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https://doi.org/10.5109/4713

出版情報：九州大学大学院農学研究院紀要. 51 (1), pp.73-81, 2006-02-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **51** (1), 73–81 (2006)

Intrasubgeneric and Interploid Cross Compatibility in Evergreen and Deciduous Azaleas

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(Received October 28, 2005 and accepted November 16, 2005)

Twenty–three diploids, six triploids, nine tetraploids and four mixoploids in evergreen azaleas and eight diploids and five tetraploids in deciduous azaleas were identified by flow cytometric analysis of ploidy in species, interspecific hybrids and cultivars. Sizes of guard cells and pollen grains tended to increase in proportion to the increase in the ploidy. Intrasubgeneric and intra– or interploid hybridizations were conducted with evergreen and deciduous azaleas. Tetraploids of evergreen azaleas had relatively high cross-ability as seed and/or pollen parents, and triploid evergreen azaleas had fertility as seed parents only. Deciduous tetraploids had high pollen fertility and, therefore, capsules set relatively frequently in the interploid crosses of 2x × 4x. No capsules set in the reciprocal crosses.

INTRODUCTION

The fundamental chromosome number in *Rhododendron* is 13 (Sax, 1930). The species of the lepidote group of the genus are highly polyploid, whereas those of the elepidote group such as subgenera of Tsutsusi and Pentanthera, which are commonly called as evergreen and deciduous azaleas respectively, are mostly diploid (Janaki Ammal, 1950). All reported species of evergreen azaleas are diploid (2n=2x=26). There are a few cultivars of triploid and tetraploid in Satsuki Hybrid (Hosoda *et al.*, 1953), and triploid, tetraploid and mixoploid in Belgian Hybrid (Pryor and Frazier, 1970; Heursel and De Roo, 1981; De Schepper *et al.*, 2001a). Deciduous azalea species contains two tetraploid species; they are *R. calendulaceum* and *R. canadense*, and the both are native of the eastern North America (Sax, 1930). Kehr (1987) pointed out that some cultivars developed from subgenus Pentanthera might contain some polyploid cultivars, but the fact has not been shown.

Hybridization within or between species has certainly played an important role in ornamental plant breeding (Horn, 2003; Van Tuyl and Lim, 2003). Intra– or interspecific cross compatibilities among evergreen and deciduous azaleas were investigated precisely (Noguchi, 1932; Akabane *et al.*, 1971; Yamaguchi and Hirata, 1986; Yamaguchi *et al.*, 1985). While the crosses between distantly related species belonging to different subgenera in *Rhododendron* were difficult or impossible, high cross compatibilities between closely related species within the same section were clarified with some exceptions. Thus, intra– or interspecific cross compatibilities in azaleas have been the objectives of studies for long time, but little is known about intra– or interspecific cross compatibilities with respect to ploidy levels. The deficient knowledge seems to be caused by the little information about polyploids in azaleas.

In this study, ploidy levels of evergreen and deciduous azalea species, interspecific hybrids and cultivars were assessed by flow cytometric analysis. Flow cytometric analysis has been introduced to determine ploidy level in *Rhododendron* as the alternative to chromosome counting (Vainola, 2000; De Schepper *et al.*, 2001a, b). The relation among the ploidy levels of the plants and sizes of their guard cells and pollen grains were also investigated to ascertain the ploidy levels determined by flow cytometry. Intrasubgeneric and interploid hybridizations in evergreen and deciduous azaleas were performed and their interploid cross compatibilities were determined.

MATERIALS AND METHODS

Plant materials

Plant materials used in this study are listed in Table 1. Ploidy levels of 13 plants of three species (*R. eriocarpum*, *R. indicum* and *R. kiusianum*), five plants of interspecific hybrids (*R. kiusianum* × *R. eriocarpum*) and 24 cultivars of evergreen azaleas were investigated. All of the species, interspecific hybrids and cultivars of evergreen azaleas used in this study belong to subgenus Tsutsusi. Ploidy levels were also determined in eight plants of *R. japonicum*, six plants of *R. japonicum* f. *flavum* and five cultivars of Knap Hill Hybrid and Mollis Hybrid. They all belong to subgenus Pentanthera, so–called deciduous azaleas. Among them, (*R. kiusianum* × *R. eriocarpum*) No. 5 and *R. japonicum* f. *flavum* No. 6 were raised from the buds treated with colchicine (chromosome doubling was expected).

Assessment of ploidy levels with flow cytometry

The assessment of ploidy levels was conducted by flow cytometry. Fully expanded young leaves or young roots were chopped with a sharp razor blade in nuclei

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Table 1. Species, interspecific hybrids and cultivars of evergreen and deciduous azaleas used for ploidy determination by flow cytometry.

