Adv. Hort. Sci., 2020 34(1): 3-10 DOI: 10.13128/ahsc-8251

AHS
Advances in Horticultural Science

Development of interspecific hybrids between *Habenaria radiata* and *Habenaria rhodocheila* complex

P. Sinumporn (*), T. Narumi-Kawasaki, S. Fukai
Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan.

Key words: apomixis, cross combination, PCR-RFLP, reciprocal crossing, seed germination.

(*) **Corresponding author:**
punpaka.sinumporn@gmail.com

Citation:
SINUMPORN P., NARUMI-KAWASAKI T., FUKAI S., 2020 - *Development of interspecific hybrids between Habenaria radiata and Habenaria rhodocheila complex* - Adv. Hort. Sci., 34(1): 3-10

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 20 October 2018
Accepted for publication 6 March 2019

Abstract: Reciprocal crosses between *Habenaria radiata* and *H. rhodocheila* complex were investigated to develop new hybrids. The fruit-setting frequency and seed germination in the cross combination of *H. radiata* × *H. rhodocheila* complex were higher than those of *H. rhodocheila* complex × *H. radiata.* The hybridity of the obtained progenies was confirmed through PCR-RFLP analysis of the rRNA gene. Cross combinations producing true hybrids, apomicts, or both were observed, indicating that both *H. radiata* and *H. rhodocheila* complex were facultative apomixis. The obtained hybrids, *H. radiata* × *H. rhodocheila* (orange flower), showed the intermediate plant form and flower shape of the parents, and both petals and lip were pale yellow.

1. Introduction

Habenaria is a large genus in the family Orchidaceae, consisting of more than 800 species distributed in tropical and subtropical areas such as Southern America, Southern and Central Africa, and East Asia (Pridgeon, 1992; Kurzweil, 2009; Pedron *et al*., 2012; Batista *et al*., 2013; Jin *et al*., 2014). *Habenaria* species show diverse plant forms, flower shapes, and petal colors. There are many *Habenaria* species having high ornamental value, but only a few species are commercialized.

In this study, we focused on two *Habenaria* species: *H. radiata* and *H. rhodocheila*. *Habenaria radiata* is a species native to Japan in the wetlands of Honshu, Shikoku, and Kyushu Islands. This species is low-temperature tolerant. The form of the flowers is unique and beautiful, and the white petals look like a white egret bird. This species has been used as ornamental pot plants (Kim *et al*., 2007, 2010; Mitoma and Kanno, 2018), but it can be used as cut flowers (Sinumporn *et al*., 2015). *Habenaria rhodocheila* is found in Southeast Asia, Laos, Myanmar, southeast China, Thailand, Malaysia, and the Philippines. The flowers of *H. rhodocheila* have a large lip and four lobes, with side lobes and oblique mid lobes. The lips show a wide range of color such as orange, pink, red, and yellow. Formerly, the pink-flowered genotype was accepted under the name *H.*

erichmichelii, and the yellow-flowered genotype was *H. xanthocheila*. The morphological characteristics are also different in each genotype, beside petal color (Kurzweil, 2009; Batista *et al*., 2013). *H. xanthocheila* is distinguished from *H. rhodocheila* in its tuber shape, i.e. *H. xanthocheila* has a crown-shaped tuber but *H. rhodocheila* has a round tuber (Cullen *et al*., 2011). Because these genotypes are very closely related, they are integrated into one species, called *H. rhodocheila* complex. In this report, we adopted the name *H. rhodocheila* complex and distinguished the genotypes only by the color of the petals. Producing hybrids between the two completely different *Habenaria* species, *H. radiata* and *H. rhodocheila* complex, could result in new hybrids having vigor, low-temperature tolerance, and beautiful flower shape with colorful petals.

Recently, many orchid species including *Habenaria* are at risk of extinction. The numbers of both *H. radiata* and *H. rhodocheila* complex are decreasing in their natural habitats, which are being destroyed through urbanization, agricultural use, ecological mismanagement of habitat, changes in climate conditions, and overcollection by people (Stewart and Kane, 2006; Mitsukuri *et al*., 2009; Tanaka *et al*., 2015). Supply of new interspecific hybrids with increased ornamental value is expected to reduce the illegal collection of the species in their habitats. There is little research on intraspecific cross breeding using these two *Habenaria* species. Only a successful of intraspecific cross between wild-type and petaloid-sepal genotypes in *H. radiata* was done (Kim *et al*., 2010; Mitoma *et al*., 2019). In this study, we carried out reciprocal crossing between *H. radiata* and *H. rhodocheila* complex, and evaluated the obtained progenies. This report is the first on successful interspecific crossing of *H. radiata* and *H. rhodocheila* complex.

