

All reciprocal F_1 hybrids by three species of *Pogonia* were flowered at two to three years after acclimatization (Figs. 22, 23, 24). There was no clear differences in flower morphology among all reciprocal F_1 hybrids.

The seeds of F_2 hybrids by self-pollination of the F_1 hybrids of *P. ophioglossoides* x *P. japonica*, *P. minor* x *P. ophioglossoides* and *P. ophioglossoides* x *P. minor* had not been formed for five seasons. Only a few seeds of F_2 hybrid by self-pollination of F_1 hybrid of *P. japonica* x *P. ophioglossoides* had been formed from a fruit capsule once. Very small amount of the F_2 seeds surface-sterilized germinated and grew on mKC medium.

Microspores of *P. japonica* x *P. minor* formed mostly normal tetrads. There were some tetrads with one or two micronuclei.

Rates of embryo formation in the F_2 reciprocal hybrids between *P. japonica* and *P. minor* were around 80% and were as high as those of both parental species (Table 13).

The non-surface-sterilized seeds from non-dehisced capsules of the reciprocal F_2 hybrids by self-pollination in the both F_1 hybrid between *P. japonica* and *P. minor* germinated very little, less than 1% (Table 13). The germination of the surface-sterilized

seeds of the self-pollination in the F_1 hybrid of *P. japonica* x *P. minor* was rather high with their germination rate of average 26.0% and clearly lower than the surface-sterilized seed germination rate of the F_1 hybrid seeds (Table 13). The germination rate of the F_2 hybrid by self-pollination in the F_1 hybrid of *P. minor* x *P. japonica* was average 4.2% (Table 13). Thus, high germination rates were not shown in the F_2 hybrid seeds even by the method of seed-sterilization in spite of high embryo formation.

A few F_2 hybrid seeds obtained by self-pollination in the F_1 hybrid between the maternal *P. japonica* and the paternal *P. ophioglossoides* germinated three plants.

Seeds in the non-dehisced capsules of *Pogonia* from Mt. Hakusan called 'Miyamatokisou' received for this study were immature and their color were green to pale brown. Embryos in seeds in the non-dehisced capsules were formed well. Although the days stage after pollination of capsules of *Pogonia* from Mt. Hakusan called 'Miyamatokisou' was not clear because it would be by natural-pollination, it was expected that the day stage after pollination would be 40-50 day stage from condition of embryo formation and seed color. Germination rate of the immature seeds of *Pogonia* called 'Miyamatokisou' was more than 60% on mKC medium.

Cytogenetic analysis

Pogonia japonica showed commonly the chromosome number of 2n=20 that verified the previous counts (Tanaka 1962a, 1965, 1971, 1974). The karyotype of *P. japonica* consists of 16 median-centromeric (eight pairs) and four (two pairs) terminal-centromeric chromosomes (Table 14; Fig. 25). *Pogonia minor* showed commonly the chromosome number of 2n=18 that verified the previous counts (Tanaka 1962a, 1965, 1971; Mutsuura and Nakahira 1960). The karyotype of *P. minor* consists of 18 median-centromeric (nine pairs) chromosomes (Table 15; Fig. 25). In contrast, *P. ophioglossoides* showed the

chromosome number of 2n=18+2-3B (Table 16; Fig. 25). The karyotype of *P. ophioglossoides* consists of 18 median-centromeric (nine pairs) chromosomes and two to three small chromosomes. Their small chromosomes were treated as B-chromosome since Oryu and Kondo (2001) reported that the *P. ophioglossoides* had 1-3 B-chromosomes. Their small chromosomes were around three μm large and had one centromere at median position each other. Baldwin and Speese (1957) reported that the chromosome number of *P. ophioglossoides* was 2n=18 and lacked any small chromosomes that could be B-chromosomes observed by the study by Oryu and Kondo (2001).

Three *Pogonia* species studied here had commonly a pair of median-centromeric chromosomes with secondary constrictions at the centromeric position (Fig. 25). However, Baldwin and Speese (1957) reported that there was a pair of median-centromeric chromosomes with secondary constrictions at the terminal position. The median-centromeric chromosomes with the secondary constriction were located at the seventh and the eighth chromosome positions in *P. japonica* and *P. minor* (Tables 14 and 15; Fig. 25), while they were located at the ninth and tenth chromosomes in *P. ophioglossoides* (Table 16; Fig. 25).

The karyotype of *P. minor* was characterized by the fifth and sixth pairs of median-centromeric chromosomes. Those chromosomes showed the relative length (RL) of 5.50-6.00 and the arm ratio (AR) 1.50-1.70 (Table 15). In contrast, the fifth and sixth pairs of *P. ophiglossoides* and *P. japonica* did not show the arm ratios more than 1.50 (Tables 14 and 16).

Two to three B-chromosomes were observed in the karyotype of *P. ophioglossoides*. Those small chromosomes have never been observed and have not yet been reported in *P. japonica* and *P. minor*.

The chromosomes of the three species of *Pogonia* except for B-chromosomes were classified into five groups: Ⅰ. Chromosomes with RL 5.50 – 7.50 and AR 1.00 – 1.50; Ⅱ. Chromosomes with RL 5.50 – 6.00 and AR 1.50 – 1.70; Ⅲ. Chromosomes with RL 4.00 – 5.00 and AR 1.00 – 1.50; Ⅳ. Chromosomes with RL 3.50 – 4.50 and AR 1.50 – 2.00; and Ⅴ. Terminal-centromeric chromosomes (Table 17). The groups Ⅰ, Ⅲ and Ⅳ were observed in three species, while the group Ⅱ was observed in two pairs of median-centromeric chromosomes of *P. minor* and the group Ⅴ was observed in two pairs of terminal-centromeric chromosomes of *P. japonica* (Fig. 26). The group Ⅰ was observed in four pairs of median-centromeric chromosomes of *P. japonica* and *P. minor* and in five pairs of median-centromeric chromosomes of *P. ophioglossoides* (Fig. 26). The median-centromeric chromosomes with a secondary constriction were included in the group Ⅰ at the three species (Fig. 26). The group Ⅲ was observed in three pairs of median-centromeric chromosomes of *P. japonica* and *P. ophioglossoides* and in two pairs of median-centromeric chromosomes of *P. minor*. The group Ⅳ was observed in one pair of the three species studied (Fig. 26). Thus, the karyotype of *P. minor* and that of *P. ophioglossoide*s was different in spite of same A-chromosome number (Fig. 26).

The chromosome number of *Pogonia* found and collected in Mt. Hakusan called ‘Miyamatokisou’ was 2n=18 (Table 18 and Fig. 27). The karyotype of *Pogonia* called ‘Miyamatokisou’ consisted of 18 median-centromeric chromosomes in which the median centromeric chromosome with a secondary constriction were located at seventh and eighth chromosomes and was similar to that of *P. minor* (Table 18 and Fig. 27). However,

the karyotype of the *Pogonia* did not involve the group Ⅲ which was usually included in that of *P. minor* (Table 18 and Fig. 27). The karyotype of *Pogonia* called 'Miyamatokisou' was also different from that of *P. ophioglossoides* because two centromeric-chromosomes with secondary constriction located as the seventh and eighth chromosomes in *Pogonia* called 'Miyamatokisou' but were located as the ninth and tenth chromosomes in *P. ophioglossoides* (Table 16, 18; Fig. 25, 27).

The chromosome number of *Pogonia* unusual individual different from *P. japonica* in Higashi-Hiroshima City was 2n=20 (Table 19 and Fig. 28). The karyotype of the *Pogonia* consisted of 16 median-centromeric chromosomes and four terminal-centromeric chromosomes (Table 19 and Fig. 28). The seventh and the eighth chromosomes had commonly a secondary constriction at the centromeric-position (Table 19 and Fig. 28). Thus, the kartyotype of the *Pogonia* unusual individual in Higashi-Hiroshima City was similar to that of *P. japonica* (Table 14, 19; Fig. 25, 28).

The chromosome number of *P. japonica* in Khasan District, Primorye Territory, Russia was 2n=20 (Fig. 29) (Takahashi *et al.* 2004). This chromosome number confirmed the previous count (Rudyka 1995) and 2n=20 is common to the chromosome member of *P. japonica* (Tanaka 1962a, 1965, 1971, 1974). The karyotype of *P. japonica* in Khasan District, Primorye Territory, Russia consisted of 16 median-centromeric and four terminal-centromeric chromosomes (Takahashi *et al.* 2004). A pair of median-centromeirc chromosomes had the secondary constriction at the centromeric position. Thus, the chromosome number, chromosome size and correlation between the relative length and the arm ratio documented here were exactly the same as those in the Japanese populations studied. However, the chromosome characteristics of the plants studied in

Khasan District, Primorye Territory, Russia as well as in Japan were obviously different from those of *P. minor*.

Reciprocal F_1 hybrids between *P. japonica* and *P. minor* displayed the chromosome number of 2n=19, exactly intermediate between 2n=20 of *P. japonica* and 2n= 18 of *P. minor* (Figs. 30 and 31). The karyotype of F_1 hybrids always consisted of 17 median-centromeric and two terminal-centromeric chromosomes (Figs. 30 and 31); two out of the 17 median-centromeric chromosomes, which had the relative lengths of 5.50-6.00 the arm ratio more than 1.5, can be correlated with those of *P. minor* and two terminal-centromeric chromosomes can be correlated with two out of four terminal-centromeric chromosomes of *P. japonica* (Figs. 30 and 31). Both reciprocal cross F_1 hybrids had eight chromosomes of the group Ⅰ, two chromosomes of the group Ⅱ, five chromosomes of the group Ⅲ, two chromosomes of the group Ⅳ, and two chromosomes of the group Ⅴ (Fig. 31). No mixoploid has been found in the F_1 hybrids and the parents during the course of investigation.

Karyotypes of the hybrids between the maternal *P. japonica* and the paternal *P. ophioglossoides* showed commonly 2n=19+1B, while those of the hybrids between the maternal *P. ophioglossoides* and the paternal *P. japonica* obtained showed commonly 2n=19+2B (Fig. 32). The reciprocal F_1 hybrids between *P. japonica* and *P. ophioglossoides* had 17 median-centromeric chromosomes and two terminal-centromeric chromosomes and consisted of nine chromosomes of group, six chromosomes of group Ⅲ, two chromosomes of group Ⅳ and two chromosomes of group Ⅴ (Fig. 33).

The karyotypes of the hybrids between the maternal *P. minor* and the paternal *P. ophioglossoides* obtained showed commonly 2n=18+2B (Fig.34), while those of the

hybrids between the maternal *P. ophioglossoides* and the paternal *P. minor* obtained showed commonly 2n=18+1B (Fig. 34).

The karyotypes of the both reciprocal-cross hybrids obtained did not show any intermediate form between the parents. For instance, a few karyotypes of the reciprocal-cross hybrids had two chromosomes with RL more than 5.50 and AR 1.50-1.70 which might be originated from the gamete of *P. minor* (Figs. 35 and 36). Also, all karyotypes observed reciprocal-cross hybrids obtained had one submedian-centromeric chromosome with AR more than 2.00 which might not be originated from the gamete of the both parents (Figs. 34, 35 and 36). The unique chromosome was characterized by arm ratio of 2.03-3.55 and relative length of 4.39-6.20 (Figs. 34, 35 and 36). However, some chromosomes could not be classified into the four groups established above due to the unique chromosome with arm ratio more than 2.00 and the chromosome number included in particular groups were depended on individual (Figs. 35 and 36).

A triploid plant (2n=27) occurred spontaneously in the hybrid between *P. ophioglossoides* x *P. minor* in seven plant of the hybrid. Most of the chromosomes in the triploid chromosome complement were median-centromeric but induced 1-2 submedian-centromeric chromosomes with arm ratio more than 2.00 (Fig. 37). The triploid karyotype did not show any B-chromosome.

The F_2 hybrids by self-pollination of the reciprocal-cross F_1 hybrids of *P. japonica* x *P. minor* displayed the chromosome numbers of 2n=19 and 20 and 2n=18, 19 and 20, respectively (Table 20 and Fig. 38). They showed slightly more plants with 2n=19 than those with 2n=20 plants. The minority of the F_2 hybrids by self-pollination of the F_1 hybrid of *P. minor* x *P. japonica* showed the chromosome number of 2n=18 (Table 20 and Fig. 38). The karyotype of the reciprocal F_2 hybrids with the chromosome number of

2n=19 consisted of 17 median-centromeric chromosomes and two terminal-centromeric chromosomes (Table 20 and Fig. 38). This karyotype of the F_2 hybrid was similar to that of F_1 hybrids. Two F_2 plant with 2n=19 had 16 median-centromeric and three terminal-centromeric chromosomes. The karyotype of the F_2 hybrids with 2n=20 consisted of 16 median-centromeric chromosomes and four terminal-centromeric chromosomes (Table 20 and Fig. 38). This karyotype of F_2 hybrid was similar to that of *P. japonica*. The karyotype of F_2 hybrid with 2n=18 consisted of 18 median-centromeric chromosomes (Table 20 and Fig. 38). This karyotype was similar to that of *P. minor*. However, it was not clear to find two median-centromeric chromosomes that had arm ratios more than 1.5 and relative lengths of 5.50-6.00 in the karyotype of the F_2 hybrids with 2n=18 and 19, that were characteristics of the F_1 hybrids and moreover *P. minor*.

Discussion

The chromosomes with the secondary constriction in *P. ophioglossoides* studied were rather different from those reported by Baldwin and Speese (1957); the secondary constrictions obtained here were located in the centromeric position (Fig. 25), however, those obtained by Baldwin and Speese (1957) were located in the sub-terminal position.

Plants described as *P. japonica* collected in Khasan District, Prymorye Territory, Russia, grew extensively wet, maritime meadow with portions of floating, luffy bogs. They produced from several hundreds to thousands partial shoots sparse over a plot counted in the population of *P. japonica* (Tatarenko 1996). In contrast, the population of the species observed in the Kunashiri Island and Honshu showed lower numbers of partial shoots, and 30-50 or 100-200 generative and vegetative shoots were recorded (Tatarenko unpublished). Size of plants at different age states in the populations of *P. japonica*

studied in Japan were not much different from those in the Far East Russia (Tatarenko 1996). Quantitative and qualitative characters of flowers of the species in the both Japanese and Far East Russian populations studied were also similar to each other (Tatarenko 1996).

Cameron and Chase (1999) stated that *P. japonica* and *P. minor* in East Asia might be differentiated from *P. ophioglossoides* in Southeast North America via Bering islands and bridges during the Tertiary period according to their molecular study. *Pogonia ophioglossoides* collected in North Carolina, U. S. A. and sent by Cameron had the chromosome number of 2n=18+2-3 B-chromosomes and the karyotype of nine pairs of median-centromeric chromosomes somewhat similar to that of *P. minor* (Takahashi and Kondo 2004). Tanaka (1962a, b) stated that the third pair of median-centromeric chromosomes in the karyotype of *P. minor* might perform a fission at the centromeric region, produced four terminal-centromeric chromosomes containing NOR's and differentiated into the karyotype of *P. japonica.*

Pogonia minor is distributed from the northmost distribution of Hokkaido (Kitamura 1964) but has not yet been found in the Chishima Isles and the Far East Russia down to the southmost distribution of the Formosa Island at the north latitude of ca 25°N (Makino 1982, Boufford *et al*. 2003, Chen 2009). In the Formosa Island *P. minor* is distributed up to 2,200-2,400m altitude (Chen 2009). *Pogonia japonica* is extended their distribution to further north region than *P. minor* is but grows in bogs in lowlands to mountainous areas (Makino 1982) and *P. minor* grows in meadows and boggy areas in hill up to high altitude of mountainous areas (Makino 1982). *Pogonia ophioglossoides* grows from subtropical, warmer temperate lowlands to four-seasons cooler temperate mountainous areas in the Southeastern U.S.A.

According to the phenomena described above, it can be speculated that the chromosome number of the northern *Pogonia* could be basically 2n=18. However, *P. japonica* (2n=20) has the northmost distribution up to the north latitude of 53°N (Nevskii 1935, Vyshin 1996). Southern distribution of *P. minor* of 2n=18 might be the result in adaptation pressure of various causes such as appearance and separation of habitat of *Pogonia* of 2n=20 mutation from *Pogonia* 2n=18, weather wave, palaeoecological activities and so on during the glacial period. During the glacial period *Pogonia* might spread their distribution to the south in East Asia. Then, in the interglacial period the one might desire to the north in the distribution and the other might desire to alpine in the distribution. *Pogonia minor* which distribute at alpine area in the Formosa Island might be the latter.

Pogonia ophioglossoides has 2 to 3 B-chromosomes with centromere (Fig. 25). It was speculated that B-chromosome might be originated in a number of ways from derivatives from A chromosomes by intra- and inter-specific crosses (Jones and Rees 1982). Appearance frequencies of B-chromosomes could be depended on population and individual and be related from a balance between transmission rate of B-chromosome and effects of B-chromosome on host condition (Camacho *et al.* 2000). B-chromosome might be related with occurrence of fission at centromeric region (White 1957). Moreover, B-chromosomes could include transposable DNA elements in some species (Beukeboom 1994). Therefore, B-chromosomes would progress genome evolution (Camacho *et al.* 2000). It is speculated that the third pair of median-centromeric chromosomes in *P. minor* might make a fission at the centromeric region to produce four terminal-centromeric chromosomes in *P. japonica*. However, existence of B-chromosomes were not reported in *P. minor* from in this study and past reports, existence of B-chromosome

in *P. ophioglossoides* which would be origin of *P. minor* in speciation increased the possibility which B-chromosomes concerned the fission at the centromeric region in *Pogonia* speciation.