Plant	Description
Evergreen azaleas	
R. eriocarpum (No. 1, 2, 3)[z]	
R. indicum (No. 1, 2, 3, 4, 5, 6, 7)	Subg. Tsutsusi.
R. kiusianum (No. 1, 2, 3)	
R. kiusianum × *R. eriocarpum* (No. 1, 2, 3, 4, 5[y])	Interspecific hybrid, subg. Tsutsusi × subg. Tsutsusi.
'Ayaka', 'Eiganohomare', 'Eiko', 'Goka', 'Gyoko', 'Hakatajiro', 'Hoshuku', 'Hoshun', 'Isshonoharu', 'Kobai', 'Koyo', 'Mibunohana', 'Miharu', 'Meicho', 'Sachinoharu', 'Shinsen', 'Suisen', 'Shunka', 'Taihei', 'Taikonotsuki', 'Daisetsuzan', 'Takarabune', 'Yuhime'	Satsuki Hybrid, an intrasubgeneric hybrid group in subg. Tsutsusi.
'Horiuchikanzaki'	Inter–group hybrid between Belgian Hybrid and Hirado Hybrid in subg. Tsutsusi.
Deciduous azaleas	
R. japonicum (No. 1, 2)	
R. japonicum f. *flavum* (No. 1, 2, 3, 4, 5, 6[y])	Subg. Pentanthera.
'Golden Flare', 'Golden Sunset', 'Klondyke', 'Melford Yellow'	Knap Hill Hybrid, an intrasubgeneric hybrid group in subg. Pentanthera.
'Directeur Moerlands'	Mollis Hybrid, an intrasubgeneric hybrid group in subg. Pentanthera.

[z]( ) = individual plant number.
[y] Seedings of (*R. kiusianum* × *R. eriocarpum*) No. 5 and *R. japonicum* f. *flavum* No. 6 were derived from buds treated with colchicine.

extraction buffer (High resolution DNA kit, Partec), and the suspension containing released nuclei was passed through a 50 μm nylon mesh filter. Then the nuclei in filtrate were stained with four times volumes of staining solution (High resolution DNA kit, Partec) containing 4'–6–diamidino–2–phenylindole (DAPI). After shaking the solution gently, samples were analyzed with a flow cytometer (PA Ploidy Analyzer, Partec). Relative DNA content was estimated according to the prominent peaks in each measurement.

Measurement of pollen grain sizes

Pollen grains were collected at the full bloom stage in each plant, and observed with an optical microscope after staining with aceto–carmine for 10 minutes. The pollen grains were classified into perfect pollen with densely stained cytoplasm or empty pollen that was not stained. The diameters of twenty perfect pollen grains in three replications were measured.

Measurement of guard cell sizes

Impressions of guard cells were made by painting the abaxial surface of three mature leaves each from diploid, triploid, tetraploid and mixoploid plants with clear fingernail polish. After the impressions had been dried, they were stripped off by a transparent adhesive tape, sealed on slide glasses, and used to measure the guard cell length under a optical microscope.

Pollen fertility

Pollen fertility of evergreen and deciduous azaleas was examined. Pollen grains were collected at their full bloom stage, and observed with an optical microscope after staining with aceto–carmine for 10 minutes. Pollen fertility was evaluated by number of perfect pollen grains (densely stained pollen grains) divided by the number of pollen grains observed. More than 400 pollen grains in three replications were observed for estimation of fertility in each accession.

Intra– and interploid crosses

Intrasubgeneric intra– and interploid crosses among evergreen and deciduous azaleas were conducted from 19 April to 9 June 2002 or from 21 April to 24 June 2003. Flowers of pistillate parents were emasculated and covered with paper bags about seven days before anthesis and pollinated at anthesis with fresh or one–year–old pollen stored at −20 °C. Viability of azalea pollen after the storage for years at −20 °C has been confirmed (Rouse, 1984; Kehr, 1987). All pollinated flowers were re–covered with paper bags to prevent them from contamination by undesirable pollen. Thirteen diploids, five triploids, four tetraploids and three mixoploids in evergreen azaleas, and six diploids and five tetraploids in deciduous azaleas were chosen as cross parents. A total of 219 pollinations in 52 cross combinations among evergreen azaleas and 202 pollinations in 28 crosses among deciduous azaleas were made. Capsule setting was observed 90 days after crossings. Capsules were collected 180 days after pollination, and number of seeds per capsule and the percentages of perfect seeds were scored.

When mixoploids produce 2x–gamate judging from the results of flow cytometric analysis and measurements of pollen grain sizes, the results obtained from crosses with the mixoploids were included in those from crosses with tetraploids in Table 6.

RESULTS

Assessment of ploidy levels

Typical histograms of flow cytometric analysis in diploid, triploid, tetraploid and mixoploid (2x+4x) plants are shown in Fig. 1. Each ploidy level was clearly identified by the analysis using young leaves. The fluorescent intensities at prominent peaks in diploid, triploid and tetraploid plants were about 100, 150 and 200, respectively. The plants showing two peaks of fluorescent intensities at 100 and 200 were judged to be mixoploids (2x+4x). Similar results were obtained from the roots in diploids, triploids and tetraploids by the flow cytometric analysis. Although 'Miharu' and 'Shinsen' were identified to be mixoploids with their leaves, their roots had only tetraploid nuclei. Twenty-three diploid, six triploid, nine tetraploid and four mixoploid evergreen azaleas, and eight diploid and five tetraploid deciduous azaleas were identified by flow cytometry (Table 2).