2. Materials and Methods

Plant materials

Tubers of *H. radiata* 'Aoba' (HRA) (Fig. 1A) were planted in April, every year, in 12 cm plastic pots (5 tubers per pot) with sphagnum moss and tubers of *H. rhodocheila* complex (orange, pink, and yellow petal genotypes, RCO, RCP, and RCY, respectively) (Fig. 1B, 1C, 1D) were planted in 12 cm plastic pots filed with a medium consisting of Growing Mix (Metro Mix 350; Sun Gro Horticulture, MA USA): Kanuma (volcanic porous soil): Vermiculite, 1:2:1. Then, *H. radiata* were placed in a greenhouse in natural temperature with solar radiation. *H. rhodocheila* complex were placed in a growth chamber controlled at a constant temperature of 20°C with solar radiation.

Fig. 1 - Plant morphology of *Habenaria* species used in this study. (A) *H. radiata* 'Aoba', (B) *H. rhodocheila* complex (orange), (C) *H. rhodocheila* complex (pink), and (D) *H. rhodocheila* complex (yellow).

Interspecific cross and in vitro germination

A preliminary crossing experiment, HRA × RCO, was carried out in 2015. HRA and two *H. rhodocheila* complexes, RCO and RCY, were then cross-pollinated reciprocally in 2017. Five plants of each genotype were used in those cross combinations. Twenty plants of HRA and RCP were also cross-pollinated reciprocally in 2017. A total of six cross combinations were made (Table 1). The pollinia of mother plants were removed in advance to prevent self-pollination and the aimed pollinia of other plants were placed on the stigma. Reciprocal crosses were also conducted. Hand-pollinated flowers were labelled individually, and the capsules were harvested before dehiscence about 2 months after pollination. The capsules were surface sterilized with 70% (v/v) ethanol for 30 s and 0.1% (v/v) sodium hypochlorite solution for 15 min

Table 1 - Reciprocal crossing between *Habenaria radiata* and *Habenaria rhodocheila* complex

Cross combination	Number of flowers pollinated	Number of pod sets (%)	Seed germination	Number of plantlets tested PCR-RFLP	Number of true hybrids (%)
HRA × RCO	13	8 (61.5)	++++	36	36 (100)
RCO × HRA	2	2 (100)	-	-	-
HRA × RCP	28	16 (57.1)	+++	21	0 (0)
RCP × HRA	41	12 (29.3)	-	-	-
HRA × RCY	11	1 (9.1)	+	18	1 (5.5)
RCY × HRA	3	1 (33.3)	+	8	0 (0)

HRA = *H. radiate*; RCO = *H. rhodocheila* (orange petal); RCP = *H. rhodocheila* (pink petal); RCY = *H. rhodocheila* (yellow petal).
*++++ = Germination well, ++ = Germination fair, + = Germination poor, - = No germination.

and then rinsed three times with sterilized water. Seeds were removed from the capsules and mixed in a petri dish, and then a batch of seeds picked up with tweezers was placed on a seed germination medium in 5 cm petri dishes. The medium was MM (Malmgren, 1996) supplemented with 20 g/l sucrose and 0.7% agar, and adjusted to pH 5.75 prior to autoclaving at 0.103 MPa pressure and 121°C for 20 min. The cultures were kept at 20°C under dark conditions. Extract numbers of seeds placed on the medium was unknown, the germination was evaluated in four stages: well (+++), fair (++), poor (+) and no-germination (-). Six months after sowing, protocorms were transplanted to 6 × 6 cm plastic culture vessels with MM medium. The protocorm cultures were kept under light inflorescence lamps (FL40S. BRN; Toshiba Lighting & Technology Co. Ltd.) for 16 h with a light intensity of 31.5 µmol m^{-2} s^{-1}at 24°C. The protocorms were subcultured every month. After 12 months, the developed plantlets were acclimatized and planted in 12 cm plastic pots using the same growing medium as for the mother plants. The obtained progenies were grown in a growth chamber controlled at 20°C.

PCR-RFLP

The total DNA of both parents and progenies was extracted from 0.1 g of leaf tissue according to a modified ABBAS DNA extraction method (Abbas *et al*., 2013). PCR was performed in a 50 µl reaction mixture containing 70 ng of total DNA, 0.2mM each of rRNA gene specific primers (5'-ACA CAC CGC CCG TCGCTC CTA-3' and 5'-ACT CGA TGG TTC ACG GGA TTC TG-3'), 2.5 mM dNTPs, 20mM of 10× PCR *Ex Taq* buffer, and 5 U/µl of *Ex Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) according to Haruki *et al*. (1997). PCR was conducted under the following thermocycling conditions: 1 cycle of 96°C, 10 s; 25 cycles of 96°C, 10 s, 55°C, 30 s, 72°C, 60 s; and 1 cycle of 72°C, 10 min.