Karyotypes of hybrids are generally maintained the independence of chromosomes and not are shown dominant/recessive and neutralization between chromosomes (Tanaka 1980). The karyotypes of the hybrids between *P. minor* and *P. japonica* studied maintained those of the parental chromosome morphology. However, the karyotypes of reciprocal hybrids between *P. minor* and *P. ophioglossoides* did not match those of parents. Variation of chromosome size and chromosome condensation through hybridization, neutral amphiplasty, were shown in *Crepis* (Navashin 1934; Langridge *et al*. 1970). Kondo *et al*. (1999) found that a hybrid had two chromosomes changed their karyotype in chromosome complement of F_1 hybrid between *Dendranthema japonica* and *Tanacetum vulgare* after reciprocal translocation in the somatic cells by using the method of the genomic *in situ* hybridization. New combination of genome would be shared with a common nucleus of hybrid plants. To be released chromosomal instability of the new combination, degree of chromosome condensation might vary and/or inactive transposable DNA might begin to work (Jones and Pasakinskiene 2005). As a result genome conflict might occur (Jones and Pasakinskiene 2005).

Seeds of F_2 hybrids got germinated with the chromosome numbers either 2n=19 or 2n=20. Following a hypothesis of Tanaka (1962a, b) these phenomena might be due to progress of speciation from *P. minor* to *P. japonica*.

Pollen formation of reciprocal F_1 hybrids between *P. ophioglossoides* and any of the Asiatic species were defective. Therefore, F_2 seeds obtained them got only a few by self-

pollination. This suggests that *P. ophioglossoides* could be distantly related to East Asiatic species.

Rate of embryo formation of F_2 seeds from reciprocal hybridization between *P. japonica* and *P. minor* was high. The F_2 seeds between *P. japonica* and *P. minor* germinated relative low in comparison to seeds of wild species and F_1 hybrids. Reciprocal F_1 hybrids between *P. japonica* and *P. minor* did not show hybrid sterility. Therefore it was expected that *P. japonica* and *P. minor* were close genetically although there were some differences about flower morphology, patterns of axenic germination and environments of their habitats.

Seedling growth of F_2 hybrids from reciprocal crosses between *P. japonica* and *P. minor* after germination was vigorous. This result suggests that F_1 hybrids between *P. japonica* and *P. minor* would be produced in nature and the F_1 hybrids could perform not only vegetative propagation but also seed propagation.

Habitats of *P. japonica* and those of *P. minor* are not always separated far from each other. For example, in the bog in Ikenodan, Mt. Hiba, *P. japonica* and *P. minor* can be seen together. However, habitats of the two species were different from each other although 10 m or less distantly grown with each other (Fig. 39). Also, in Higashi-Hiroshima City, plants of *P. japonica* grow in a bog while those of *P. minor* grow in not the bog but a bank with relatively dry soil far along the bog where the plants of *P. japonica* grow. The distance between the two habitats was about 5 m. Since the flowering periods of two species were the same to each other, they have had some chance to make hybridization. However, there is no record about natural hybrids between the two species in Ikenodan and Higashi-Hiroshima City.

Hybrids between *P. japonica* and *P. minor* have not yet been discovered in spite of that they exist face to face in nature. This might be caused by that the flower of *P. minor* does not fully open. As the flower of *P. minor* is close, there may be very few opportunities that pollen may be carried by insects being inserted into the stigma. It was suggested that reproductive isolation was shown by difference of their flower morphology. *Pogonia minor* in nature often set fruits, while it in cultivated state also often set fruits without any cross-pollination treatment. Thus, *P. minor* may have a self-pollination mechanism. As for *P. japonica*, growth of rhizomes for their elongation is well and vegetative propagation is vigorous. In contrast, *P. minor* has poor rhizome elongation. *Pogonia minor* would have been depended on seed propagation by flower unopened to prosperity of descendants. Even if hybrids between *P. japonica* and *P. minor* appear in nature, they might disappear by natural selection.

Pogonia called 'Miyamatokisou' which is different from *P. japonica* and *P. minor* according to their flower morphology has newly discovered in Akita Pref. (Takahashi 1987). Features of individual plants of *Pogonia* are described as follows: The lip of new *Pogonia* is slenderer than that of the standard *P. japonica*. There is a faded red-purple line on sepals of the new type of *Pogonia*. It has specific fragrance during the daytime. The karyotype of *Pogonia* called 'Miyamatokisou' from fanciers from Mt. Hakusan had the chromosome number of 2n=18 same number as *P. minor* and *P. ophioglossoides*. However, karyotype of the 'Miyamatokisou' was somewhat different from that of the two *Pogonia*.

A plant of *Pogonia* with a flower shape different from *P. japonica* and *P. minor* were also found in Higashi-Hiroshima City for the first time: The flower was half-open like *P. japonica* but lip of the flower was slenderer built and poorer fimbriate than that of *P.*

japonica and was rather similar to that of *P. minor* (Fig. 21). Flower shape of the *Pogonia* was similar to *Pogonia* called ‘Miyamatoksou’. In the *Pogonia*’s habitat, both *P. japonica* and *P. minor* can be seen sometimes. The *Pogonia* lives near *P. minor* at relatively dried area. The distance from *P. minor* population edge to the *Pogonia* was ca 0.5 m. It was expected that this *Pogonia* in Higashi-Hiroshima City was a natural hybrid between *P. japonica* and *P. minor*. However, the karyotype of this *Pogonia* was 2n=20 and quite same as that of *P. japonica*. It was expected that it would be a variety of *P. japonica*. The karyotype of F_2 hybirids showed 2n=18, 19 and 20. Therefore, the feasibility on this unusual *Pogonia* is also still remains. Molecular genetic analysis was necessary to clarify to the origin of the two new forms of *Pogonia*.

Table 11. Rates of embryo formation and seed germination in *Pogonia japonica*, *P. minor*, *P. ophioglossoides* and their F_1 hybrids

Species and F_1 hybrid	Embryo formation (%)	Seed germination (%)
P. japonica	93.7	90.6
P. minor	87.7	84.2
P. ophioglossoides	90.2	89.7
P. japonica x *P. minor*	91.5	81.3
P. japonica x *P. ophioglossoides*	93.5	89.6
P. minor x *P. japonica*	89.1	80.0
P. minor x *P. ophioglossoides*	88.4	82.8
P. ophioglossoides x *P. japonica*	93.7	91.5
P. ophioglossoides x *P. minor*	87.4	85.6

[1] Rates of embryo formation were measured just before sowing.
[2] Rates of seed germination were measured at the 150-day-stage after sowing.

Table 12. Rates of embryo formation and seed germination in *Pogonia japonica*, *P. minor* and their F_1 hybrids

Taxon	Sterilization treatment*	Embryo formation %	Seed germination %	(no. of germinated seeds / no. of seeds sowed)
P. japonica	non-seed sterilization	92.5	61.3	(761/1,241)
	seed sterilization		82.7	(1,283/1,551)
P. minor	non-seed sterilization	86.1	0.7	(3/ 434)
	seed sterilization		80.3	(159/ 198)
P. japonica × *P. minor*	non-seed sterilization	87.6	56.1	(952/1,697)
	seed sterilization		86.8	(520/ 599)
P. minor × *P. japonica*	non-seed sterilization	85.9	1.7	(7/ 410)
	seed sterilization		84.3	(231/ 274)

*Seed sterilization = seeds were sterilized by 1.2% sodium hypochlorite solution.
Seeds at 75-90 days stage after reciprocal-cross pollination were sown on KC medium with no hormone.
Rates of seed germination were measured at the 150-day-stage after sowing.

Table 13. Rates of embryo formation and seed germination of F_2 hybrids in the F_1 hybrids between *Pogonia japonica* and *P. minor*

F_2 hybrid	Sterilization treatment*	Embryo formation %	Seed germination %	(no. of germinated seeds / no. of seeds sowed)
P. japonica × *P. minor* self-pollination	non-seed sterilization	87.6	0.7	(18/2,766)
	seed sterilization		26.0	(485/1,867)
P. minor × *P. japonica* self-pollination	non-seed sterilization	84.3	0.2	(2/ 835)
	seed sterilization		4.2	(18/ 431)

Seed sterilization = seeds were sterilized by 1.2% sodium hypochlorite solution.
Seeds at 75-90 days stage after self-pollination were sown on KC medium with no hormone.
Rates of seed germination were measured at the 150-day-stage after sowing.

Table 14. Measurements of somatic chromosomes of *Pogonia japonica* at metaphase, 2n=20

No.	Relative length	Long arm (μm)	Short arm (μm)	Arm ratio (L/S)
1	7.08	12.50	11.63	1.07
2	6.97	12.69	11.06	1.15
3	6.72	11.50	11.38	1.01
4	6.62	11.69	10.88	1.07
5	6.29	11.44	10.00	1.14
6	6.24	10.81	10.44	1.04
7*	6.60	11.81	10.69	1.10
8*	5.69	10.13	9.25	1.10
9	4.83	9.38	7.07	1.33
10	4.64	8.75	7.07	1.24
11	4.51	8.75	6.63	1.32
12	4.48	8.19	7.06	1.16
13	4.18	8.00	6.25	1.28
14	4.17	8.06	6.13	1.31
15	4.20	9.13	5.19	1.76
16	3.76	8.00	4.81	1.66
17	3.38	11.50	-	-
18	3.36	11.40	-	-
19	3.16	10.75	-	-
20	3.12	10.63	-	-

*The chromosome with a secondary constriction at median region.

Table 15. Measurements of somatic chromosomes of *Pogonia minor* at metaphase, 2n=18

No.	Relative length	Long arm (μm)	Short arm (μm)	Arm ratio (L/S)
1	6.92	12.81	11.88	1.08
2	6.69	13.88	10.00	1.39
3	6.59	12.75	10.75	1.19
4	6.38	13.38	9.38	1.43
5	6.36	12.13	10.56	1.15
6	6.36	12.25	10.44	1.17
7*	5.89	10.69	10.31	1.04
8*	5.59	10.06	9.88	1.02
9	5.90	12.63	8.44	1.50
10	5.89	12.81	8.19	1.56
11	5.89	13.19	7.81	1.69
12	5.59	12.00	7.94	1.51
13	4.70	9.38	7.38	1.27
14	4.61	9.13	7.31	1.25
15	4.34	8.75	6.75	1.30
16	4.15	8.13	6.69	1.22
17	4.31	9.69	5.69	1.70
18	3.86	8.88	4.88	1.82

*The chromosome with a secondary constriction at median region.

Table 16. Measurements of somatic chromosomes of *Pogonia ophioglossoides* at metaphase, 2n=18+3B

No.	Relative length	Long arm (μm)	Short arm (μm)	Arm ratio (L/S)
1	7.35	13.94	12.54	1.11
2	7.23	13.60	12.45	1.09
3	7.01	13.14	12.14	1.08
4	6.25	11.93	10.60	1.13
5	6.16	11.26	10.94	1.03
6	6.14	11.40	10.74	1.06
7	6.14	12.54	9.60	1.31
8	6.11	11.00	11.00	1.00
9*	6.07	11.66	10.20	1.14
10*	6.01	11.66	10.00	1.17
11	5.28	10.80	8.24	1.31
12	4.92	10.34	7.40	1.40
13	4.98	10.86	7.06	1.54
14	4.61	9.40	7.20	1.31
15	4.16	8.06	6.94	1.16
16	4.11	8.14	6.66	1.22
17	3.81	8.40	5.34	1.57
18	3.65	7.73	5.40	1.43
19	0.63	1.14	1.14	1.00
20	0.63	1.14	1.14	1.00
21	0.59	1.06	1.06	1.00

*The chromosome with a secondary constriction at median region.

Table 17. Category classification of karyotype of *Pogonia*.

	Category				
	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ
Relative length	5.50~7.50	5.50~6.00	4.00~5.50	3.50~4.50	3.00~3.50
Arm ratio	1.00~1.50	1.50~1.70	1.00~1.50	1.50~2.00	-

Table 18. Measurements of somatic chromosomes of *Pogonia* from Mt. Hakusan called 'Miyamatokisou' at metaphase, 2n=18

No.	Relative length	Long arm (μm)	Short Arm (μm)	Arm ratio (L/S)
1	6.97	3.71	3.26	1.14
2	6.77	3.42	3.35	1.02
3	6.76	3.46	3.30	1.05
4	6.67	3.88	2.79	1.39
5	6.58	3.74	2.84	1.32
6	6.57	3.35	3.22	1.04
7*	6.43	3.45	2.98	1.16
8*	5.95	3.14	2.81	1.12
9	5.80	3.01	2.79	1.08
10	5.67	2.85	2.82	1.01
11	5.41	2.82	2.59	1.09
12	5.06	2.81	2.25	1.25
13	4.95	2.70	2.25	1.20
14	4.72	2.69	2.03	1.33
15	4.56	2.76	1.80	1.54
16	4.15	2.31	1.84	1.25
17	3.52	2.11	1.41	1.50
18	3.44	2.30	1.14	2.02

*The chromosome with a secondary constriction at median region.

Table 19. Measurements of somatic chromosomes of *Pogonia* from Higashi-Hiroshima City at metaphase, 2n=20

No.	Relative length	Long arm (μm)	Short Arm (μm)	Arm ratio (L/S)
1	7.43	16.15	15.55	1.04
2	7.05	16.11	14.09	1.14
3	6.89	15.76	13.66	1.15
4	6.86	15.24	14.03	1.09
5	6.18	13.30	13.09	1.02
6	5.91	12.95	12.60	1.06
7*	6.08	13.35	12.62	1.06
8*	5.57	12.16	11.61	1.05
9	5.21	13.20	9.03	1.46
10	4.95	10.99	10.15	1.08
11	4.56	10.60	8.88	1.19
12	4.53	10.92	8.40	1.30
13	4.28	10.51	7.75	1.35
14	3.79	8.14	8.03	1.01
15	3.72	10.42	5.45	1.91
16	3.57	9.64	5.60	1.72
17	3.49	14.89	-	-
18	3.40	14.49	-	-
19	3.38	14.41	-	-
20	3.31	13.35	-	-

*The chromosome with a secondary constriction at median region.

Table 20. Chromosome numbers of *Pogonia japonica*, *P. minor*, F_1 hybrids and F_2 hybrids

Taxon	No. of plants studied	No. of plants which have chromosome number of :			No. of terminal-centromeric chromosome per cell
		2n=18	2n=19	2n=20	
P. japonica	15			15	4
P. minor	10	10			0
P. japonica × *P. minor*	10		10		2
P. minor × *P. japonica*	10		10		2
(*P. japonica* × *P. minor*) self-pollinated F_2 hybrids	34		19		2-3*
				15	4
(*P. minor* × *P. japonica*) self-pollinated F_2 hybrids	27	4			0
			15		2-3*
				8	4

* Only one plant had three terminal-centromeric chromosomes while the other plants had two terminal-centromeric chromosomes.

Fig. 20. Flower of *Pogonia* from Mt. Hakusan, Gifu Pref. called ‘Miyamatokisou’.

Fig. 21. Flower of *Pogonia* in Higashi-Hiroshima City, Hiroshima Pref. The distance between the *Pogonia* and *P. minor* was about 0.5m. The distance between the *Pogonia* and *P. japonica* was about 5m.

Fig. 22. Flower of F_1 hybrids between *P. japonica* and *P. minor*. A. *P. japonica* × *P. minor*. B. *P. minor* × *P. japonica.*

Fig. 23. Flower of F_1 hybrids between *P. japonica* and *P. ophioglossoides*. A. *P. japonica* × *P. ophioglossoides*. B. *P. ophioglossoides* × *P. japonica*.

Fig. 24. Flower of F_1 hybrids between *P. minor* and *P. ophioglossoides*. A. *P. minor* × *P. ophioglossoides*. B. *P. ophioglossoides* × *P. minor*.

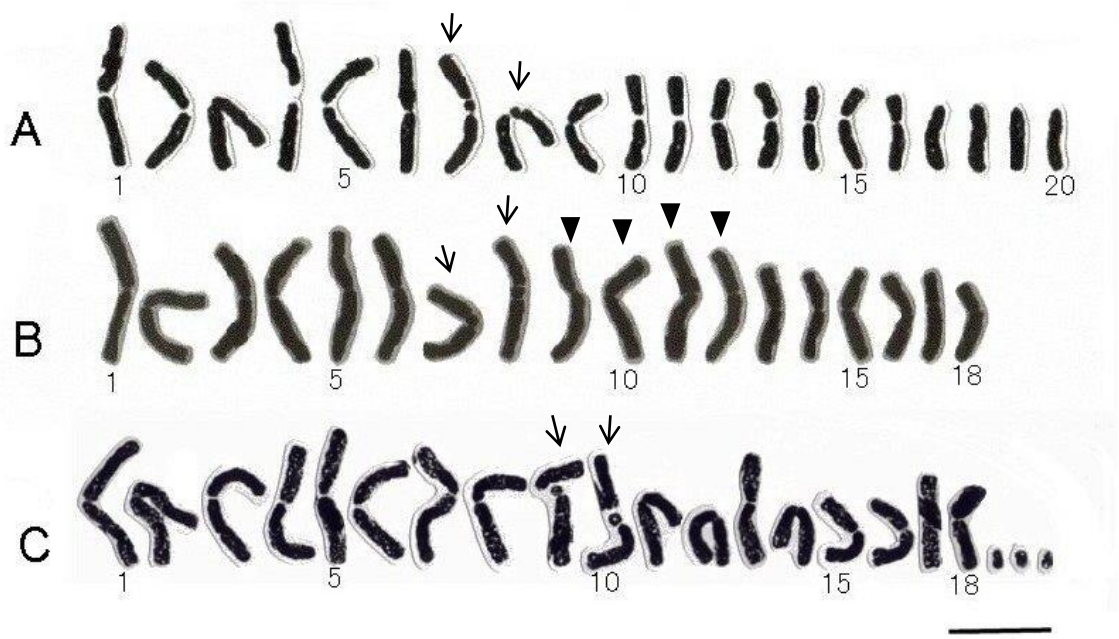

Fig. 25. Chromosome alignments at mitotic metaphase in complements in *Pogonia*. A. *Pogonia japonica.* B. *P. minor.* C. *P. ophioglossoides.* Arrows show chromosomes with a secondary constriction. Arrow heads show chromosomes with RL at 5.50-6.00 and AR at 1.50-1.70. Bar = 20μm. (Chromosome alignments of *P. japonica* and *P. minor* in this figure were used original pictures at Tanaka et al. 1996.)