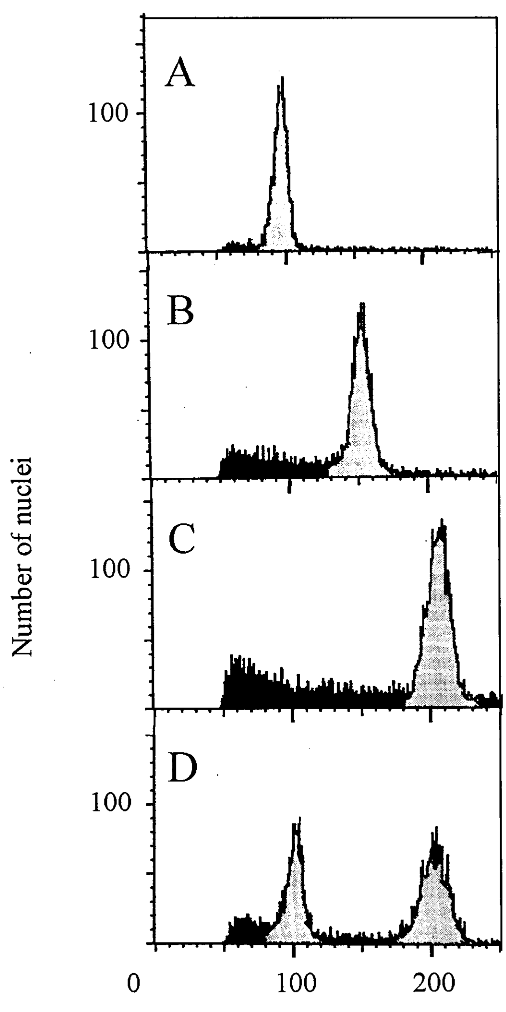

Fig. 1. Flow cytometric histogram patterns of diploid, triploid, tetraploid and mixoploid. A: diploid evergreen azalea *R. indicum* No. 1, B: triploid evergreen azalea 'Daisetsuzan', C: tetraploid azalea 'Hoshun' and D: mixoploid azalea 'Miharu'.

Pollen grain sizes

Most of the pollen grains from the diploid azaleas were well stained and plump, while empty pollen grains were found in triploid, tetraploid and mixoploid azaleas in relatively high frequencies (Fig. 2). The sizes of well–stained pollen grains tended to increase in proportion to the increase in ploidy levels (Table 3). Stained pollen grains from the mixoploid evergreen azaleas were as large as those from the tetraploid evergreen azalea.

Guard cell sizes

Stomatal guard cells of the tetraploid 'Hoshun' were longer than those of the diploids (Fig. 3, Table 4). Although the guard cells of mixoploid 'Shinsen', a Satsuki Hybrid, were smaller than those of the diploids, 'Miharu' had much larger guard cells than diploid plants.

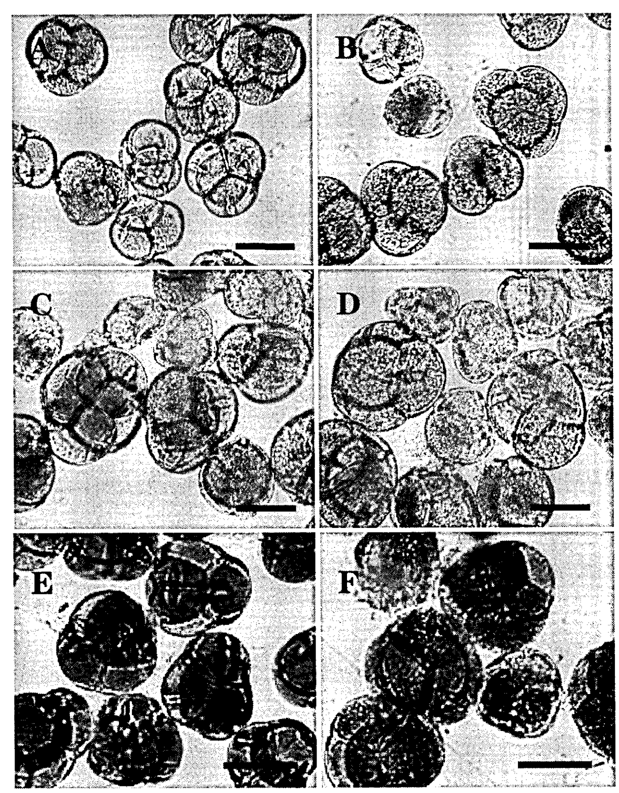

Fig. 2. Pollen grains of diploid, triploid, tetraploid and mixoploid plants. A: diploid evergreen azalea 'Mibunohana', B: triploid evergreen azalea 'Goka', C: tetraploid evergreen azalea 'Hoshun', D: mixoploid evergreen azalea 'Miharu', E: diploid deciduous azalea *R. japonicum* f. *flavum* (No. 1) and F: tetraploid deciduous azalea 'Klondyke'. Scale=50 μm.