The amplified products of both the parents and the progenies were digested with selected restriction endonucleases (*Alu* I, *Hha* I, *Rsa* I, and *Sty* I; Nippon Gene Co. Ltd., Toyama, Japan) at 37°C for 1 h. The digested products were separated by electrophoresis in 1.8% agarose gels (Invitrogen, Carlsbad, California, USA) containing 0.1 µl/ml ethidium bromide solution and photographed.

3. Results

Pod set and seed germination in the reciprocal crossings

Six reciprocal cross combinations were made, and the pod set frequencies varied depending on both cross combinations and the ovule parents. The HRA × RCO cross combination resulted in 8 pod sets from 13 flowers (61.5%), and the opposite cross of RCO × HRA resulted in 2 pod sets from 2 flowers (100%). HRA × RCP had 16 pod sets from 28 flowers (57.1%), and the opposite cross RCP × HRA had 12 from 41 flowers (29.3%). HRA × RCY had only 1 pod set from 11 flowers (9.1%), and the opposite cross RCY × HRA had 1 from 3 flowers (33.3%) (Table 1).

All pods were harvested before dehiscence, and the seeds were cultured on MM medium without plant growth regulators. The seeds from the cross combination of HRA × RCO germinated well, but the seeds from RCO × HRA did not germinate. The seeds of HRA × RCP also had rather high germination, but the seeds from RCP × HRA did not germinate. Both cross combinations of HRA × RCY and RCY × HRA had poor seed germination (Table 1).

The sown seeds from HRA × RCO (Fig. 2A) swelled within 50 days of culture (Fig. 2B), developed protocorms around 90 days of culture (Fig. 2B), and the protocorms produced rhizoids (Fig. 2C). After being

subjected to light, the protocorms turned green (Fig. 2D), produced the first leaf within 120 days of culture (Fig. 2E), and then developed plantlets around 150 days of culture (Fig. 2F).

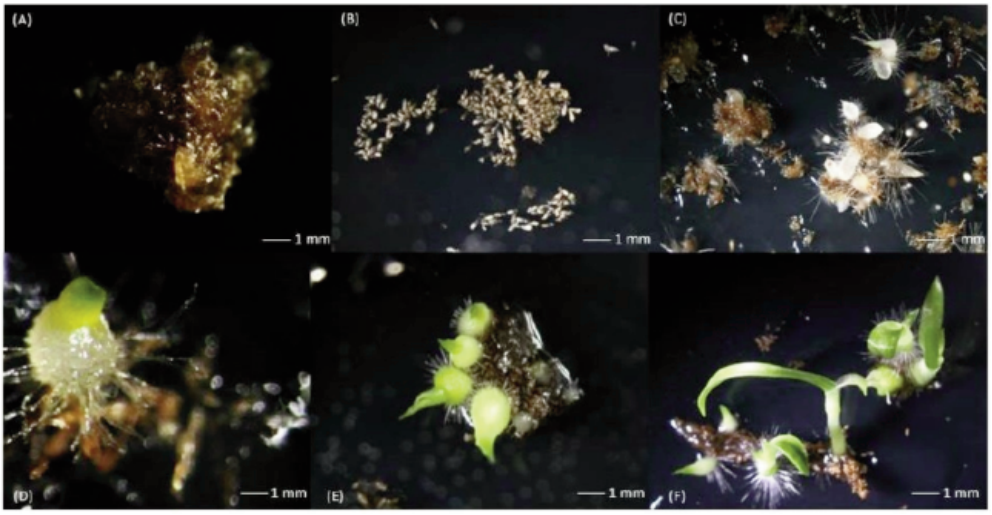

Fig. 2 - Seed development of *Habenaria radiate* × *Habenaria rhodocheila* on MM medium. (A) Sown seeds (day 0), (B) Protocorm development (50 days), (C) Enlarged embryo rupture testa and developed protocorm (3 months), (D) Green protocorm with protomeristem (3 months), (E) Emergence of first leaf (4 month), (F) Plantlets (5 months).