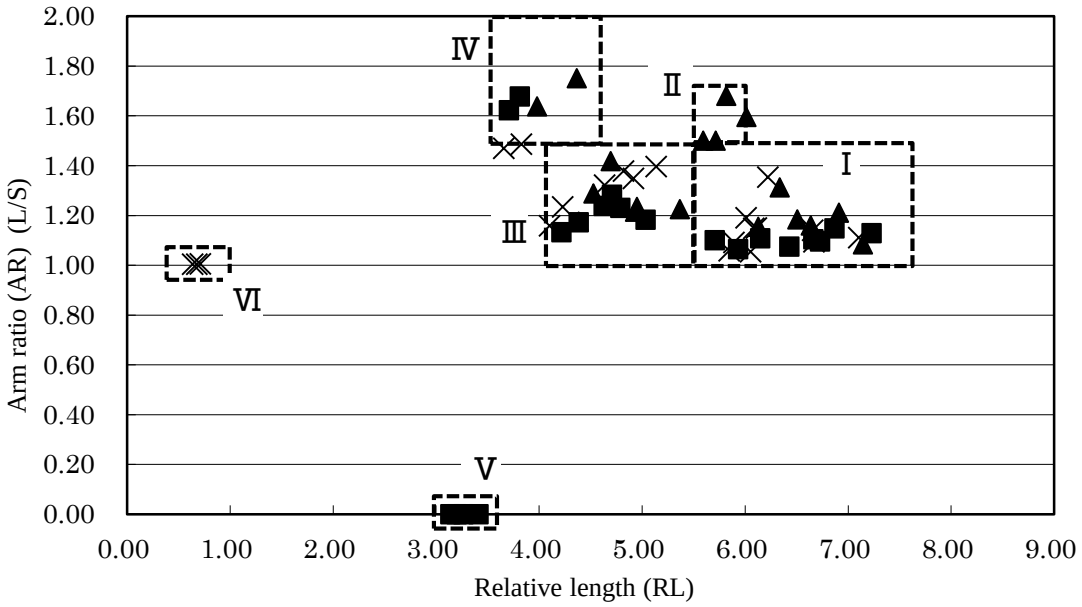

Fig. 26. Correlation between relative length and arm ratio of somatic chromosomes of three species of *Pogonia.* ■. *P. japonica*. ▲. *P. minor*. × *P. ophioglossoides*. Ⅰ. Chromosomes with RL 5.50 - 7.50 and AR 1.00 - 1.50. Ⅱ. Chromosomes with RL 5.50 - 6.00 and AR 1.50 – 1.70. Ⅲ. Chromosomes with RL 4.00 - 5.50 and AR 1.00 - 1.50. Ⅳ. Chromosomes with RL 3.50 - 4.50 and AR 1.50 - 2.00. Ⅴ. Terminal-centromeric chromosomes. Ⅵ. B-chromosomes.

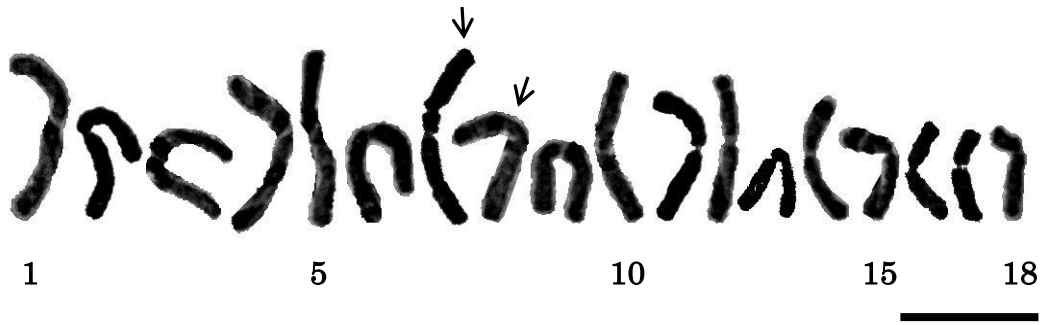

Fig. 27. Chromosome alignment at mitotic metaphase in complements in *Pogonia* from Mt. Hakusan called ‘Miyamatokisou’. Bar = 20μm. Arrows show chromosomes with a secondary constriction. (Chromosome alignment in this figure was used original pictures at Takahashi *et al*. 2014.)

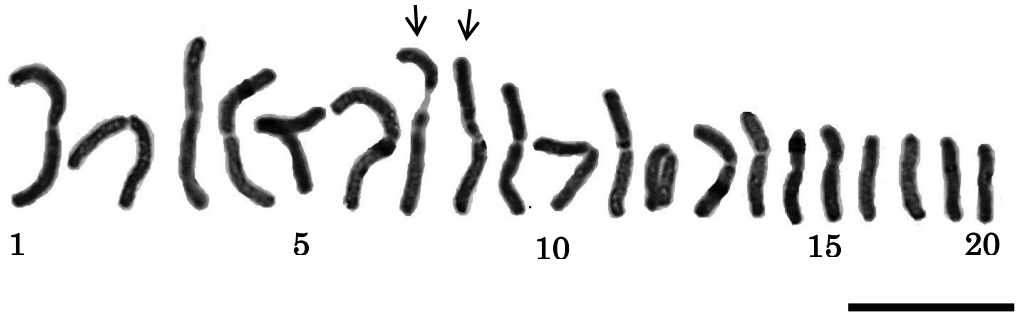

Fig. 28. Chromosome alignment at mitotic metaphase in complements in *Pogonia* from Higashi-Hiroshima City. Bar = 20μm. Arrows show chromosomes with a secondary constriction.

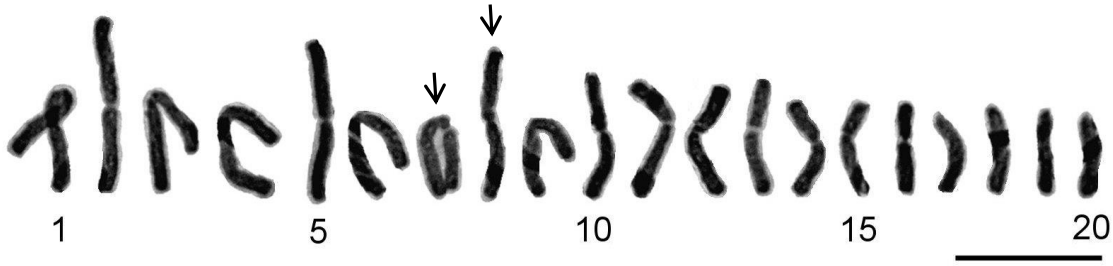

Fig. 29. Chromosome alignment at mitotic metaphase in complements in *Pogonia* from the Far Eastern Russia. Bar = 20μm. Arrows show chromosomes with a secondary constriction. (The chromosome alignment in this figure was used original pictures at Takahashi et al. 2004.)

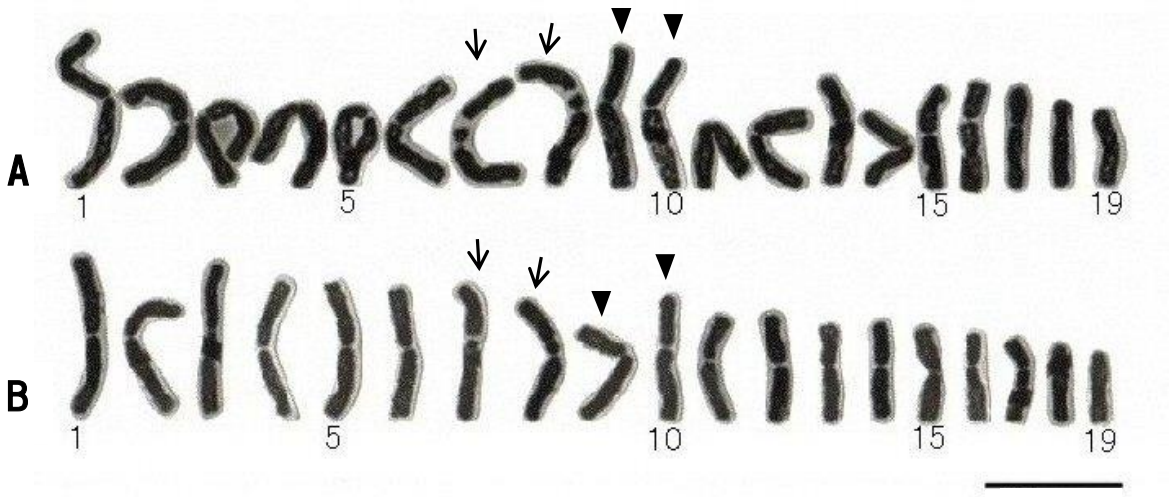

Fig 30. Chromosome alignments at mitotic metaphase in complements in F_1 hybrids between *Pogonia japonica* and *P. minor*. A. *P. japonica* × *P. minor*. B. *P. minor* × *P. japonica*. Arrows show chromosomes with a secondary constriction. Bar = 20μm. . (Chromosome alignments of *P. japonica* and *P. minor* in this figure were used original pictures at Tanaka *et al*. 1996.)

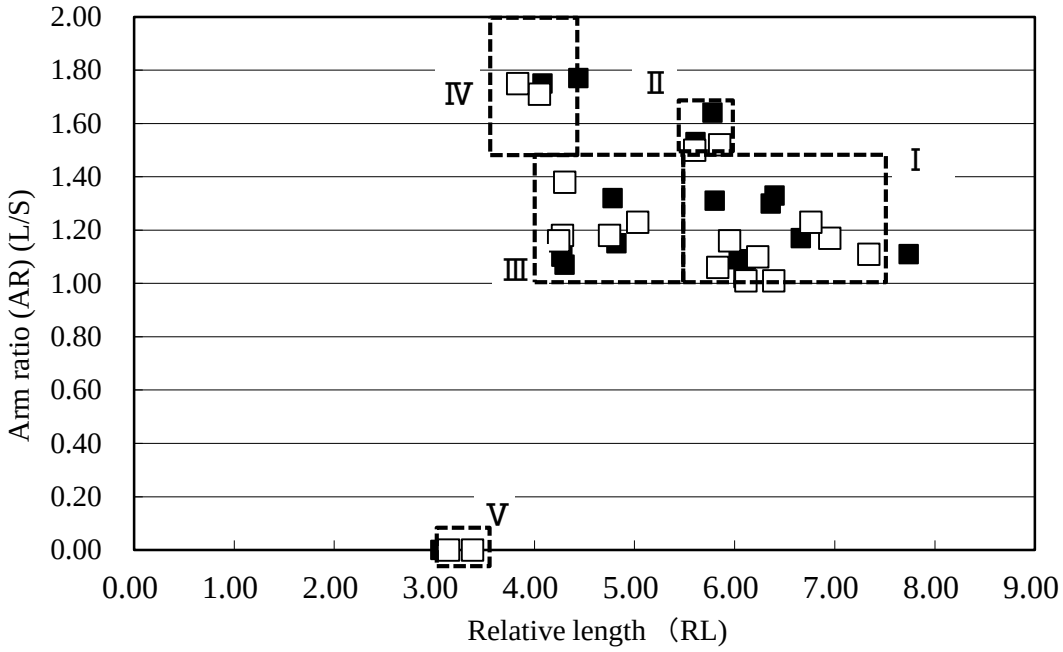

Fig. 31. Correlation between relative length and arm ratio of somatic chromosomes of F_1 reciprocal hybrids between *P. japonica* and *P. minor*. ■. *P. japonica* × *P. minor*. □. *P. minor* × *P. japonica*. . I. Chromosomes with RL 5.50 - 7.50 and AR 1.00 - 1.50. Ⅱ. Chromosomes with RL 5.50 - 6.00 and AR 1.50 – 1.70. Ⅲ. Chromosomes with RL 4.00 - 5.50 and AR 1.00 - 1.50. Ⅳ. Chromosomes with RL 3.50 - 4.50 and AR 1.50 - 2.00. Ⅴ. Terminal-centromeric chromosomes.

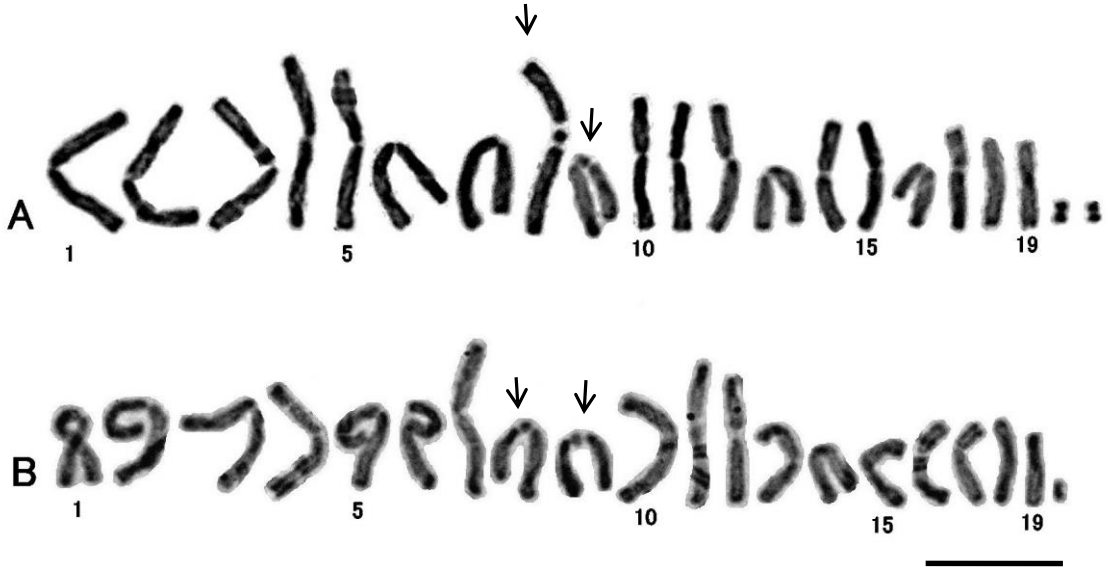

Fig. 32. Chromosome alignments at mitotic metaphase in complements in F_1 hybrids between *Pogonia japonica* and *P. ophioglossoides*. A. *P. ophioglossoides* × *P. japonica.* B. *P. japonica* × *P. ophioglossoides.* Arrows show chromosomes with a secondary constriction. Bar = 20μm.

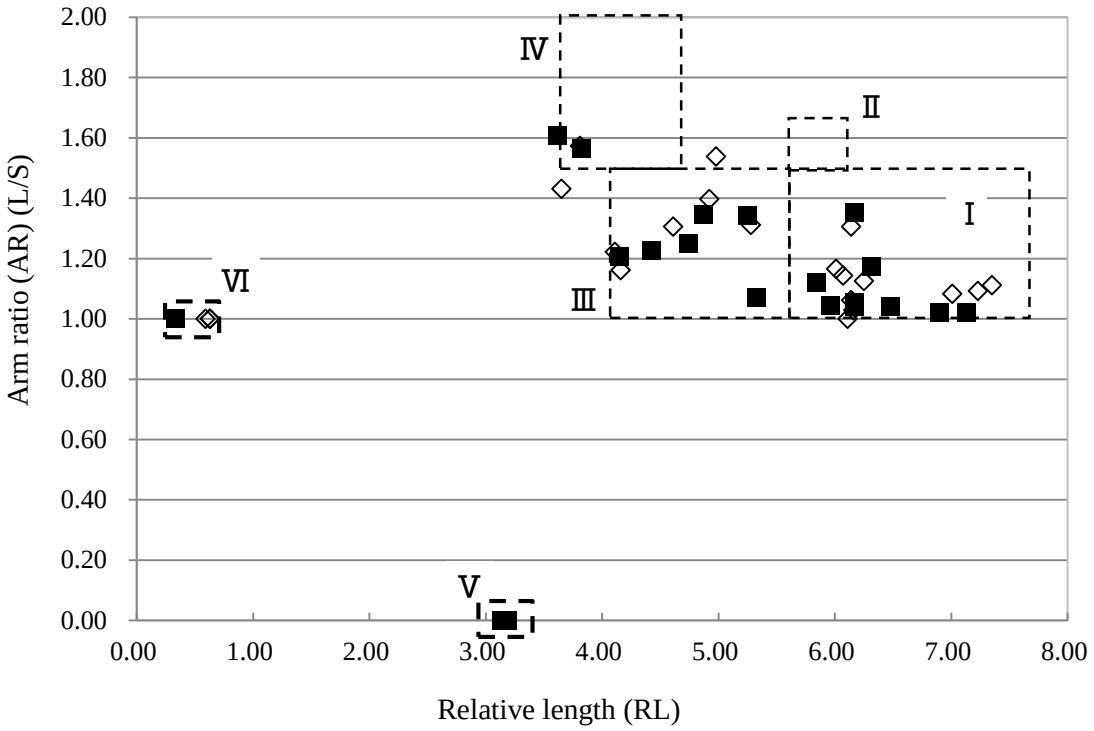

Fig. 33. Correlation between relative length and arm ratio of somatic chromosomes of F_1 reciprocal hybrids between *P. japonica* and *P. minor*. ■. *P. japonica* × *P. ophioglossoides*. ◇. *P. ophioglossoides* × *P. japonica*. Ⅰ. Chromosomes with RL 5.50 - 7.50 and AR 1.00 - 1.50. Ⅱ. Chromosomes with RL 5.50 - 6.00 and AR 1.50 – 1.70. Ⅲ. Chromosomes with RL 4.00 - 5.50 and AR 1.00 - 1.50. Ⅳ. Chromosomes with RL 3.50 - 4.50 and AR 1.50 - 2.00. Ⅴ. Terminal-centromeric chromosomes. Ⅵ. B-chromosomes.