Table 2. Ploidy levels of evergreen and deciduous azaleas determined by flow cytometry.

Plant	Ploidy level
Evergreen azaleas	
R. eriocarpum No. 1, 2, 3	2x
R. indicum No. 1, 2, 3, 4, 5, 6, 7	
R. kiusianum No. 1, 2, 3	
R. kiusianum × *R. eriocarpum* No. 1, 2, 3, 4	
'Eiganohomare', 'Gyoko', 'Hakatajiro', 'Kobai', 'Mibunohana', 'Takarabune'	
'Daisetsuzan', 'Goka', 'Horiuchikanzaki', 'Isshonoharu', 'Meicho', 'Yuhime'	3x
R. kiusianum × *R. eriocarpum* No. 5	4x
'Ayaka', 'Eiko', 'Hoshuku', 'Hoshun', 'Sachinoharu', 'Shunka', 'Taihei', 'Taikonotsuki'	
'Koyo', 'Miharu', 'Shinsen', 'Suisen'	2x+4x
Deciduous azaleas	
R. japonicum No. 1, 2	2x
R. japonicum f. *flavum* No. 1, 2, 3, 4, 5	
'Directoeur Moerlands'	
R. japonicum f. *flavum* No. 6	4x
'Golden Flare', 'Golden Sunset', 'Klondyke', 'Melford Yellow'	

Table 3. Mean diameter of pollen grains well-stained with aceto-carmine in various ploids of evergreen and deciduous azaleas.

Plant	Ploidy level	Mean diameter of pollen grains (μm)
Evergreen azaleas		
R. indicum No. 3	2x	39.1
R. indicum No. 5	2x	35.0
'Mibunohana'	2x	40.7
'Daisetsuzan'	3x	42.2
'Goka'	3x	44.6
'Hoshun'	4x	48.7
'Miharu'	2x+4x	47.9
'Shinsen'	2x+4x	50.0
Deciduous azaleas		
R. japonicum f. *flavum* No. 1	2x	41.3
'Klondyke'	4x	44.8

Table 4. Sizes of guard cells in various ploids of evergreen and deciduous azaleas.

Plant	Ploidy level	Mean diameter of guard cells (μm)
Evergreen azaleas		
R. indicum No. 3	2x	19.2
R. indicum No. 5	2x	19.3
'Mibunohana'	2x	18.2
'Daisetsuzan'	3x	16.1
'Goka'	3x	19.8
'Hoshun'	4x	23.9
'Miharu'	2x+4x	21.3
'Shinsen'	2x+4x	15.8
Deciduous azaleas		
R. japonicum f. *flavum* No. 1	2x	10.2
'Klondyke'	4x	16.3

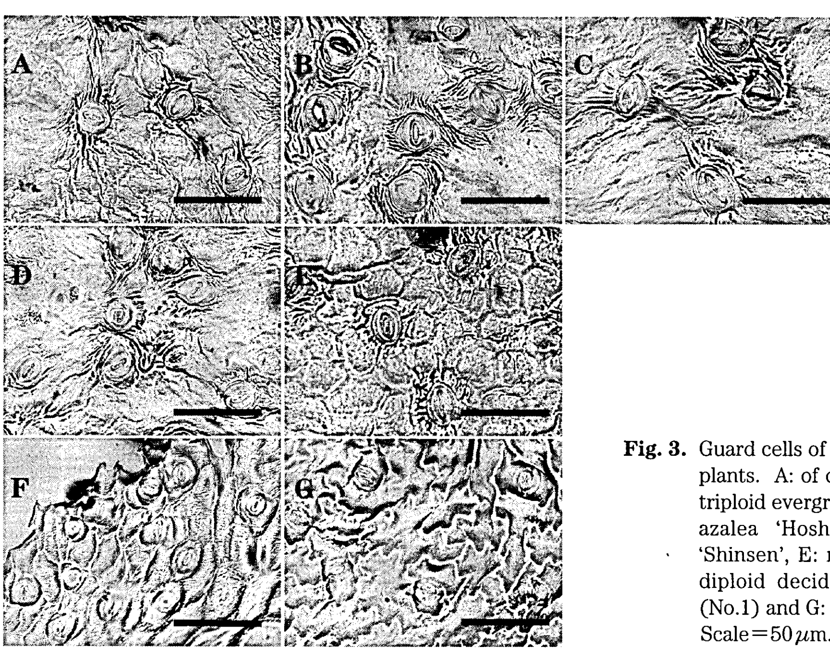

Fig. 3. Guard cells of diploid, triploid, tetraploid and mixoploid plants. A: of diploid evergreen azalea 'Mibunohana', B: triploid evergreen azalea 'Goka', C: tetraploid evergreen azalea 'Hoshun', D: mixoploid evergreen azalea 'Shinsen', E: mixoploid evergreen azalea 'Miharu', F: diploid deciduous azalea *R. japonicum* f. *flavum* (No.1) and G: tetraploid deciduous azalea 'Klondyke'. Scale=50 μm.