Fig. 34. Chromosome alignments at mitotic metaphase in complements in the two species and their artificial hybrids in *Pogonia*. A. *P. ophioglossoides* × *P. minor*. B. *P. minor* × *P. ophioglossoides*. Bar = 20μm. Arrow heads show chromosomes with arm ratio more than 2.00. Arrows show chromosomes with a secondary constriction. (Chromosome alignments in this figure were used original pictures at Takahashi and Kondo 2004.)

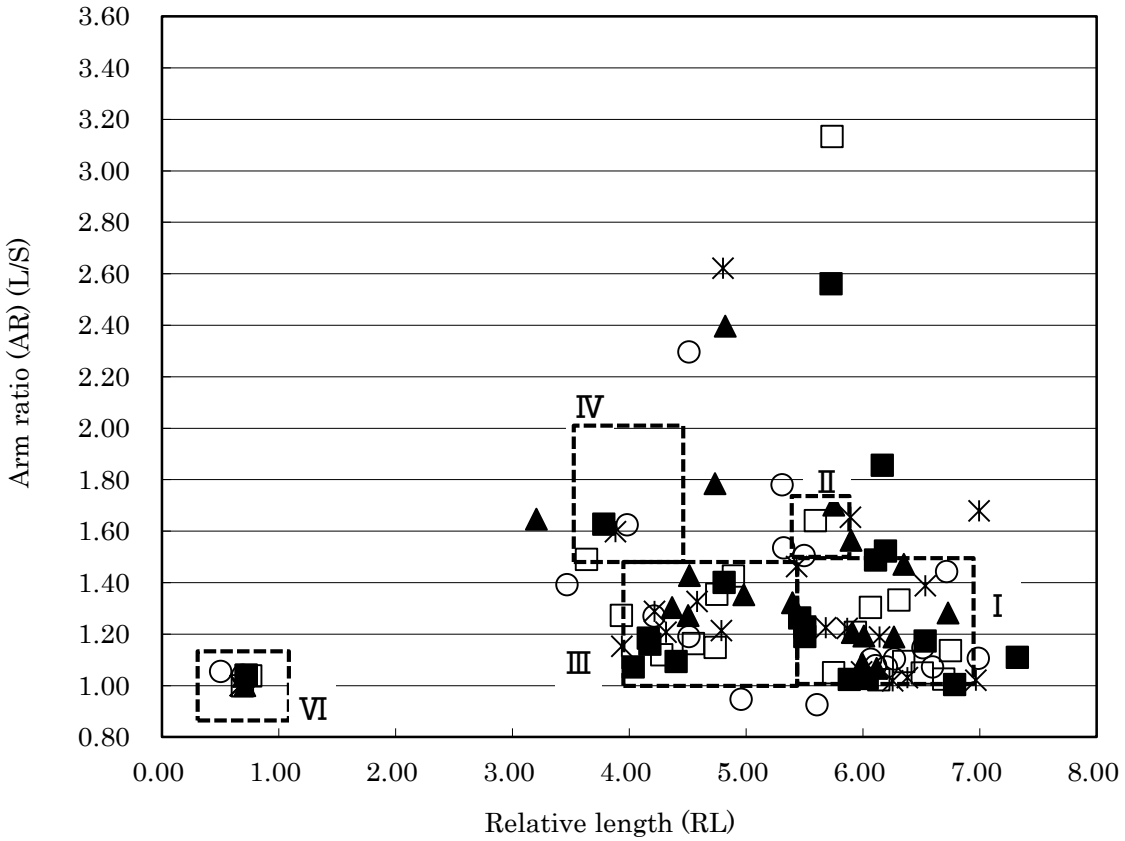

Fig. 35. Correlation between relative length and arm ratio of somatic chromosomes of *P. minor* × *P. ophioglossoides.* ▲. Hybrid 1. ■. Hybrid 2. ○. Hybrid 3. □. Hybrid 4. ∗. Hybrid 5. Ⅰ. Chromosomes with RL 5.50 - 7.50 and AR 1.00 - 1.50. Ⅱ. Chromosomes with RL 5.50 - 6.00 and AR 1.50 - 1.70. Ⅲ. Chromosomes with RL 4.00 - 5.50 and AR 1.00 - 1.50. Ⅳ. Chromosomes with RL 3.50 - 4.50 and AR 1.50 - 2.00. Ⅵ. B-chromosomes.

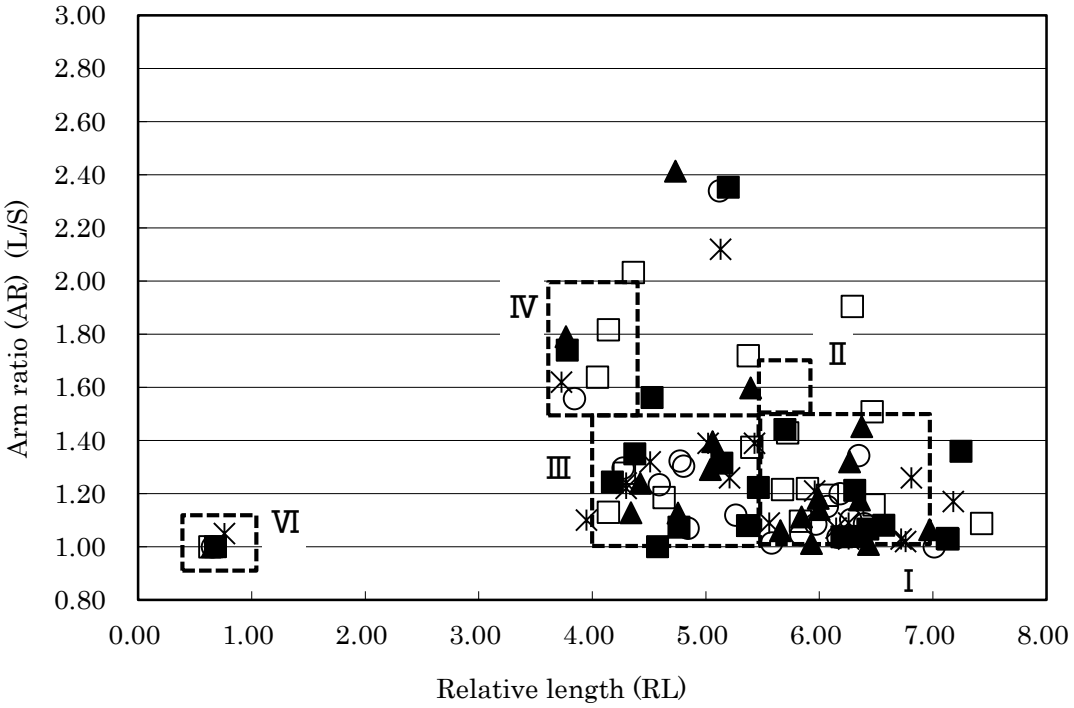

Fig. 36. Correlation between relative length and arm ratio of somatic chromosomes of *P. ophioglossoides* × *P. minor.* ▲. Hybrid 1. ■. Hybrid 2. ○. Hybrid 3. □. Hybrid 4. ✻. Hybrid 5. I. Chromosomes with RL 5.50 - 7.50 and AR 1.00 - 1.50. Ⅱ. Chromosomes with RL 5.50 - 6.00 and AR 1.50 - 1.70. Ⅲ. Chromosomes with RL 4.00 - 5.50 and AR 1.00 - 1.50. Ⅳ. Chromosomes with RL 3.50 - 4.50 and AR 1.50 - 2.00. Ⅴ. Terminal-centromeric chromosomes. Ⅵ. B-chromosomes.

Fig. 37. Chromosome alignment at mitotic metaphase in complement of triploid hybrid between *Pogonia ophioglossoides* and *P. minor*. Bar = 20μm. Arrows show chromosomes with a secondary constriction. An arrow head shows a chromosome with AR of more than 2.00. (The chromosome alignments in this figure was used original pictures at Takahashi and Kondo 2004.)

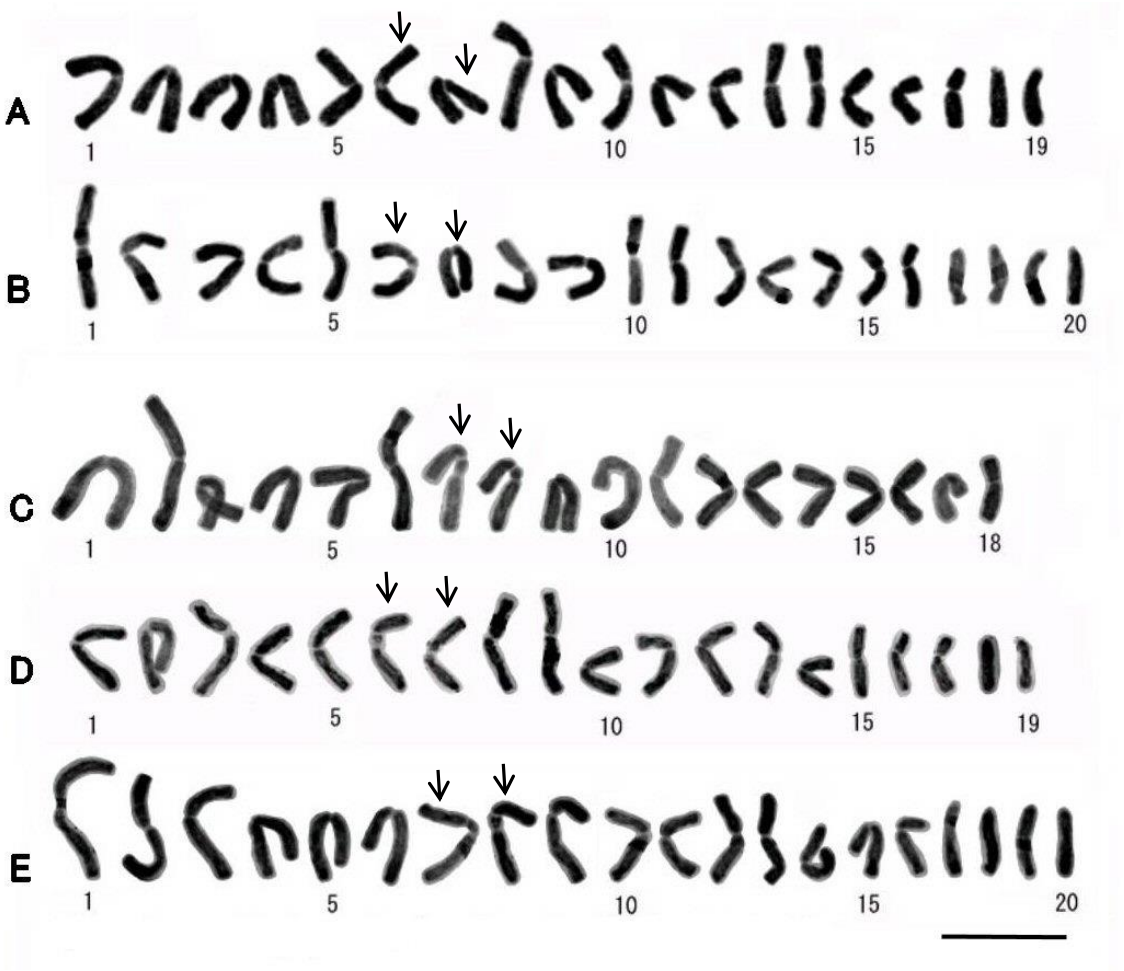

Fig. 38. Alignments of the chromosomes in the somatic-metaphase complements in the F_2 reciprocal hybrids between *Pogonia japonica* and *P. minor*. A and B. (*P. japonica* × *P. minor*) self-pollinated F_2 hybrids. C-E. (*P. minor* × *P. japonica*) self-pollinated F_2 hybrids. Bar = 20μm. Arrows show chromosomes with a secondary constriction.

Fig. 39. Habitats of *Pogonia* in Ikenodan, Mt. Hiba (Alt. 1,279.5m). A: The habitat of *P. japonica*. B: The habitat of *P. minor*.

Chapter 5 RAPD (Random Amplified Polymorphic DNA) Molecular characteristics of newly found *Pogonia* different from *P. japonica* and *P. minor*

Materials and methods

Plant materials

Some plants of *Pogonia japonica* and *P. minor* were collected in Ikenodan High-Moore, alt. 1,279.5 m, Mt. Hiba. Morphologically unusual type so-called 'Miyamatokisou' different from the former two species (Fig. 20) was collected in Mt. Hakusan, Gifu Prefecture. Additionally, some individual plants of the commercialized *P. japonica* and *P. minor* were purchased from some nurseries for standardizing the experimental materials. The present research-centered 'Miyamatokisou' were a little bit different in flower shape from both *P. japonica* and *P. minor*.

On the other hand, a few plants collected in a bog in Higahi-Hiroshima City (Fig. 21) were morphologically somewhat different from the standardized *P. japonica* and *P. minor* and a little bit similar to 'Miyamatokisou' found in Mt. Hakusan (Fig. 21).

Artificial F_1 hybrids between *P. japonica* and *P. minor* were made for the purpose to get known what is the true characteristics of the interspecific hybrid between the two species.

DNA extraction

Total genomic DNA of each taxon of *Pogonia* studied was extracted from fresh leaves by using the CTAB method described by Doyle and Doyle (1987).

RAPD amplification

Four hundred primers [RAPD primer series (Operon); KitA – KitT] were used for this analysis (Table 18). Then, 10 µl reaction mixture included 5 µl of 2xPCR buffer for KOD FX Neo, 1.0µl 2mM dNTP, 0.25µl 0.2µM RAPD primer, 0.1µl KOD FX Neo (TOYOBO), 10 ng/µl template DNA. PCR was performed using gene Amp PCR System 9700 (PE Applied Biosystems) and TaKaRa PCR Thermal Cycler Dice (TAKARA BIO Inc.) and DNA amplification were carried out with one cycle of 94ºC for 3 min, followed by 45 cycles of denature temperature 94ºC for 1 min, annealing temperature 42ºC for 1 min and 68ºC for 1.5 min, and concluded by one cycle of 68ºC for 10 min. PCR products was electrophoresed on 1.5% agarose gel with DNA size marker (200bp DNA Ladder, TaKaRa) at 100V for 30 min in Mupid-2 plus (ADVANCE) filled by filled by 1 x TAE Buffer. After staining of the electrophoresed gel with Ethidium bromide solution for 30 min observation and photography of the band were performed using Printgraphy AE-6933 FXCF-U (ATTO) under the ultraviolet exposure.

Data analysis

All amplifications were repeated thrice in order to confirm the reproducible amplification of scored fragments. Second reproducible and clear amplification bands were scored for the construction of the data matrix. The marked changes observed in RAPD profiles (disappearance and/or appearance of bands in comparison with untreated control treatments) were evaluated. Each gel of RAPD was analyzed by scoring present (1) or absent (0) bands. The RAPD and pooled data matrices were entered into the cluster analysis soft (http://aoki2.si.gunma-u.ac.jp/BlackBox). A dendrogram was constructed by using the Ward method.

Results

In this study, 400 primer were used to analyze genetic identity and closely related (Table 21). Polymorphic bands were obtained in 237 primers from 400 primers used (Table 21). Band patterns of *P. ophioglossoides* were different from those of other species clearly (Tables 22, 23 and 24).

DNA fragments generated OPO-02 was *P. japonica* specific (Table 22 and Fig. 40). The DNA fragment for *P. japonica* was found around 1000bp and found on F_1 hybrid between *P. minor* and *P. japonica* using OPO-02 primer (Fig. 40). Species specific DNA fragments for *P. minor* was unclear during the course of investigation. Species specific DNA fragment for *P. japonica* and *P. minor* were only one and zero, respectively. DNA arrangement of the two wild species would be similar to each other.

DNA fragments generated OPE-02 was species specific for *Pogonia* 'Miyamatokisou' collected in Mt. Hakusan (Table 22 and Fig. 41). A species specific DNA fragment was found on 1100bp. A DNA fragment on 800bp using OPO-04 found in *P. japonica*, *P. minor* and F_1 hybrid between the two species was not found in *Pogonia* 'Miyamatokisou' collected in Mt. Hakusan as well as the new type of *Pogonia* collected in Higahi-Hiroshima City (Table 22 and Fig. 42).

Those clusters were analyzed on the basis of those specific DNA fragments in RAPD using 237 primer (Fig. 43). Genic relationship between *P. japonica* collected in Mt. Hiba and that cultivated and propagated in nursery were quite closely related and nearly the same in RAPD analysis to each other since they were the same species, while genic relationship between *P. minor* collected in Mt. Hiba and that cultivated in nursery for sale were also closely related to each other since they were the two species placed as the

respective species. On the other hand, RAPD bands between *Pogonia* 'Miyamatokisou' and a specific strain minority of *Pogonia* grown in Higashi-Hiroshima City were also nearly the same, while their flower shapes were similar to each other. However, *Pogonia* 'Miyamatokisou' of Mt. Hakusan seemed to be another strain different from that of Higashi-Hiroshima City.

Discussion

Progress of evolution, biosystematics and expansion of habitats in *Pogonia* distributed in East Asia and Eastern North America were studied, clarified and justified by using modern methodologies such as molecular approaches.

These unusual types grown together with normal types of the species either *P. japonica* or *P. minor* are expected being species variabilities or hybridities between the two species since artificial hybridization between *P. japonica* (2n=20) and *P. minor* (2n=18) were successfully made although their chromosome numbers were different from each other (Tanaka *et al*., 1996) and F_2 hybrids by self-pollination of the F_1 hybrids could be formed (Takahashi 2001).

The chromosome number of *Pogonia* 'Miyamatokisou' and that of *P. minor* were the same as 2n=18. However, the karyotypes of those taxa were not the same. The chromosome number and the karyotype of *Pogonia* unnamed taxon from Higashi-Hiroshima City and that of *P. japonica* were the same, 2n=20. However, a species specific fragment of *P. japonica* used OPO-02 primer in RAPD was not shown in *Pogonia* from Higashi-Hiroshima City. Therefore, it was suggested that two types of unusual *Pogonia* would not be species variabilities in this study.

It is suggested that the feasibility of natural hybridization could exist in their natural habitats and their surrounding areas. However, until now it has not yet been reported

natural hybrids. Higashi-Hiroshima City has both populations of *P. japonica* and *P. minor*: A hybrid-like individual was located about 0.3 m distance far from a population of *P. minor* and about 5 m distance far from a population of *P. japonica*. Thus, it was expected that the hybrid-like *Pogonia* could be a natural hybrid between *P. japonica* and *P. minor*. However, a species specific fragment of *P. japonica* used OPO-02 primer in RAPD was not only shown in *Pogonia* from Higashi-Hiroshima City but also shown in *Pogonia* called 'Miyamatokisou'. Also a common fragment of *P. japonica*, *P. minor* and *P. minor* x *P. japonica* used OPO-04 primer in RAPD was not shown in the two type of unusual *Pogonia*.

During the course of investigation, the studied individuals of *Pogonia* called 'Miyamatokisou' and *Pogonia* from Higashi-Hiroshima City could not be the same as *P. japonica*, *P. minor* and F_1 hybrids between them.

Pogonia ophioglossoides has fragrance (Radford *et al*. 1968), while there have been no information which *P. japonica* and *P. minor* have fragrance. It was mentioned that *Pogonia* called 'Miyamatokisou' had fragrance during the daytime by Takahashi （1987）. If all 'Miyamatokisou' have fragrance, they should be closely related to *P. ophioglossoides*.

Pogonia japonica and *P. ophioglossoides* generally grow in sunny bogs, although *P. minor* grows not only in mountainous bogs but also in relative dried grass fields. *Pogonia minor* exists various places such as relative dried soil in the edge of bogs and paddies in Higashi-Hiroshima City, such boggy area as in Mt. Hiba and at dried fields growing mainly *Pleioblastus chino* var. *viridis* in Akiyoshidai (Karst Plateau), Yamaguchi Prefecture. Takahashi (1987) mentioned that *Pogonia* called 'Miyamatokisou' at Akita Pref. grew in certain bogs. *Pogonia* called 'Miyamatokisou' from Mt. Hakusan

transferred and studied grows in relatively humid grass field. Habitats of *Pogonia* called ‘Miyamatokisou’ might be similar to those of *P. minor* somewhat.

Cameron and Chase (1999) reported that the two species of *Pogonia* in East Asia might be originated and differentiated from *P. ophioglossoides* in Southeast North America. *Pogonia ophioglossoides* was closer to *Pogonia* ‘Miyamatokisou’ than to *P. japonica* and *P. minor* by the present cluster analysis. *Pogonia* ‘Miyamatokisou’ might be an intermediate species to evolve from *P. ophioglossoides* to *P. minor* and *P. japonica*, from their points of cluster analysis, their fragrance, their habitats and their shape of lip.

To clarify the origin of *Pogonia* ‘Miyamatokisou’ and *Pogonia* from Higashi-Hiroshima City, many more studies in cytogenetics and molecular genetics are necessary to clarify their species relationships and speciation.

Table 21. Primer sequences with presence of polymorphic bands

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPA-01	CAGGCCCTTC	1
OPA-02	TGCCGAGCTG	1
OPA-03	AGTCAGCCAC	0
OPA-04	AATCGGGCTG	1
OPA-05	AGGGGTCTTG	0
OPA-06	GGTCCCTGAC	1
OPA-07	GAAACGGGTG	1
OPA-08	GTGACGTAGG	0
OPA-09	GGGTAACGCC	1
OPA-10	GTGATCGCAG	1
OPA-11	CAATCGCCGT	1
OPA-12	TCGGCGATAG	1
OPA-13	CAGCACCCAC	0
OPA-14	TCTGTGCTGG	1
OPA-15	TTCCGAACCC	1
OPA-16	AGCCAGCGAA	1
OPA-17	GACCGCTTGT	0
OPA-18	AGGTGACCGT	1
OPA-19	CAAACGTCGG	1
OPA-20	GTTGCGATCC	1
OPB-01	GTTTCGCTCC	1
OPB-02	TGATCCCTGG	1
OPB-03	CATCCCCCTG	1
OPB-04	GGACTGGAGT	1
OPB-05	TGCGCCCTTC	0
OPB-06	TGCTCTGCCC	1
OPB-07	GGTGACGCAG	1
OPB-08	GTCCACACGG	1
OPB-09	TGGGGGACTC	1
OPB-10	CTGCTGGGAC	1
OPB-11	GTAGACCCGT	1
OPB-12	CCTTGACGCA	1
OPB-13	TTCCCCCGCT	1
OPB-14	TCCGCTCTGG	0
OPB-15	GGAGGGTGTT	1
OPB-16	TTTGCCCGGA	1
OPB-17	AGGGAACGAG	1
OPB-18	CCACAGCAGT	1
OPB-19	ACCCCCGAAG	1
OPB-20	GGACCCTTAC	1
OPC-01	TTCGAGCCAG	0
OPC-02	GTGAGGCGTC	0

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPC-03	GGGGGTCTTT	0
OPC-04	CCGCATCTAC	0
OPC-05	GATGACCGCC	1
OPC-06	GAACGGACTC	1
OPC-07	GTCCCGACGA	1
OPC-08	TGGACCGGTG	0
OPC-09	CTCACCGTCC	1
OPC-10	TGTCTGGGTG	0
OPC-11	AAAGCTGCGG	1
OPC-12	TGTCATCCCC	1
OPC-13	AAGCCTCGTC	0
OPC-14	TGCGTGCTTG	0
OPC-15	GACGGATCAG	1
OPC-16	CACACTCCAG	1
OPC-17	TTCCCCCCAG	0
OPC-18	TGAGTGGGTG	1
OPC-19	GTTGCCAGCC	1
OPC-20	ACTTCGCCAC	0
OPD-01	ACCGCGAAGG	0
OPD-02	GGACCCAACC	0
OPD-03	GTCGCCGTCA	0
OPD-04	TCTGGTGAGG	1
OPD-05	TGAGCGGACA	1
OPD-06	ACCTGAACGG	1
OPD-07	TTGGCACGGG	1
OPD-08	GTGTGCCCCA	1
OPD-09	CTCTGGAGAC	1
OPD-10	GGTCTACACC	1
OPD-11	AGCGCCATTG	0
OPD-12	CACCGTATCC	0
OPD-13	GGGGTGACGA	1
OPD-14	CTTCCCCAAG	1
OPD-15	CATCCGTGCT	0
OPD-16	AGGGCGTAAG	1
OPD-17	TTTCCCACGG	1
OPD-18	GAGAGCCAAC	0
OPD-19	CTGGGGACTT	0
OPD-20	ACCCGGTCAC	0
OPE-01	CCCAAGGTCC	0
OPE-02	GGTGCGGGAA	1
OPE-03	CCAGATGCAC	0
OPE-04	GTGACATGCC	0
OPE-05	TCAGGGAGGT	0
OPE-06	AAGACCCCTC	0
OPE-07	AGATGCAGCC	1
OPE-08	TCACCACGGT	0

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPE-09	CTTCACCCGA	0
OPE-10	CACCAGGTGA	0
OPE-11	GAGTCTCAGG	0
OPE-12	TTATCGCCCC	1
OPE-13	CCCGATTCGG	1
OPE-14	TGCGGCTGAG	1
OPE-15	ACGCACAACC	0
OPE-16	GGTGACTGTG	0
OPE-17	CTACTGCCGT	1
OPE-18	GGACTGCAGA	0
OPE-19	ACGGCGTATG	1
OPE-20	AACGGTGACC	0
OPF-01	ACGGATCCTG	1
OPF-02	GAGGATCCCT	1
OPF-03	CCTGATCACC	1
OPF-04	GGTGATCAGG	1
OPF-05	CCGAATTCCC	1
OPF-06	GGGAATTCGG	1
OPF-07	CCGATATCCC	1
OPF-08	GGGATATCGG	1
OPF-09	CCAAGCTTCC	0
OPF-10	GGAAGCTTGG	1
OPF-11	TTGGTACCCC	0
OPF-12	ACGGTACCAG	1
OPF-13	GGCTGCAGAA	1
OPF-14	TGCTGCAGGT	1
OPF-15	CCAGTACTCC	1
OPF-16	GGAGTACTGG	1
OPF-17	AACCCGGGAA	1
OPF-18	TTCCCGGGTT	1
OPF-19	CCTCTAGACC	1
OPF-20	GGTCTAGAGG	1
OPG-01	CTACGGAGGA	0
OPG-02	GGCACTGAGG	1
OPG-03	GAGCCCTCCA	0
OPG-04	AGCGTGTCTG	1
OPG-05	CTGAGACGGA	1
OPG-06	GTGCCTAACC	0
OPG-07	GAACCTGCGG	1
OPG-08	TCACGTCCAC	1
OPG-09	CTGACGTCAC	1
OPG-10	AGGGCCGTCT	1
OPG-11	TGCCCGTCGT	1
OPG-12	CAGCTCACGA	0
OPG-13	CTCTCCGCCA	0
OPG-14	GGATGAGACC	1

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPG-15	ACTGGGACTC	1
OPG-16	AGCGTCCTCC	0
OPG-17	ACGACCGACA	0
OPG-18	GGCTCATGTG	1
OPG-19	GTCAGGGCAA	0
OPG-20	TCTCCCTCAG	0
OPH-01	GGTCGGAGAA	1
OPH-02	TCGGACGTGA	1
OPH-03	AGACGTCCAC	1
OPH-04	GGAAGTCGCC	0
OPH-05	AGTCGTCCCC	1
OPH-06	ACGCATCGCA	0
OPH-07	CTGCATCGTG	0
OPH-08	GAAACACCCC	0
OPH-09	TGTAGCTGGG	0
OPH-10	CCTACGTCAG	0
OPH-11	CTTCCGCAGT	1
OPH-12	ACGCGCATGT	1
OPH-13	GACGCCACAC	0
OPH-14	ACCAGGTTGG	1
OPH-15	AATGGCGCAG	0
OPH-16	TCTCAGCTGG	1
OPH-17	CACTCTCCTC	0
OPH-18	GAATCGGCCA	0
OPH-19	CTGACCAGCC	1
OPH-20	GGGAGACATC	0
OPI-01	ACCTGGACAC	1
OPI-02	GGAGGAGAGG	0
OPI-03	CAGAAGCCCA	0
OPI-04	CCGCCTAGTC	0
OPI-05	TGTTCCACGG	0
OPI-06	AAGGCGGCAG	0
OPI-07	CAGCGACAAG	0
OPI-08	TTTGCCCGGT	1
OPI-09	TGGAGAGCAG	0
OPI-10	ACAACGCGAG	0
OPI-11	ACATGCCGTG	1
OPI-12	AGAGGGCACA	0
OPI-13	CTGGGGCTGA	0
OPI-14	TGACGGCGGT	1
OPI-15	TCATCCGAGG	1
OPI-16	TCTCCGCCCT	1
OPI-17	GGTGGTGATG	0
OPI-18	TGCCCAGCCT	0
OPI-19	AATGCGGGAG	0
OPI-20	AAAGTGCGGG	1

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPJ-01	CCCGGCATAA	0
OPJ-02	CCCGTTGGGA	0
OPJ-03	TCTCCGCTTG	1
OPJ-04	CCGAACACGG	0
OPJ-05	CTCCATGGGG	0
OPJ-06	TCGTTCCGCA	1
OPJ-07	CCTCTCGACA	1
OPJ-08	CATACCGTGG	0
OPJ-09	TGAGCCTCAC	0
OPJ-10	AAGCCCGAGG	1
OPJ-11	ACTCCTGCGA	0
OPJ-12	GTCCCGTGGT	0
OPJ-13	CCACACTACC	1
OPJ-14	CACCCGGATG	1
OPJ-15	TGTAGCAGGG	1
OPJ-16	CTGCTTAGGG	0
OPJ-17	ACGCCAGTTC	1
OPJ-18	TGGTCGCAGA	0
OPJ-19	GGACACCACT	0
OPJ-20	AAGCGGCCTC	1
OPK-01	CATTCGAGCC	1
OPK-02	GTCTCCGCAA	0
OPK-03	CCAGCTTAGG	0
OPK-04	CCGCCCAAAC	0
OPK-05	TCTGTCGAGG	0
OPK-06	CACCTTTCCC	1
OPK-07	AGCGAGCAAG	0
OPK-08	GAACACTGGG	0
OPK-09	CCCTACCGAC	1
OPK-10	GTGCAACGTG	1
OPK-11	AATGCCCCAG	1
OPK-12	TGGCCCTCAC	1
OPK-13	GGTTGTACCC	0
OPK-14	CCCGCTACAC	1
OPK-15	CTCCTGCCAA	0
OPK-16	GAGCGTCGAA	0
OPK-17	CCCAGCTGTG	0
OPK-18	CCTAGTCGAG	1
OPK-19	CACAGGCGGA	1
OPK-20	GTGTCGCGAG	0
OPL-01	GGCATGACCT	1
OPL-02	TGGGCGTCAA	0
OPL-03	CCAGCAGCTT	1
OPL-04	GACTGCACAC	0
OPL-05	ACGCAGGCAC	0
OPL-06	GAGGGAAGAG	1

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPL-07	AGGCGGGAAC	1
OPL-08	AGCAGGTGGA	0
OPL-09	TGCGAGAGTC	1
OPL-10	TGGGAGATGG	0
OPL-11	ACGATGAGCC	1
OPL-12	GGGCGGTACT	1
OPL-13	ACCGCCTGCT	1
OPL-14	GTGACAGGCT	1
OPL-15	AAGAGAGGGG	0
OPL-16	AGGTTGCAGG	0
OPL-17	AGCCTGAGCC	1
OPL-18	ACCACCCACC	0
OPL-19	GAGTGGTGAC	0
OPL-20	TGGTGGACCA	0
OPM-01	GTTGGTGGCT	0
OPM-02	ACAACGCCTC	1
OPM-03	GGGGGATGAG	0
OPM-04	GGCGGTTGTC	0
OPM-05	GGGAACGTGT	0
OPM-06	CTGGGCAACT	1
OPM-07	CCGTGACTCA	1
OPM-08	TCTGTTCCCC	0
OPM-09	GTCTTGCGGA	1
OPM-10	TCTGGCGCAC	0
OPM-11	GTCCACTGTG	1
OPM-12	GGGACGTTGG	0
OPM-13	GGTGGTCAAG	0
OPM-14	AGGGTCGTTC	1
OPM-15	GACCTACCAC	0
OPM-16	GTAACCAGCC	1
OPM-17	TCAGTCCGGG	1
OPM-18	CACCATCCGT	0
OPM-19	CCTTCAGGCA	0
OPM-20	AGGTCTTGGG	0
OPN-01	CTCACGTTGG	0
OPN-02	ACCAGGGGCA	1
OPN-03	GGTACTCCCC	1
OPN-04	GACCGACCCA	1
OPN-05	ACTGAACGCC	0
OPN-06	GAGACGCACA	0
OPN-07	CAGCCCAGAG	0
OPN-08	ACCTCAGCTC	0
OPN-09	TGCCGGCTTG	0
OPN-10	ACAACTGGGG	1
OPN-11	TCGCCGCAAA	0
OPN-12	CACAGACACC	1
OPN-13	AGCGTCACTC	1

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPN-14	TCGTGCGGGT	1
OPN-15	CAGCGACTGT	0
OPN-16	AAGCGACCTG	1
OPN-17	CATTGGGGAG	1
OPN-18	GGTGAGGTCA	1
OPN-19	GTCCGTACTG	1
OPN-20	GGTGCTCCGT	1
OPO-01	GGCACGTAAG	1
OPO-02	ACGTAGCGTC	1
OPO-03	CTGTTGCTAC	1
OPO-04	AAGTCCGCTC	1
OPO-05	CCCAGTCACT	1
OPO-06	CCACGGGAAG	1
OPO-07	CAGCACTGAC	1
OPO-08	CCTCCAGTGT	0
OPO-09	TCCCACGCAA	0
OPO-10	TCAGAGCGCC	1
OPO-11	GACAGGAGGT	0
OPO-12	CAGTGCTGTG	0
OPO-13	GTCAGAGTCC	1
OPO-14	AGCATGGCTC	1
OPO-15	TGGCGTCCTT	1
OPO-16	TCGGCGGTTC	1
OPO-17	GGCTTATGCC	1
OPO-18	CTCGCTATCC	1
OPO-19	GGTGCACGTT	1
OPO-20	ACACACGCTG	1
OPP-01	GTAGCACTCC	0
OPP-02	TCGGCACGCA	0
OPP-03	CTGATACGCC	1
OPP-04	GTGTCTCAGG	1
OPP-05	CCCCGGTAAC	1
OPP-06	GTGGGCTGAC	1
OPP-07	GTCCATGCCA	0
OPP-08	ACATCGCCCA	0
OPP-09	GTGGTCCGCA	1
OPP-10	TCCCGCCTAC	1
OPP-11	AACGCGTCGG	0
OPP-12	AAGGGCGAGT	0
OPP-13	GGAGTGCCTC	0
OPP-14	CCAGCCGAAC	1
OPP-15	GGAAGCCAAC	1
OPP-16	CCAAGCTGCC	1
OPP-17	TGACCCGCCT	1
OPP-18	GGCTTGGCCT	1
OPP-19	GGGAAGGACA	0
OPP-20	GACCCTAGTC	1