Table 5. Pollen fertility in evergreen and deciduous azaleas with various ploidy.

Plant	Ploidy level	Pollen fertility (%)
Evergreen azaleas		
R. indicum No. 3	2x	88.8
R. indicum No. 5	2x	99.3
'Mibunohana'	2x	84.0
'Daisetsuzan'	3x	72.0
'Goka'	3x	66.6
'Hoshun'	4x	60.5
'Miharu'	2x+4x	23.4
'Shinsen'	2x+4x	90.6
Deciduous azaleas		
R. japonicum f. *flavum* No. 1	2x	95.9
'Klondyke'	4x	89.1

Pollen fertility

Pollen fertility of diploid evergreen azaleas was higher (84.0–99.3%) than that of triploids (66.6 and 72.0%) and tetraploid (60.5%) evergreen azaleas, while in two mixoploid evergreen azaleas it was different (23.4 and 90.6%) (Table 5). Both diploid and tetraploid deciduous azaleas had relatively high pollen fertility; 95.9 and 89.1%, respectively.

Intra– and interploid crosses

In intraploid crosses with evergreen azaleas, 2x × 2x crosses brought higher capsule set (95.2%) than 4x × 4x crosses (48.7%) (Table 6). No capsules set in any of the crosses with triploids when used as pollen parents. The capsule set was the highest in 3x × 2x (72.2%) among interploid crosses with evergreen azaleas (Table 6).

Capsules from 2x × 2x crosses were also obtained with higher rate (87.3%) than those from 4x × 4x (6.8%) among the intraploid crosses with deciduous azaleas, (Table 7). Although capsules were obtained from 2x × 4x crosses, no capsules set in the reciprocal crosses, 4x × 2x (Table 7).

More than 500 seeds per capsule were obtained from the 2x × 2x crosses with evergreen azaleas and relatively many seeds (more than 300 per capsule) were produced in the crosses of 4x × 4x on an average. Most of them were perfect seeds (97.6 for the 2x × 2x and 93.2% for the 4x × 4x) (Table 6). Average number of seeds per capsule obtained from the interploid crosses ranged from 62.1 for the 3x × 4x to 268.2 for the 3x × 2x. Perfect seeds were obtained relatively frequently (more than 80%) from the interploid crosses, on an average, except for the 3x × 4x crosses (72.2%). In the

Table 6. Capsule set rates, number of seeds and percentages of perfect seeds in intra– and interploid crosses with evergreen azaleas.

Seed parent	Pollen parent	No. of pollinated flowers	No. of capsules	% of capsules set	No. of seeds per capsule	% of perfect seeds
Intra–ploid crosses						
[2x]	**[2x]**					
R. eriocarpum No. 1	*R. eriocarpum* No. 2	6	6	100	903.8	99.2
R. eriocarpum No. 2	*R. indicum* No. 1	8	8	100	609.1	98.1
R. eriocarpum No. 2	'Eiganohomare'	2	2	100	66.0	95.5
R. eriocarpum No. 2	'Hakatajiro'	5	5	100	332.4	96.5
R. indicum No. 1	*R. eriocarpum* No. 2	5	5	100	652.8	97.0
R. indicum No. 2	*R. eriocarpum* No. 2	5	4	80	800.5	97.0
R. indicum No. 3	*R. eriocarpum* No. 2	3	3	100	392.3	99.2
R. indicum No. 4	*R. indicum* No. 1	3	3	100	654.0	98.0
'Mibunohana'	*R. indicum* No. 3	5	4	80	449.5	94.3
2x × 2x Subtotal		42	40	95.2	587.3	97.6
[3x]	**[3x]**					
'Goka'	'Isshonoharu'	2	0	0	–	–
'Horiuchikanzaki'	'Goka'	4	0	0	–	–
3x × 3x Subtotal		6	0	0	–	–
[4x]	**[2x+4x]**					
'Hoshuku'	'Shinsen'	6	1	16.6	138.0	93.5
'Hoshun'	'Miharu'	5	0	0	–	–
'Hoshun'	'Shinsen'	5	4	80	410.7	85.5

Table 6. Continued

Seed parent	Pollen parent	No. of pollinated flowers	No. of capsules	% of capsules set	No. of seeds per capsule	% of perfect seeds
"Sachinoharu'	'Suisen'	2	0	0	–	–
[2x+4x]	**[4x]**					
'Miharu'	'Hoshun'	5	4	80	310.3	97.2
'Miharu'	'Sachinoharu'	5	3	60	194.3	97.3
'Shinsen'	'Hoshun'	5	4	80	495.0	95.5
'Shinsen'	'Sachinoharu'	6	3	50	289.3	94.1
4x × 4x Subtotal		39	19	48.7	339.6	93.2
Inter-ploid crosses						
[2x]	**[3x]**					
R. eriocarpum No. 2	'Goka'	9	0	0	–	–
R. eriocarpum No. 2	'Daisetsuzan'	5	0	0	–	–
R. eriocarpum No. 2	'Horiuchikanzaki'	5	0	0	–	–
R. indicum No. 6	'Daisetsuzan'	5	0	0	–	–
R. indicum No. 1	'Horiuchikanzaki'	5	0	0	–	–
'Mibunohana'	'Daisetsuzan'	2	0	0	–	–
2x × 3x Subtotal		31	0	0	–	–
[2x]	**[4x]**					
R. eriocarpum No. 1	'Taikonotsuki'	2	2	100	35.0	100
R. eriocarpum No. 3	'Ayaka'	4	0	0	–	–
'Mibunohana'	'Hoshun'	5	5	100	136.0	84.7
[2x]	**[2x+4x]**					
R. eriocarpum No. 2	'Shinsen'	5	5	100	456.6	95.7
R. eriocarpum No. 2	'Suisen'	5	1	20	35.0	88.6
R. indicum No. 7	'Shinsen'	3	1	33.3	48.0	89.6
R. indicum No. 7	'Miharu'	5	5	100	35.8	98.3
2x × 4x Subtotal		29	19	65.5	173.4	93.5
[3x]	**[2x]**					
'Daisetsuzan'	*R. indicum* No. 3	1	1	100	201.0	74.1
'Daisetsuzan'	*R. indicum* No. 5	1	0	0	–	–
'Daisetsuzan'	*R. indicum* No. 6	5	3	60	201.3	92.7
'Daisetsuzan'	*R. eriocarpum* No. 2	4	3	75	239.3	91.5
'Goka'	*R. indicum* No. 3	2	2	100	145.0	70.7
'Horiuchikanzaki'	*R. indicum* No. 3	5	4	80	418.3	86.2
3x× 2x Subtotal		18	13	72.2	268.2	86.4
[3x]	**[4x]**					
'Goka'	'Hoshun'	2	2	100	103.5	60.9
[3x]	**[2x+4x]**					
'Daisetsuzan'	'Miharu'	5	5	100	45.5	82.4
'Horiuchikanzaki'	'Miharu'	5	0	0	–	–
3x × 4x Subtotal		12	7	58.3	62.1	72.2
[4x]	**[2x]**					
'Hoshuku'	*R. indicum* No. 6	5	0	0	–	–
'Hoshun'	*R. indicum* No. 5	5	1	20	65.0	73.8
[2x+4x]	**[2x]**					
'Miharu'	*R. indicum* No. 5	5	5	100	52.0	88.5
'Miharu'	'Eiganohomare'	2	0	0	–	–
'Shinsen'	*R. indicum* No. 5	5	0	0	–	–
'Shinsen'	*R. indicum* No. 6	5	2	40	115.0	81.7
'Shinsen'	*R. eriocarpum* No. 2	2	0	0	–	–
4x × 2x Subtotal		29	8	27.6	69.4	84.0
[4x]	**[3x]**					
'Hoshun'	'Goka'	2	0	0	–	–
'Sachinoharu'	'Horiuchikanzaki'	1	0	0	–	–
[2x+4x]	**[3x]**					
'Shinsen'	'Horiuchikanzaki'	5	0	0	–	–
'Shinsen'	'Daisetsuzan'	5	0	0	–	–
4x × 3x Subtotal		13	0	0	–	–
Total		219	106	48.4	355.8	75.0

Table 7. Capsule set rates, number of seeds and percentages of perfect seeds in intra- and interploid crosses with deciduous azaleas.