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPQ-01	GGGACGATGG	0
OPQ-02	TCTGTCGGTC	0
OPQ-03	GGTCACCTCA	0
OPQ-04	AGTGCGCTGA	1
OPQ-05	CCGCGTCTTG	1
OPQ-06	GAGCGCCTTG	1
OPQ-07	CCCCGATGGT	1
OPQ-08	CTCCAGCGGA	1
OPQ-09	GGCTAACCGA	0
OPQ-10	TGTGCCCGAA	1
OPQ-11	TCTCCGCAAC	0
OPQ-12	AGTAGGGCAC	0
OPQ-13	GGAGTGGACA	0
OPQ-14	GGACGCTTCA	1
OPQ-15	GGGTAACGTG	1
OPQ-16	AGTGCAGCCA	1
OPQ-17	GAAGCCCTTG	1
OPQ-18	AGGCTGGGTG	1
OPQ-19	CCCCCTATCA	0
OPQ-20	TCGCCCAGTC	1
OPR-01	TGCGGGTCCT	1
OPR-02	CACAGCTGCC	1
OPR-03	ACACAGAGGG	0
OPR-04	CCCGTAGCAC	0
OPR-05	GACCTAGTGG	0
OPR-06	GTCTACGGCA	1
OPR-07	ACTGGCCTGA	1
OPR-08	CCCGTTGCCT	0
OPR-09	TGAGCACGAG	1
OPR-10	CCATTCCCCA	1
OPR-11	GTAGCCGTCT	1
OPR-12	ACAGGTGCGT	1
OPR-13	GGACGACAAG	0
OPR-14	CAGGATTCCC	1
OPR-15	GGACAACGAG	1
OPR-16	CTCTGCGCGT	1
OPR-17	CCGTACGTAG	1
OPR-18	GGCTTTGCCA	1
OPR-19	CCTCCTCATC	1
OPR-20	ACGGCAAGGA	1
OPS-01	CTACTGCGCT	1
OPS-02	CCTCTGACTG	1
OPS-03	CAGAGGTCCC	1
OPS-04	CACCCCCTTG	0
OPS-05	TTTGGGGCCT	0
OPS-06	GATACCTCGG	1
OPS-07	TCCGATGCTG	1

Table 21. Continued

Primer	Sequence	Presence of polymorphic bands present(1) and absent(0)
OPS-08	TTCAGGGTGG	1
OPS-09	TCCTGGTCCC	1
OPS-10	ACCGTTCCAG	1
OPS-11	AGTCGGGTGG	0
OPS-12	CTGGGTGAGT	1
OPS-13	GTCGTTCCTG	1
OPS-14	AAAGGGGTCC	0
OPS-15	CAGTTCACGG	1
OPS-16	AGGGGGTTCC	1
OPS-17	TGGGGACCAC	0
OPS-18	CTGGCGAACT	1
OPS-19	GAGTCAGCAG	1
OPS-20	TCTGGACGGA	1
OPT-01	GGGCCACTCA	1
OPT-02	GGAGAGACTC	1
OPT-03	TCCACTCCTG	1
OPT-04	CACAGAGGGA	0
OPT-05	GGGTTTGGCA	0
OPT-06	CAAGGGCAGA	0
OPT-07	GGCAGGCTGT	1
OPT-08	AACGGCGACA	0
OPT-09	CACCCCTGAG	1
OPT-10	CCTTCGGAAG	0
OPT-11	TTCCCCGCGA	1
OPT-12	GGGTGTGTAG	0
OPT-13	AGGACTGCCA	1
OPT-14	AATGCCGCAG	1
OPT-15	GGATGCCACT	0
OPT-16	GGTGAACGCT	1
OPT-17	CCAACGTCGT	1
OPT-18	GATGCCAGAC	1
OPT-19	GTCCGTATGG	1
OPT-20	GACCAATGCC	1

Table 22. Existence of species specific DNA fragments

Primer	*Pogonia japonica* from Mt. Hiba	*Pogonia japonic*a cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
OPA-01	0	0	0	0	0	0	0
OPA-02	0	0	0	0	0	0	0
OPA-04	1	1	1	1	1	1	0
OPA-06	0	0	0	0	0	0	0
OPA-07	0	0	0	0	0	0	0
OPA-09	0	0	0	0	0	0	0
OPA-10	0	0	0	0	0	0	0
OPA-11	1	1	1	1	1	1	0
OPA-12	0	0	0	0	0	0	0
OPA-14	1	1	1	1	1	1	0
OPA-15	1	0	1	1	0	0	1
OPA-16	0	0	0	0	0	0	0
OPA-18	0	0	0	0	0	0	0
OPA-19	0	0	0	0	0	0	1
OPA-20	0	0	0	0	0	1	1
OPB-01	0	0	0	0	0	0	0
OPB-02	0	0	0	0	0	0	0
OPB-03	0	0	0	0	0	0	0
OPB-04	0	0	1	0	0	0	1
OPB-06	0	0	0	0	0	0	0
OPB-07	0	0	0	0	0	0	1
OPB-08	0	0	0	0	0	0	0
OPB-09	0	0	0	0	1	1	1
OPB-10	0	0	0	0	0	0	1
OPB-11	0	0	0	0	0	0	1
OPB-12	0	0	0	0	0	0	0
OPB-13	0	0	0	0	1	1	1
OPB-15	1	1	1	1	1	1	0
OPB-16	0	0	0	0	0	0	1
OPB-17	0	0	1	0	1	1	1
OPB-18	0	0	0	0	0	0	1
OPB-19	0	0	0	0	0	0	1
OPB-20	0	0	0	0	1	1	1
OPC-05	1	1	1	1	1	1	0
OPC-06	1	1	0	0	1	1	0
OPC-07	0	0	0	0	0	0	1
OPC-09	1	1	1	1	1	1	0
OPC-11	0	0	0	0	0	0	1
OPC-12	0	0	0	0	0	0	0

Table 22. Continued

Primer	*Pogonia japonica* from Mt. Hiba	*Pogonia japonic*a cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
OPC-15	0	0	0	0	0	0	1
OPC-16	0	0	1	0	1	1	1
OPC-18	0	0	0	0	0	0	1
OPC-19	0	0	0	0	0	0	0
OPD-04	0	0	0	1	1	1	0
OPD-05	0	0	0	0	0	1	1
OPD-06	0	0	0	0	0	0	0
OPD-07	0	0	0	0	0	0	0
OPD-08	0	0	0	0	0	0	1
OPD-09	1	1	1	1	1	1	0
OPD-10	1	1	1	0	1	1	0
OPD-13	0	0	0	0	0	0	0
OPD-14	0	0	0	0	0	0	1
OPD-16	0	0	0	0	0	0	1
OPD-17	1	1	1	1	1	1	0
OPE-02	0	0	0	0	1	1	0
OPE-07	1	1	0	0	1	1	1
OPE-12	0	0	0	0	0	0	0
OPE-13	0	0	0	0	0	0	0
OPE-14	0	0	0	0	0	1	0
OPE-17	0	0	0	0	0	0	0
OPE-19	0	0	0	0	0	0	0
OPF-01	0	0	0	0	0	0	0
OPF-02	0	0	0	0	0	0	0
OPF-03	1	1	1	1	1	1	0
OPF-04	0	1	1	1	1	1	0
OPF-05	1	1	1	1	1	1	0
OPF-06	1	1	1	1	0	1	1
OPF-07	0	0	0	0	0	0	0
OPF-08	0	0	0	0	0	0	0
OPF-10	1	1	1	1	1	1	0
OPF-12	0	0	0	0	0	0	0
OPF-13	1	1	1	1	1	1	0
OPF-14	0	0	0	0	0	0	1
OPF-15	0	0	0	0	0	0	0
OPF-16	1	1	1	1	1	1	0
OPF-17	0	0	0	0	0	0	1
OPF-18	0	1	1	0	0	0	1
OPF-19	0	0	0	0	0	0	1
OPF-20	0	0	0	0	0	0	1
OPG-02	0	0	0	1	0	0	0
OPG-04	1	1	1	1	1	1	0

Table 22. Continued

Primer	*Pogonia japonica* from Mt. Hiba	*Pogonia japonica* cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
OPG-05	0	0	0	0	0	0	0
OPG-07	0	0	0	0	0	0	1
OPG-08	0	0	0	0	0	0	0
OPG-09	0	0	0	0	0	0	0
OPG-10	0	0	0	0	0	0	0
OPG-11	1	1	1	1	0	0	0
OPG-14	1	1	1	1	1	1	0
OPG-15	0	0	0	0	0	0	1
OPG-18	0	0	0	0	0	0	0
OPH-01	1	1	1	1	1	1	0
OPH-02	0	0	0	0	0	0	1
OPH-03	1	1	1	1	0	0	0
OPH-05	1	1	1	1	1	1	0
OPH-11	1	1	1	1	1	1	0
OPH-12	0	0	0	0	0	0	1
OPH-14	0	0	0	0	0	0	0
OPH-16	1	1	1	1	1	1	0
OPH-19	0	0	0	0	0	0	0
OPI-01	0	0	0	0	0	0	0
OPI-08	1	1	1	1	1	1	0
OPI-11	0	0	0	0	0	0	0
OPI-14	0	0	0	0	0	0	0
OPI-15	1	1	1	1	1	1	0
OPI-16	1	1	1	1	0	0	1
OPI-20	0	0	0	0	0	0	0
OPJ-03	0	0	0	0	0	0	0
OPJ-06	0	0	0	0	0	0	0
OPJ-07	0	0	0	0	0	0	0
OPJ-10	0	0	0	0	0	0	0
OPJ-13	0	0	0	0	0	0	0
OPJ-14	1	1	1	1	1	1	0
OPJ-15	0	0	0	0	0	0	0
OPJ-17	1	1	1	1	1	1	0
OPJ-20	0	0	0	0	0	0	0
OPK-01	0	1	1	1	1	1	1
OPK-06	0	0	0	0	0	0	0
OPK-09	0	0	0	0	0	0	0
OPK-10	1	1	1	1	1	1	0
OPK-11	1	1	1	1	1	1	0
OPK-12	0	0	0	0	0	0	0
OPK-14	0	0	0	0	0	0	0
OPK-18	0	0	0	0	0	0	0
OPK-19	0	0	0	0	0	0	0

Table 22. Continued

Primer	*Pogonia japonica* from Mt. Hiba	*Pogonia japonic*a cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
OPL-01	1	1	1	1	1	1	0
OPL-03	1	1	1	1	1	1	0
OPL-06	0	0	0	0	0	0	0
OPL-07	1	1	0	0	0	0	0
OPL-09	1	1	1	1	1	1	0
OPL-11	1	1	1	1	1	1	0
OPL-12	0	0	0	0	0	0	0
OPL-13	0	0	0	0	0	0	0
OPL-14	1	1	1	1	1	1	0
OPL-17	0	0	0	0	0	0	1
OPM-02	0	0	0	0	0	0	0
OPM-06	0	0	0	0	0	0	0
OPM-07	0	0	0	0	0	0	0
OPM-09	0	0	1	0	1	1	1
OPM-11	0	0	0	0	0	0	0
OPM-14	1	1	0	0	0	1	1
OPM-16	0	0	0	0	1	1	1
OPM-17	0	0	0	0	0	0	1
OPN-02	0	0	0	0	0	1	1
OPN-03	0	0	0	0	1	1	0
OPN-04	1	0	1	0	0	1	0
OPN-10	1	1	1	1	1	1	0
OPN-12	0	0	0	0	0	0	0
OPN-13	0	0	0	0	0	0	1
OPN-14	0	0	0	0	0	0	1
OPN-16	1	1	1	1	1	1	0
OPN-17	0	0	0	0	0	0	0
OPN-18	0	1	0	0	1	1	0
OPN-19	0	0	0	0	0	0	1
OPN-20	1	0	1	0	0	1	1
OPO-01	0	0	0	0	0	1	0
OPO-02	1	1	0	0	0	0	0
OPO-03	0	0	0	0	0	0	0
OPO-04	1	1	1	1	0	0	0
OPO-05	1	1	1	0	0	0	0
OPO-06	1	1	0	0	1	1	1
OPO-07	0	0	0	0	0	0	0
OPO-10	0	0	0	0	0	0	0
OPO-13	0	0	0	0	0	0	0
OPO-14	0	0	0	0	0	0	0
OPO-15	0	0	0	0	0	0	0
OPO-16	1	1	1	1	1	1	0

Table 22. Continued

Primer	*Pogonia japonica* from Mt. Hiba	*Pogonia japonica* cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
OPO-17	1	1	1	1	1	1	0
OPO-18	0	0	0	0	0	0	0
OPO-19	0	0	0	0	0	0	0
OPO-20	0	0	1	0	0	1	1
OPP-03	0	0	0	0	0	0	0
OPP-04	0	0	0	0	0	0	0
OPP-05	0	0	0	0	0	0	1
OPP-06	0	1	0	0	0	1	1
OPP-09	0	0	0	0	0	0	1
OPP-10	0	0	0	0	0	0	1
OPP-14	0	0	0	0	0	0	0
OPP-15	1	1	1	1	1	1	0
OPP-16	1	0	0	0	0	0	1
OPP-17	0	0	0	0	0	1	0
OPP-18	0	0	0	0	0	0	0
OPP-20	0	0	0	0	0	0	0
OPQ-04	0	0	0	0	0	0	0
OPQ-05	0	0	0	0	0	0	1
OPQ-06	0	0	0	0	0	1	1
OPQ-07	0	0	0	0	0	1	1
OPQ-08	0	0	0	0	1	1	1
OPQ-10	1	1	1	1	1	1	0
OPQ-14	0	0	0	0	0	0	1
OPQ-15	1	1	1	1	1	1	0
OPQ-16	1	1	1	1	1	1	0
OPQ-17	0	0	1	1	1	1	1
OPQ-18	1	1	1	1	1	1	0
OPQ-20	1	1	1	1	1	1	0
OPR-01	0	0	0	0	0	0	1
OPR-02	1	1	1	1	1	1	0
OPR-06	0	0	0	0	0	0	0
OPR-07	1	1	1	1	1	1	0
OPR-09	1	1	1	1	1	1	0
OPR-10	0	0	0	0	0	0	0
OPR-11	1	1	1	1	1	1	0
OPR-12	0	0	0	0	0	0	1
OPR-14	0	0	0	0	0	0	0
OPR-15	1	1	1	1	1	1	0
OPR-16	1	1	1	1	1	1	0
OPR-17	1	1	1	1	1	1	0
OPR-18	1	1	1	1	1	1	0
OPR-19	1	1	1	1	1	1	0
OPR-20	0	0	0	0	0	1	1

Table 22. Continued

Primer	*Pogonia japonica* from Mt. Hiba	*Pogonia japonic*a cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
OPS-01	0	0	1	0	0	0	0
OPS-02	1	1	1	1	1	1	0
OPS-03	0	0	0	0	0	1	1
OPS-06	0	0	0	0	1	1	1
OPS-07	1	1	1	1	1	1	0
OPS-08	0	0	0	1	1	1	0
OPS-09	0	0	0	0	0	0	0
OPS-10	1	1	1	1	0	0	1
OPS-12	0	0	0	0	0	0	1
OPS-13	0	0	0	0	0	0	1
OPS-15	0	0	0	0	0	0	0
OPS-16	0	1	1	1	1	1	1
OPS-18	0	0	0	0	0	0	0
OPS-19	0	0	0	0	0	0	1
OPS-20	0	0	0	0	0	0	1
OPT-01	1	1	1	1	1	1	0
OPT-02	0	0	1	0	0	0	1
OPT-03	0	0	0	0	0	0	1
OPT-07	0	0	0	0	0	0	1
OPT-09	1	1	1	1	1	1	0
OPT-11	0	0	0	0	0	0	1
OPT-13	1	1	1	1	1	1	0
OPT-14	1	1	1	1	1	1	0
OPT-16	0	0	0	0	0	0	0
OPT-17	0	0	0	0	0	0	1
OPT-18	1	1	0	0	1	1	1
OPT-19	0	0	0	0	0	0	0
OPT-20	1	1	1	1	1	1	0

Table 23. Number of markers shown different bands in interspecific species

	Pogonia japonica from Mt. Hiba	*Pogonia japonica* cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivate states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
Pogonia japonica from Mt. Hiba	0	11	20	25	44	79	110
Pogonia japonica cultivated states	-	0	19	28	47	72	113
Pogonia minor from Mt. Hiba	-	-	0	21	48	83	106
Pogonia minor cultivate states	-	-	-	0	35	80	115
Pogonia from Mt. Hakusan called 'Miyamatokisou'	-	-	-	-	0	73	116
Pogonia from Higashi-Hiroshima City	-	-	-	-	-	0	105
Pogonia ophioglossoides	-	-	-	-	-	-	0