Seed parent	Pollen parent	No. of pollinated flowers	No. of capsules	% of capsules set	No. of seeds per capsule	% of perfect seeds
Intra-ploid crosses						
[2x]	**[2x]**					
R. japonicum f. *flavum* No. 1	*R. japonicum* f. *flavum* No. 2	15	11	73.3	343.7	93.4
R. japonicum f. *flavum* No. 2	*R. japonicum* f. *flavum* No. 1	4	2	50.0	192.5	84.4
R. japonicum f. *flavum* No. 1	*R. japonicum* No. 2	40	38	95.0	379.3	94.1
R. japonicum No. 1	*R. japonicum* f. *flavum* No. 1	4	4	100	493.7	94.6
2x × 2x Subtotal		63	55	87.3	373.7	93.8
[4x]	**[4x]**					
'Golden Flare'	'Klondyke'	6	0	0	–	–
'Golden Flare'	'Golden Sunset'	6	0	0	–	–
'Golden Sunset'	'Klondyke'	5	1	20.0	126.0	80.2
'Golden Sunset'	'Golden Flare'	4	2	50.0	235.0	90.9
'Klondyke'	'Golden Flare'	5	0	0	–	–
'Klondyke'	'Golden Sunset'	3	0	0	–	–
'Melford Yellow'	*R. japonicum* f. *flavum* No. 6	5	0	0	–	–
'Melford Yellow'	'Klondyke'	10	0	0	–	–
4x × 4x Subtotal		44	3	6.8	198.7	88.6
Inter-ploid crosses						
[2x]	**[4x]**					
R. japonicum f. *flavum* No. 1	*R. japonicum* f. *flavum* No. 6	10	1	10.0	20.0	60.0
R. japonicum f. *flavum* No. 1	'Golden Flare'	17	11	64.7	131.7	78.5
R. japonicum f. *flavum* No. 1	'Klondyke'	10	6	60.0	192.0	85.6
R. japonicum f. *flavum* No. 1	'Melford Yellow'	7	4	57.1	123.3	75.1
R. japonicum f. *flavum* No. 2	'Golden Flare'	4	0	0	–	–
R. japonicum f. *flavum* No. 2	'Klondyke'	5	5	100	75.7	59.9
R. japonicum f. *flavum* No. 4	'Golden Sunset'	5	5	100	413.0	62.8
R. japonicum No. 1	'Klondyke'	5	5	100	388.3	82.0
2x × 4x Subtotal		63	37	58.7	202.7	75.0
[4x]	**[2x]**					
'Golden Flare'	*R. japonicum* f. *flavum* No. 1	4	0	0	–	–
'Golden Flare'	*R. japonicum* f. *flavum* No. 2	4	0	0	–	–
'Golden Sunset'	*R. japonicum* f. *flavum* No. 1	4	0	0	–	–
'Golden Sunset'	*R. japonicum* f. *flavum* No. 4	4	0	0	–	–
'Klondyke'	*R. japonicum* f. *flavum* No. 1	4	0	0	–	–
'Klondyke'	*R. japonicum* f. *flavum* No. 2	4	0	0	–	–
'Klondyke'	*R. japonicum* No. 2	4	0	0	–	–
'Melford Yellow'	*R. japonicum* f. *flavum* No. 1	4	0	0	–	–
4x × 2x Subtotal		32	0	0	–	–
Total		202	95	47.0	301.6	88.8

intraploid crosses with deciduous azaleas, more perfect seeds were obtained from the 2x × 2x crosses than the 4x × 4x crosses (Table 7). Relatively many perfect seeds were produced in the interploid crosses of 2x × 4x.

DISCUSSION

Polyploids in evergreen and deciduous azaleas

Numerous studies about ploidy levels in *Rhododendron* revealed that most evergreen and deciduous azalea species are diploid (Sax, 1930; Janaki Ammal, 1950; Li, 1957; De Schepper *et al.*, 2001a). Ploidy levels of many cultivars were also investigated. Most of them were diploid and a few were polyploids. De Schepper *et al.* (2001a) determined the ploidy levels of 88 evergreen azalea cultivars by flow cytometry, and found three triploid (2n=3x=39) and one mixoploid (2n=2x+4x) cultivars of Belgian Hybrid. Tetraploid sports from diploid cultivars were identified in Belgian Hybrid (De Schepper *et al.*, 2001b). Tetraploid and triploid cultivars of Satsuki Hybrid were reported, i.e., 'Banka', 'Taihei' and 'Wako' were identified to be tetraploid and 'Bangaku' was identified to be triploid by chromosome counting (Hosoda *et al.*, 1953). Most deciduous species are diploids except that *R. calendulaceum* and *R. canadense* are tetraploids (Sax, 1930; Janaki Ammal, 1950; Li, 1957). Although some studies about ploidy levels of deciduous azalea species have been made, ploidy level of their cultivars is little known.

In the present study, many polyploid cultivars were identified in both evergreen and deciduous azaleas. This is the first description of tetraploids in Knap Hill Hybrid, triploid in inter–group hybrid between Belgian Hybrid and Hirado Hybrid and mixoploids in Satsuki Hybrid. Satsuki Hybrid 'Taihei', which had been identified to be a tetraploid by chromosome counting, also showed tetraploidy through the present flow cytometric analysis. Although the origin of these polyploids was not studied, unreduced gametes have been considered to be indispensable in the formation of polyploid azalea cultivars (Pryor and Frazier. 1970; Heursel and De Roo, 1981). On the other hand, there are some reports of tetraploid sports generating from diploid cultivars in Satsuki Hybrid and Belgian Hybrid (Hosoda *et al.*, 1953; De Schepper *et al.*, 2001b).

Four mixoploid cultivars in Satsuki Hybrid, of which the leaves consisted of both diploid and tetraploid cells, were identified through flow cytometric analysis with young leaves. It is known that guard cells on the epidermal layer originate from L1, pollen grains from L2 and adventitious roots from L3. Since the mixoploid 'Shinsen' had (1) slightly smaller guard cells than diploid evergreen azaleas, (2) pollen grains as large as those of tetraploid 'Hoshun' and (3) only one tetraploid (4C) peak in flow cytometric analysis of the roots, it must be diploid in the L1 and tetraploid in the L2 and L3. With the same approach, it is judged that the mixoploid 'Miharu' must include diploid cells in the L1 and/or L2 and tetraploid cells in the L1, L2 and/or L3.

Mixoploid 'Shinsen' is a sport from diploid evergreen azalea 'Gyoko'. 'Shinsen' has rounder leaves and thicker petals than 'Gyoko'. Since 'Shinsen' produced triploid 'Goka' in the cross with diploid 'Takarabune', it would produce 2x gamete. Although 'Koyo' and 'Miharu' are the sports from 'Suisen' and have different color patterns in their flowers, they have the same ploidy patterns as the original cultivar 'Suisen'. Considering that the tetraploid cultivars of 'Hoshuku', 'Hoshun' and 'Taikonotsuki' are the hybrids between mixoploid 'Suisen' and tetraploid 'Eiko', 'Suisen' and its sports ('Koyo' and 'Miharu') would also produce 2x gamete.

Ornamental plants often contain triploid and tetraploid cultivars, in general, because polyploids have favorable characters for consumers such as vigorous growth, thick stems, large flowers and firm petals. Since some triploids are more vigorous and have bigger flowers than tetraploids, triploid cultivars have been bred in various ornamental plants such as tulips, freesias and cannas (Ukai, 2003). The same may be said in triploid Satsuki Hybrid 'Meicho', which has the largest flowers among Satsuki Hybrid cultivars, and other triploid cultivars also tend to have larger flowers than tetraploids. The same observation applies to triploid 'Euratom', a remarkably vigorous Belgian Hybrid and the most widely used as a root stock for grafting in Europe (Heursel and De Roo, 1981).

Appearance of polyploids has heavily influenced on the evolution of cultivated plants. Changing ploidy levels is followed by various morphological, physiological and genetic rearrangements. Hybridization with tetraploids of *Primula malacoides*, which was originally a diploid with low variability, produced attractive plants with attractive characters such as more flowers, larger flowers and more seeds per flower than diploids (Horn, 2003). Flower color variability in tetraploids of *P. malacoides* was explained by dosage effects. De Schepper *et al.* (2001b) revealed the close relationships among somatic polyploidy, flower coloration and flower morphology in azalea. Although only few attempts have so far been made in interploid crossings among azaleas, this suggests that hybridization with polyploids plays an important role in the expansion of plant characteristics.

Intrasubgeneric and interploid crossability in evergreen and deciduous azaleas

Many diploids, triploids, tetraploids and mixoploids were identified among azalea cultivars in this study. Crossability of polyploids in interploid crosses was clarified through intrasubgeneric crossings with evergreen and deciduous azaleas. High fertility in evergreen azaleas and high cross compatibilities among them had been described in previous reports so far as diploids were concerned (Noguchi, 1932; Akabane *et al.*, 1971; Yamaguchi *et al.*, 1985). Yamaguchi *et al.* (1985) suggested that it was better to use large–flowered species for the pollen parents when the sizes of parental flowers were apparently different. Michishita *et al.* (2003) proved the positive relationship between male/female style length ratio (SLR) and number of seeds per capsule in interspecific crosses among evergreen azaleas. In the present study, the degree of fertility was distinctive in different ploidy levels and cross compatibility differed individually among interploid crosses. Tetraploid evergreen azaleas were fertile as seed or pollen parents, while tetraploid deciduous azaleas had low crossability as seed parents. Higher capsule set and more perfect seeds were obtained from the 2x × 4x crosses than those from the 4x × 2x crosses with evergreen or deciduous azaleas. There would be a room for arguing whether tetraploids with large flowers affect on fertilization in 4x × 2x crosses.

Since no capsules set in the crosses with triploids evergreen azaleas as pollen parents, it is considered that triploids are male sterile or have very low pollen fertility. Crosses of the 3x × 2x set capsules most frequently and produced the largest number of seeds among interploid crossings of evergreen azaleas. It may be judged from these results that triploid evergreen azaleas have relatively high female fertility as seeds parents. The reason for the high fertility is not obvious at present. In highbush blueberries, close relatives to *Rhododendron*, crossabilities of triploids were very low when they were used as pollen parents (Vorsa and Ballington, 1991). However, one triploid plant was relatively fertile as a female parent in the 3x × 4x and the 3x × 6x crosses. The female fertility has been considered to attribute to 2x gamete production in the triploid.

Ploidy levels of the progenies obtained in the present study remain to be proved. Since polyploids tend to form gametes with asymmetric chromosome set more frequently than diploids, the progenies obtained from interploid crosses with polyploids might have more allele combinations than diploid progenies from intraploid crosses with diploids. Correlation between allele dosage effects and flower colors has been observed in tetraploids of *Primura malacoides* (Horn, 2003). The present intrasubgeneric and interploid crosses with evergreen and deciduous azaleas must have produced various ploids or aneuploids with high heterozygosity. They might have variegated phenotypes and be useful materials to clarify the relationships between allele dosage effects and various characteristics.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Masaru Akabane for his donation of the white–flowered evergreen azalea. We also thank to Associate Professor Koichiro Gyokusen in the Laboratry of Silviculture, Faculty of Agriculture, Kyushu University for cooperation in flow cytometric analysis.

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