Table 24. RAPD similarity matrix among seven *Pogonia*

	Pogonia japonica from Mt. Hiba	*Pogonia japonica* cultivated states	*Pogonia minor* from Mt. Hiba	*Pogonia minor* cultivated states	*Pogonia* from Mt. Hakusan called 'Miyamatokisou'	*Pogonia* from Higashi-Hiroshima City	*Pogonia ophioglossoides*
Pogonia japonica from Mt. Hiba	0.00	0.04	0.09	0.08	0.15	0.18	0.80
Pogonia japonica cultivated states		0.00	0.09	0.07	0.12	0.16	0.80
Pogonia minor from Mt. Hiba			0.00	0.07	0.14	0.17	0.75
Pogonia minor cultivated states				0.00	0.11	0.18	0.81
Pogonia called from Mt. Hakusan called 'Miyamatokisou'					0.00	0.07	0.74
Pogonia from Higashi-Hiroshima City						0.00	0.68
Pogonia ophioglossoides							0.00

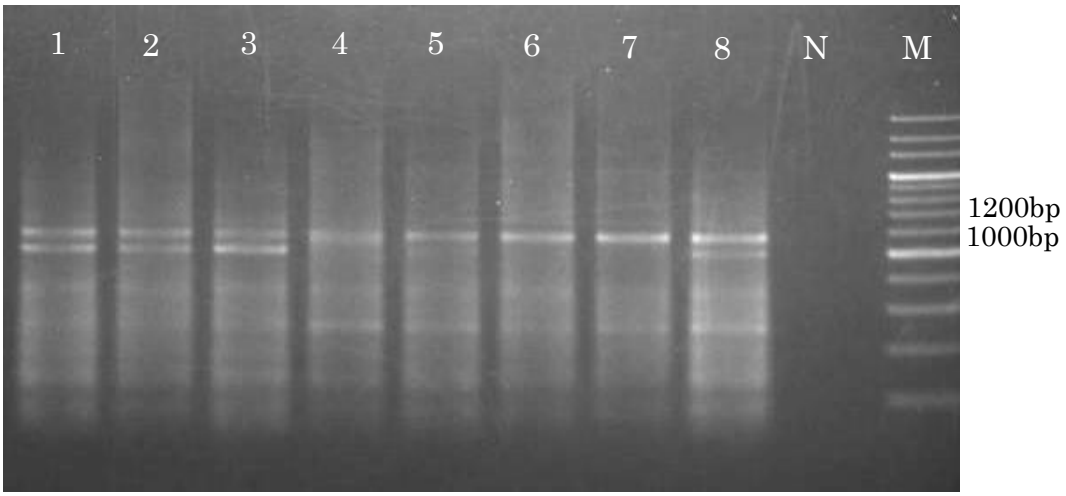

Fig. 40. RAPD production from total DNAs after using OPO-02. 1: *P. japonica* from Mt. Hiba1, 2: *P. japonica* from Mt. Hiba2. 3: *P. japonica* cultivated status. 4: *P. minor* from Mt. Hiba. 5: *P. minor* cultivated status. 6: *Pogonia* from Mt. Hakusan called 'Miyamatokisou'. 7: *Pogonia* from Higashi-Hiroshima City. 8: *P. minor* × *P. japonica*. N: Negative control. M: 1Kb plus DNA ladder was used for molecular maker. (Takahashi *et al*. 2014.)

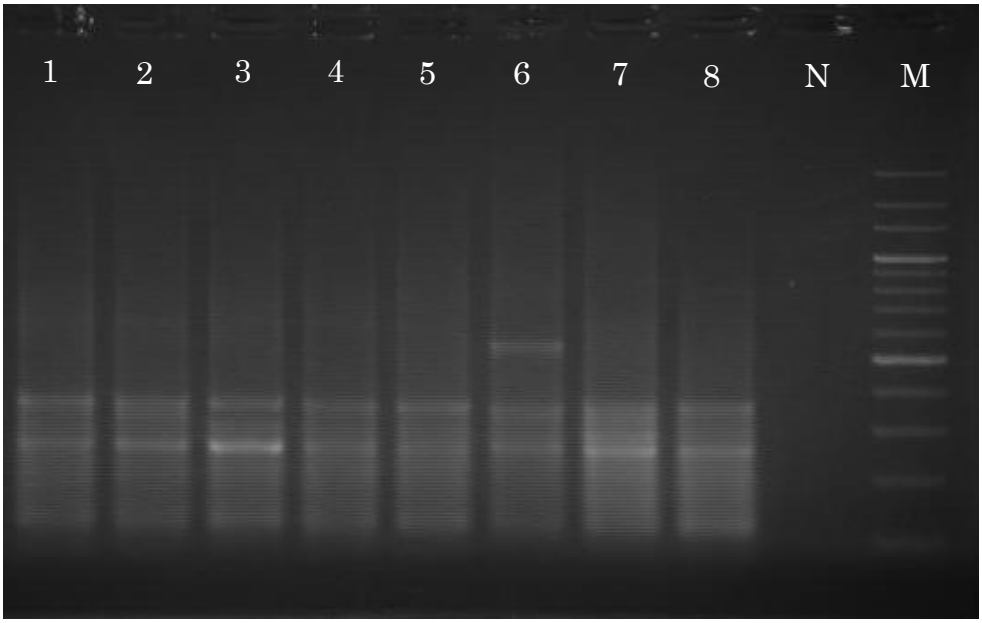

Fig. 41. RAPD production from total DNAs after using OPE-02. 1: *P. japonica* from Mt. Hiba1, 2: *P. japonica* from Mt. Hiba2. 3: *P. japonica* cultivated status. 4: *P. minor* from Mt. Hiba. 5: *P. minor* cultivated status. 6: *Pogonia* from Mt. Hakusan called 'Miyamatokisou'. 7: *Pogonia* from Higashi-Hiroshima City. 8: *P. minor* × *P. japonica*. N: Negative control. M: 1Kb plus DNA ladder was used for molecular maker. (Takahashi *et al*. 2014.)

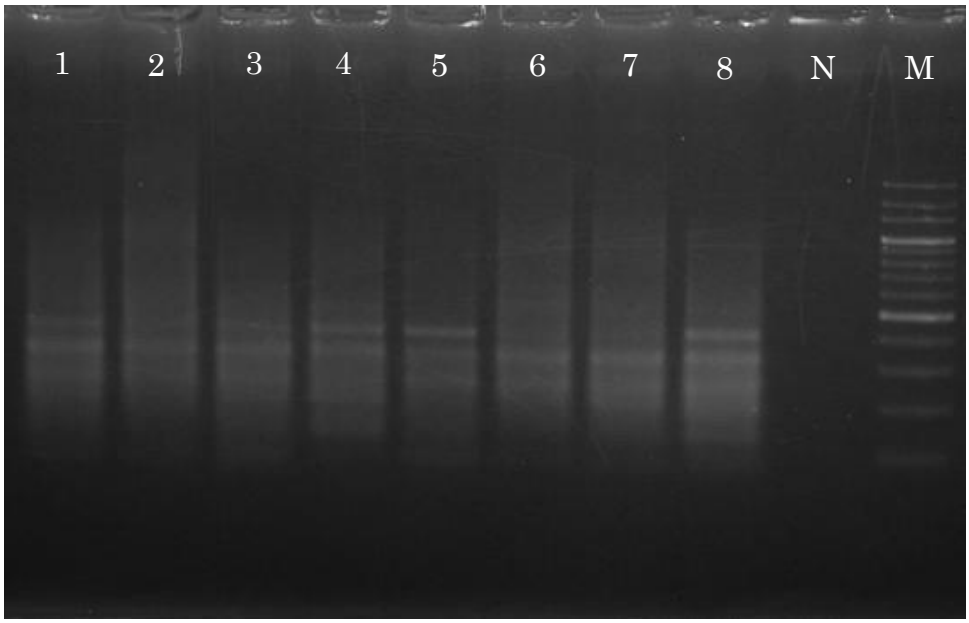

Fig. 42. RAPD production from total DNAs after using OPO-04. 1: *P. japonica* from Mt. Hiba1, 2: *P. japonica* from Mt. Hiba2. 3: *P. japonica* cultivated status. 4: *P. minor* from Mt. Hiba. 5: *P. minor* cultivated status. 6: *Pogonia* from Mt. Hakusan called 'Miyamatokisou'. 7: *Pogonia* from Higashi-Hiroshima City. 8: *P. minor* × *P. japonica*. N: Negative control. M: 1Kb plus DNA ladder was used for molecular maker. (Takahashi *et al*. 2014.)

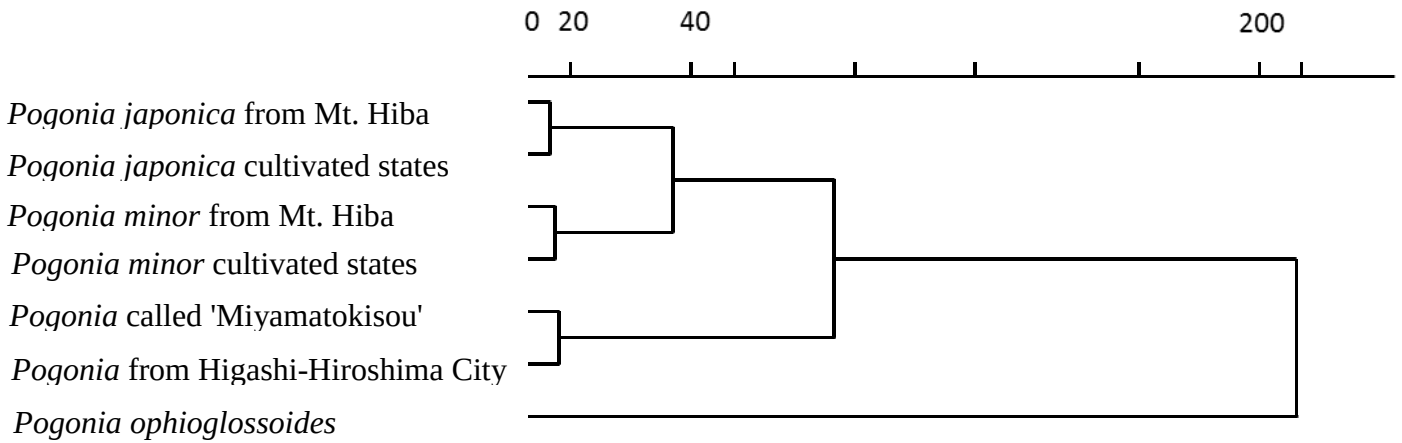

Fig. 43. Dendrogram of genetic relationships among seven *Pogonia* species using 400 primers. Scale indicates the genetic distance derived from RAPD markers data. Cluster tree was determined with the Ward method. (Takahashi *et al*. 2014.)

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Abstract

Among the four species existed in *Pogonia*, there are distributed in East Asia and one species is distributed in the southeastern part of North America. In this study, *P. japonica* and *P. minor* distributed in East Asia and *P. ophioglossoides* distributed in eastern North America were used as plant materials.

Since *Pogonia japonica* and *P. minor* distributed in Japan are threatened or locally extinct due to degradation and decrease of their habitats by land reclamation and overharvest, they are very necessary to be conserved by *in vitro* propagation. Effects of nutrition in culture media for plant growth of *P. japonica* were investigated. Effects of symbiotic fungus for plant growth from rhizome tips of *P. japonica* and *P. ophioglossoides* were tested. Additionally, the establishment for micropropagation of *P. japonica* were attempted.

Among the orchidaceous plants *Pogonia* has the largest chromosomes which are the distinct characteristics of the most primitiveness of the family. The karyotypes of *P. japonica. P. minor, P. ophioglossoides*, reciprocal F_1 hybrids and F_2 hybrids among the three species were analyzed and compared.

Two new types of the forms of *Pogonia* different from *P. japonica* and *P. minor* were found in the wild status in Japanese Archipelago. To clarify their identification, their information were obtained by kartyotype analysis and RAPD molecular analysis.

1 Seed germination of *Pogonia* (*in vitro*)

Effects of seed aging on axenic germination of *P japonica* and *P. minor* were investigated. Immature seeds of *Pogonia japonica* at the 45-day-stage showed the best seed-germination rate of 76.9% cultured on modified Knudson C (mKC) medium.

Germination rates of the seeds at and after the 60-day-old stages in the non-dehisced capsules of *P. japonica* on mKC medium were between 40-60% and decreased in compared with those at 45-day-stage. Germination rate of the immature seeds at the 45-day-old stage in non-dehisced capsules of *P. japonica* was 57.1% on Hyponex medium, while that of the immature seeds at and after the 60-day-old stages was very little on the medium. Thus, axenic seed germination of *P. japonica* was seemed to be correlated with seed maturation. In contrast, the highest germination rate of seeds in non-dehisced capsule of *P. minor* at the 75-day-stage was 1.4% on mKC medium. However, immature seeds in non-dehisced capsules of *P. minor* did not germinate at all on Hyponex medium. Thus, the patterns of axenic seed germination of *P. japonica* and *P. minor* were different massively from each other.

The surface-sterilized seeds of *P. japonica* as well as *P. minor* from the matured, dehisced capsules obtained showed high germination rates on mKC medium in spite of more aged seeds and their germination rates were 83.3% and 10.2%, respectively.

When immature seeds of *P. minor* were sowed and cultured on mKC medium under 4℃ for a month and then shifted to 24℃, their germination rate was 8.5% and got slightly high.

In symbiotic germination with 12 orchid fungi of *P. japonica* using surface-sterilized seeds, high germination rates of more than 90% were shown on all symbiotic oat-powdered agar (OPA) media. When surface-sterilized seeds of *P. japonica* were sowed on symbiotic OPA media with fungi isolated and obtained from *Spiranthes sinensis* and *Cymbidium goeringii*, their germination and the plant growth were especially well. The aspect of symbiotic germination and plant growth on OPA media with fungi was better than that of axenic germination and plant growth on mKC medium.

2 Plant growth

Rhizome tips of juvenile plants of *P. japonica* and *P. ophioglossoides* grown separately on B5, Vacin and Went (VW), modified Hyponex, mKC, Murashige and Skoog (MS), 1/2MS, 1/4MS solid media without any growth regulators produced one adventitious bud per rhizome tip on all media used within seven days. Plant growth from rhizome-tips of the two species on MS and VW media was well, although that of their species on B5 and MS media diluted was poor. However, growth of leaves and rhizomes in plantlets seemed to be affected by the nitrogen source in medium nutrient: growth of leaves was inhibited and occurred chlorosis in the leaves if grown on the MS Gelrite medium modified by containing only NO_3-N (only KNO_3 was added to MS medium as inorganic nitrogen nutrient) as compared to the medium containing NH_4-N [NH_4NO_3 or $(NH_4)_2SO_2$] were added as inorganic nitrogen nutrient to MS medium).

Rhizome tips of *P. japonica* and *P. ophioglossoides* were cultured on OPA media with symbiotic fungus. When rhizome tips of both species were cultured on symbiotic OPA media with fungus isolated and obtained from *Spiranthes sinensis* and *Cymbidium goeringii*, the plant growth were especially well.

3 Micropropagation of *Pogonia japonica*

Rhizome-derived protocorm-like bodies (RPLBs) were induced in either MS or B5 liquid media supplemented with BA≧0.02mg/l and NAA≦0.2 mg/l by shaking at 2 rpm on a rotary culture equipment with continuous illumination, whereas abnormal shoot-tip aggregations (ASTAs) were induced only in MS liquid media. The RPLBs proliferated and produced numerous rhizome tips in the same medium and environment and thus, may

be an excellent culture line for micropropagation of *P. japonica*. They generated multiple shoots which grew up to form plantlets and juvenile plants suitable for weaning. Their juvenile plants were acclimatized and were cultivated outside, then were flowered at two to three years after acclimatization.

4 Cytogenetic analysis of *Pogonia*

The karyotype of *P. japonica* was 2n=20=16 median-centromeric chromosomes + 4 terminal-centromeric chromosomes; while that of *P. minor* was 2n=18=18 median-centromeric chromosome. In this study, all karyotyspes of *P.ophioglossoides* included two to three small chromosomes, around 3 μm each, and were treated as B-chromosomes. *Pogonia ophioglossoides* was 2n=18+2-3B=18 median-centromeric chromosomes+2-3B-chomosomes. A distinctive feature of *P. minor* was to have four median-centromeric chromosomes with the arm ratio of 1.50-1.70 and the relative lengths of 5.50-6.00. Three *Pogonia* species studied had commonly a pair of median-centromeric chromosomes with secondary constrictions at the centromeric position.

Seventy-five to 90-days-stage seeds of the reciprocal cross F_1 hybrids among three species of *Pogonia* were harvested and were sowed on mKC medium. Embryo formation rates and germination rates of the all reciprocal F_1 hybrids were ca 90% and more than 80%, respectively.

The chromosome numbers of the F_1 hybrids between *P. japonica* and *P. minor* showed constantly 2n=19, intermediate chromosome number between those of the parents. The karyotypes of the F_1 hybrids were exactly karyomorpholgically intermediate between those of the parents commonly consisted of 17 median-centromeric chromosomes including two median-centromeric chromosomes with arm ratios of 1.50-

1.70 and relative lengths of 5.50-6.00 which must be originated from the gamete of *P. minor* and two terminal-centromeric chromosomes which must be originated from the gamete of *P. japonica* .

Karyotypes of the artificial hybrids between the maternal *P. japonica* and the paternal *P. ophioglossoides* were commonly 2n=19+1B, whereas those between the maternal *P. ophioglossoides* and the paternal *P. japonica* were commonly observed as 2n=19+2B. Thus, the karyotypes of F_1 hybrids were intermediate between those of the parents. They commonly consisted of two terminal-centromeric chromosomes and 17 median-centromeric chromosomes and the two terminal-centromeric chromosomes could be originated from the gamete of *P. japonica*.

Karyotypes of the artificial hybrids between the maternal *P. minor* and the paternal *P. ophioglossoides* were commonly 2n=18+2B, whereas those between the maternal *P. ophioglossoides* and the paternal *P. minor* were commonly 2n=18+1B. However, the karyotypes of the both reciprocal cross hybrids did not show any morphologically intermediate form between those of the parents. All karyotypes observed in the reciprocal cross hybrids obtained had a submedian-centromeric chromosome with arm ratio more than 2.00 which had not been visualized in those observed in the gamete from the both parents. Also the number of median-centromeric chromosomes with arm ratios of 1.50-1.70 and relative lengths of 5.50-6.00 were different accordingly an individual and were zero to two.

All reciprocal F_1 hybrids were acclimatized and were cultivated. They were flowered at two to three years stage after acclimatization. There was no clear differences in flower morphology among all reciprocal F_1 hybrids.

Whole sets of the reciprocal F_1 hybrids flowered were cross-pollinated to make reciprocal cross F_2 hybrids by self-pollination among the F_1 hybrids. Pollens of reciprocal F_1 hybrids between *P. ophioglossoides* and *P. japonica* and between *P. ophioglossoides* and *P. minor* had not been formed perfectly. Therefore, seeds of F_2 hybrids were obtained only extremely from those F_1 hybrids. Those reciprocal F_1 hybrids showed hybrid sterility and it would suggest that *P. ophioglossoides* distributed in eastern North America was genetically distant relationships to *P. japonica* and *P. minor* distributed in East Asia.

Germination rates of the F_2 hybrids by self-pollination of the F_1 hybrids *P. japonica* x *P. minor* and *P. minor* x *P. japonica* were 26.0% and 4.0%, respectively. The F_2 plants produced by self-pollination of the F_1 hybrids of reciprocal crosses between the maternal *P. japonica* and the paternal *P. minor* showed the chromosome numbers of 2n=19 and 20 whereas those between the maternal *P. minor* and the paternal *P. japonica* showed the chromosome numbers of 2n=18, 19 and 20.

The two unusual forms of *Pogonia* whose flower shapes were slightly different from either *P. japonica* or *P. minor* were isolated and collected to detect their origins. One temporally was called ‘Miyamatokisou’ was collected in Mt. Hakusan, Gifu Prefecture and the other one still unnamed was collected in Higashi-Hiroshima City, Hiroshima Prefecture. The karyotype of *Pogonia* called ‘Miyamatokisou’ was 2n=18 and consisted of 18 median-centromeric chromosomes but was a little bit different from that of *P. minor* (2n=18); the karyotype of the ‘Miyamatokisou’ did not have median-centromeric chromosome with arm ratios of 1.50-1.70 and relative lengths of 5.50-6.00 which were a distinctive feature in *P. minor*. The karyotype of the unusual *Pogonia* collected in Higashi-Hiroshima City was 2n=20 and consisted of 16 median-centromeric

chromosomes and four terminal-centromeric chromosomes, and was similar to that of *P. japonica* (2n=20).

5 RAPD molecular characteristics of newly found *Pogonia* different from *P. japonica* and *P. minor*

Origin and relationships of those two new forms were analyzed by RAPD (the randomly amplified polymorphic DNA) technique. DNA band patterns of *P. ophioglossoides* were quite different from other *Pogonia*. A species specific fragment of *P. japonica* used OPO-02 primer in RAPD was not visualized in *Pogonia* from Mt. Hakusan called 'Miyamatokisou' and *Pogonia* from Higashi-Hiroshima City. A DNA fragment using OPO-04 found in *P. japonica*, *P. minor* and F_1 hybrid between the two species was not found in *Pogonia* called 'Miyamatokisou' and taxonomically unknown *Pogonia* from Higashi-Hiroshima City. Therefore, according to the results of RAPD obtained here, *Pogonia* generally called 'Miyamatokisou' and a taxonomically unknown *Pogonia* from Higashi-Hiroshima City had different DNA fragment-patterns from *P. japonica*, *P. minor* and their F_1 hybrids. Their two new form were not hybrids between *P. japonica* and *P. minor* and varieties of their two species. Additionally, DNA fragments generated OPE-02 was species specific for *Pogonia* called 'Miyamatokisou'. It was shown that *Pogonia* called 'Miyamatokisou' was different from *Pogonia* originated in Higashi-Hiroshima City although their flower shapes were similar to each other.

According to the Cluster analysis, genetic relationship between *P. ophioglossoides* and Asian *Pogonia, P. japonica* and *P. minor* was not closely related. Genetic relationship between *P. japonica* and *P. minor* and that between *Pogonia* called 'Miyamatokisou' and the taxonomically unknown *Pogonia* from Higashi-Hiroshima City were quite closely

related. *Pogonia ophioglossoides* was closer to *Pogonia* called ‘Miyamatokisou’ and *Pogonia* from Higashi-Hiroshima City than to *P. japonica* and *P. minor*.

It was confirmed that newly naturally derived *Pogonia* from *P. japonica* and *P. minor* existed in nature according to the RAPD analysis and that their *Pogonia* were relatively close to *P. ophioglossoides* in genetic relationship according to the cluster analysis. They might be an intermediate species to evolve from *P. ophioglossoides* to *P. minor* and *P. japonica*.

摘　要

ラン科 *Pogonia*（トキソウ）属は 4 種からなり、東アジアに 3 種、北東アメリカに 1 種が自生する。本研究では、東アジアに分布する *Pogonia japonica*（トキソウ）及び *Pogonia minor*（ヤマトキソウ）、北アメリカ南東部に分布する *Pogonia ophioglossoides* を材料として実施した。

日本には *P. japonica* 及び *P. minor* が自生しているが、自生地の崩壊や生態系の変化及び乱獲により、両種ともに絶滅が危ぶまれている。そこで、種の保全、増殖、栽培園芸化を目的に *in vitro* 培養を確立するため、両種の *in vitro* での発芽を分析した。また、*P. japonica* を材料に、培地の栄養分が形態形成に及ぼす影響を調査した。さらに、*P. japonica* の大量増殖系の確立を目指した。

Pogonia 属の染色体は、ラン科植物の中では最大で、最も原始的である。*P. japonica*、*P. minor* 及び *P. ophioglossoides* の 3 種の核型分析、さらに *Pogonia* 属の種間雑種の作出を試み、染色体の動向を観察した。また、自然の中に生育する上記３種とは花の形態が異なる *Pogonia* 2 系統を発見し、核型及び RAPD 分析を行った。

１　*Pogonia* 属の *in vitro* での種子発芽

Pogonia japonica 及び *P. minor* において、種子の熟度が無菌発芽に及ぼす影響を調べた。*P. japonica* の受粉後 45 日目の未裂開果実中の未熟種子について、果実を殺菌し、果実中の種子を掻き出して Knudson C 培地に播種した場合に、76.9%と最も良い発芽率を示した。受粉後 60 日目以降の未裂開果実中の未熟種子の Knudson C 培地上での発芽率は 40-60％だった。また、Hyponex 培地を用いた場合、受粉後 45 日目の未裂開果実中の未熟種子を播種した場合に、57.1％の発芽率を示したが、受粉後 60 日目以降の未裂開果実中の未熟種子の発芽はご

くわずかだった。*P. japonica* においては、種子の熟度が増すと、発芽率が下がる傾向が認められた。未裂開果実中の *P. minor* の種子発芽について、Knudson C 培地上での発芽率が最も高かった試験区においては 1.4%、Hyponex 培地上では全く発芽が認められず、*P. japonica* の種子発芽と大きな差が認められた。

完熟裂開果実から得た *P. japonica* と *P. minor* の完熟種子を表面殺菌して Knudson C 培地に蒔いた場合、種子の熟度が高いにも関わらず、*P. japonica* は 83.3%、*P. minor* は 10.2%と高い発芽率を示した。

また、*Pogonia minor* の未裂開果実中の種子を Knudson C 培地に播き、4℃の低温処理を 30 日間行った場合にも、無処理区と比較して、8.5%のいくらか高い発芽率を得ることができた。

Pogonia japonica の共生発芽について、共生菌を置床したすべての処理区において、90%以上の高い発芽率を得ることができた。特に OPA 培地に *Spiranthes sinensis*（ネジバナ）及び *Cymbidium goeringii*（シュンラン）より単離した共生菌を置床して播種した場合に、発芽及びその後の生長が良好で、それらは、Knudson C 培地上のものと比較してより良好であった。

２　植物体の成長

MS 培地上で生長している *P. japonica* 及び *P. ophioglossoides* の地下茎頂を、植物ホルモンを含まない B5、VW、Hyponex、Knudson C、MS、1/2MS 及び 1/4MS 固形培地上に植え付けたところ、7 日以内にすべての培地上で、1 地下茎頂から 1 不定芽が形成された。続いて植え付け 60 日後に植物体の生長の様子を観察したところ、MS 及び VW 培地に植えた個体の生長が最も良く、これに対して B5、1/2MS 及び 1/4MS 培地に植えた個体は生長が緩慢だった。また、B5、Knudson C 及び Hyponex 培地で生長した個体の葉はクロロシスを起こした。培地中の多量無機窒素成分を比較したとき、B5 培地は NO_3-N（硝酸態窒素）の割合が高いとい

う特徴があることから、培地成分の多量無機窒素形態に着目し、*P. japonica* を材料として研究を進めたところ、MS 培地の多量無機窒素を NO_3-N（KNO_3）のみにした場合に、NH_4-N（アンモニア態窒素）〔NH_4NO_3、$(NH_4)_2SO_4$〕を含む培地と比較して、植物体の生長が阻害され、クロロシスを示した。

Pogonia japonica 及び *P. ophioglossoides* の地下茎頂からの植物体の生長に対する共生菌の影響を調べた。両種とも、共生発芽と同様に、OPA 培地に *Spiranthes sinensis*（ネジバナ）及び *Cymbidium goeringii*（シュンラン）より単離した共生菌を置床して地下茎頂を植え付けた場合に、生長が良好だった。

３　*Pogonia japonica* の大量増殖

Pogonia japonica の増殖体を形成させるため、地下茎頂を NAA(ナフタレン酢酸)及び BA(ベンジルアデニン)を含む MS 及び B5 液体培地に植え付け、2rpm、2,000～10,000 lux、24±1℃の条件で回転培養を行った。BA 0.02mg/l 以上、NAA 0.02～0.2mg/l を含む MS 及び B5 液体培地に地下茎頂を植え付けた時、2 種類の増殖体が形成された。一つはプロトコーム様体（Protocorm-like body）（PLB）で、地下茎由来の PLB という意味でライゾーム由来 PLB（Rhizome-origined protocorm-like body）（RPLB）と名付けた。RPLB は MS 及び B5 培地で形成された。もう一つは薄緑の多数の葉芽が集まったような増殖体で、奇形の葉芽の集合体という意味で ASTA（Abnormal shoot tip aggregation）と名付けた。ASTA は MS 培地でのみ形成された。RPLB は同じ培養条件下で培養することにより、よく増殖したが、ASTA はほとんど増殖しなかった。RPLB 及び ASTA を植物ホルモン無添加の MS 固形培地に移植すると、多芽体を形成し、その後それらは幼植物体に生長した。さらにそれらを馴化し、野外で栽培したところ、2～3 年後には花を咲かせた。

４　*Pogonia* 属の細胞遺伝学的分析

P. japonica、*P. minor* 及び *P. ophioglossoides* を核形態学的に比較した。*P. japonica* の核型は 2n=20=16m+4t であった。*P. minor* の核型は 2n=18=18m であった。その特徴は、相対長 5.50～6.00、腕比 1.50～1.70 の染色体を 4 個含んでいることであった。*P. ophioglossoides* の核型は 2n=18+2-3B で、*P. minor* と同様にすべてが中部動原体型染色体であった（18m）。しかし *P. minor* に含まれる相対長 5.50～6.00、腕比 1.50～1.70 の中部動原体型染色体は観察されなかった。さらに今回の研究で観察した *P. ophioglossoides* のすべての核型は、2～3 個の約 3μm の小さな染色体を含んでおり、B 染色体として取り扱った。3 種全ての核型で、1 対の二次狭窄を持つ中部動原体染色体が観察された。

Pogonia 3 種間で正逆人工交雑を行い、形成された受粉後 75-90 日目の種子を Knudson C 培地上に無菌的に播種した。すべての F_1 雑種の種子の胚形成率は 90%前後、それらの発芽率は 80%だった。

Pogonia japonica と *P. minor* 間 F_1 雑種の染色体数は 2n=19 で、明らかに *P. japonica* と *P. minor* の中間の染色体数であった。核型について、F_1 雑種は *P. japonica* 由来の 2 個の端部動原体染色体及び *P. minor* 由来の相対長 5.50～6.00、腕比 1.50～1.70 の中部動原体型染色体 2 個を含む 17 個の中部動原体染色体を持ち、両親の中間型を示した。

Pogonia japonica と *P. ophioglossoides* 間の F_1 雑種の染色体数について、*P. japonica* × *P. ophioglossoides* は 2n=19+1B、*P. ophioglossoides* × *P. japonica* は 2n=19+2B で、常染色体については 17 個の中部動原体型染色体及び 2 個の端部動原体型染色体から成り、両親の中間型を示した。

Pogonia minor × *P. ophioglossoides* の F_1 雑種の染色体数については 2n=18+2B、*P. ophioglossoides* ×P. *minor* については 2n=18+1B であった。核型は、両親の中間型を示さなかった。観察したすべての核型は、両親に含まれな

い腕比 2.00 以上の次中部動原体型染色体を 1 個必ず含んでいた。また、*P. minor* の特徴である相対長 5.50～6.00、腕比 1.50～1.70 の中部動原体染色体の個数は、個体により異なり、その数は 0～2 個であった。

屋外で 2～3 年間栽培したところ、すべての F_1 雑種において花が咲いた。F_1 雑種間で、花の形態に大きな違いはなかった。

Pogonia japonica と *P. minor* の正逆 F_1 雑種からは多くの種子を得ることができた。しかし *P. ophioglossoides* を片親とすると、花粉の形成が悪く、*P. japonica* × *P. ophioglossoides* の F_1 雑種の自家受粉により、F_2 種子をわずかに得ることができたが、他 3 パターンの F_1 雑種においては、F_2 種子を全く得ることができず、雑種不稔性を示した。北東アメリカに自生する *P. ophioglossoides* は、東アジアに自生する *P. japonica* 及び *P. minor* と遺伝的に遠縁であることが示唆された。

P. minor × *P. japonica* の F_2 種子の発芽率は 1%以下であった。*P. japonica* × *P. minor* の F_2 種子の発芽率はいくぶん高く、26.0%であった。

F_2 雑種の染色体数は、*P. japonica* ×*P. minor* の F_2 雑種で、2n=19 および 20、*P. minor* × *P. japonica* の F_2 雑種では 2n=18、19、20 であった。

一方、野生で自生する *Pogonia* で、花の形態が *P. japonica* や *P. minor* といくらか異なる 2 つの野生タイプの *Pogonia* を入手したため、それらの核型分析を行った。一つは、趣味家が‘ミヤマトキソウ’と呼んでいる *Pogonia* で、白山に自生する個体である。もう一つは、広島県東広島市の湿地で偶然に発見した個体である。‘ミヤマトキソウ’と呼ばれる *Pogonia* は、核型は 2n=18 であるが、*P. minor* の特徴である相対長 5.50～6.00、腕比 1.50～1.70 の染色体は含まれなかった。*P. ophioglossoides* の核型とも、二次狭窄を持つ中部動原体染色体の順番が異なっていた。東広島市産の *Pogonia* は 2n=20 で、染色体数及び核型は *P. japonica* と同様であった。

５　*Pogonia japonica* 及び *P. minor* とは異なる 2 系統の RAPD 分析

Pogonia japonica と *P. minor* の両者の花とも異なる花の形態をもつ 2 タイプの *Pogonia* について、起源や原種との関係を知るため、RAPD 手法を用いて DNA 多型を解析した。400 種類の RAPD プライマーを用いて分析を行った結果、*P. ophioglossoides* のバンドパターンは、他の *Pogonia* と大きく異なるものだった。*P. japonica* の特異的バンドが現れたのは OPO-02 のみで、*P. minor* については特異バンドを示すプライマーは見つからなかった。OPO-02 プライマーを用いた時に得られた *P. japonica* の特異バンドは、‘ミヤマトキソウ’や東広島産の *Pogonia* では見られなかった。また、OPO-04 により、*P. japonica*、*P. minor* に共通に見られたバンドは、‘ミヤマトキソウ’及び東広島産の *Pogonia* では見られなかった。‘ミヤマトキソウ’と東広島市産の *Pogonia* は、*P. japonica* や *P. minor* とは異なる DNA パターンを示した。このことから、‘ミヤマトキソウ’及び東広島市産の *Pogonia* が、*P. japonica* 及び *P. minor* の変種や 2 種間の雑種ではない可能性が示された。また、‘ミヤマトキソウ’と東広島市産の *Pogonia* は、花の形態は類似していたが、OPE-02 において異なる DNA パターンを示した。

クラスター分析により、 *P. ophioglossoides* は *P. japonica* や *P. minor* とは遠縁であり、*P. japonica* と *P. minor* は非常に近縁であることが分かった。また、‘ミヤマトキソウ’と東広島市産の *Pogonia* の間の関係も近縁であった。‘ミヤマトキソウ’と東広島市で発見された *Pogonia* は、*P. japonica* 及び *P. minor* の関係と比較すると、いくらか遠縁であった。*P. ophioglossoides* との関係については、*P. japonica* 及び *P. minor* との関係よりも、‘ミヤマトキソウ’及び東広島市産の *Pogonia* との関係の方が近く、*P. japonica* や *P. minor* より早く分化した系統であることが推測された。

Published papers