Confirmation of hybridity through PCR-RFLP analysis

To confirm the hybridity of the obtained progenies, PCR-RFLP analysis targeting the ribosomal RNA gene was used according to Haruki *et al*. (1997). The early growth stage of two progenies of HRA × RCO, named WXO1 and WXO2, were used. Expected single PCR product was amplified in all the tested plants. Three restriction enzymes (*Alu* I, *Hha* I and *Rsa* I) that showed polymorphism in the digested PCR products between both parents were applied. The band pattern of both WXO1 and WXO2 was intermediate between both parents (HRA and RCO), indicating that both WXO1 and WXO2 were true hybrids. Thirty-six HRA × RCO progenies in the *in vitro* stage were chosen randomly and their hybridity was tested in the same manner. The results showed that all the tested progenies were true hybrids (Table1) (Fig. 3A). In contrast, an early growth-stage progeny of HRA × RCP, named WXP1, showed a band pattern the same as the female parent (HRA) when the PCR products were digested with *Alu* I, *Hha* I, *Rsa* I, and *Sty* I, suggesting that the plants were apomicts. Twenty-one HRA × RCP progenies in the *in vitro* stage were tested in the same manner. The results showed all the tested progenies were apomicts (Fig.3B). One rapidly grown apomict was designed WXY1 and the one that was judged to be a true hybrid was designed WXY2. Moreover, the results of HRA × RCY (WXY) showed that the progenies included both apomixis and true hybrids (Fig. 3C). The progenies of the reciprocal cross RCY × HRA (YXW) showed the same band pattern as the female parent (RCY), suggesting that they were apomicts.

Morphological characteristics of the obtained progenies

The obtained progenies WXO1 and WXO2 (confirmed as hybrids through PCR-RFLP analysis), WXY1 (assumed to be an apomict), and the parent RCO were grown in a growth chamber controlled at 20°C. HRA grown in a greenhouse without heating was used for morphological comparison.

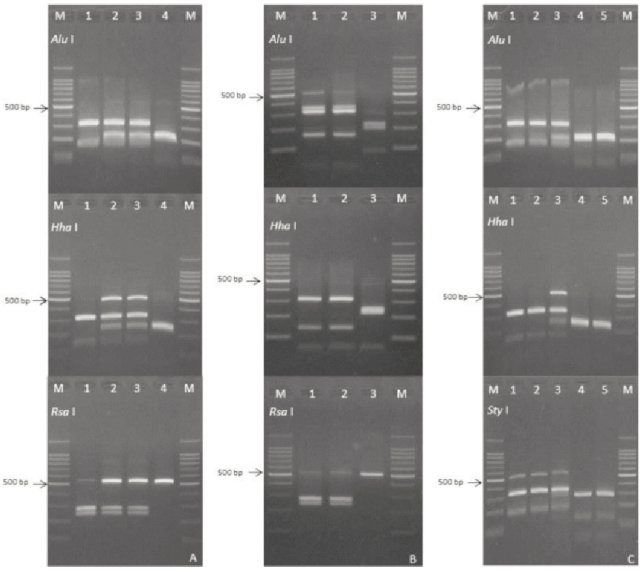

Fig. 3 - PCR-RFLP profile of parents and progenies. A: HRA × RCO. 1. HRA, 2. WXO1, 3.WXO2, 4. RCO. B: HRA × RCP. 1. HRA, 2. WXP, 3. RCP. C: HRA × RCY. 1. HRA, 2. WXY1, 3.WXY2, 4.YXW, 5. RCY.

Both WXO1 and WXO2 grew vigorously, and the first flowering was observed in WXO1 one year after transfer to *ex vitro* and in WXO2 two years after transfer to *ex vitro*. Both WXO1 and WXO2 showed an intermediate plant form (Fig. 4A, 4B, respectively) and leaf morphology of theirs parents. HRA had narrow light green leaves (Fig. 5A). RCO had wide lanceolate leaves with an undulate leaf margin, and the leaves were green or greyish green, sometimes with red-brown spots (Fig. 5D). WXO1 had lanceolate light green leaves, and WXO2 had lanceolate light green leaves with an undulate leaf margin (Fig. 5B, 5C, respectively). The inflorescence morphology of WXO1 and WXO2 was intermediate between the parents. WXO1 had an inflorescence with two flowers, and WXO2 had an inflorescence with eight flowers. *Habenaria radiata* produced two to four flowers (average 2.8 flowers) per inflorescence. RCO had eight to ten flowers (average 9.4 flowers) per inflorescence (Fig. 1B). The inflorescence morphology of WXY1 was similar to HRA, although it could not be accurately determined due to the poor growth of WXY1. HRA had a flower consisting of two pure white petals and a lip with three green ovate sepals. The lip had three main lobes; the two lateral lobes were highly fringed and the center lobe was simple (Fig. 6A). RCO had a flower consisting of two grey-brown orange petals and an orange lip and three sepals. The dorsal sepal was egg-shaped and the slanted lateral sepals sometimes rolled-in. The lip had three main lobes, with the two side lobes elliptical and the middle lip had two ovate-oblong lobes (Fig. 6B). The petals of both WXO1 and WXO2 were pale yellowish, which was an intermediate characteristic of the parents. The sepals of WXO1 and WXO2 were green, with the dorsal sepal being egg-shaped, the same as the female parent (HRA), and the lateral sepals were slanted and rolled-in, the same as the male parent (RCO). In addition, two petals were attached, forming an egg-shaped hood with the dorsal sepal. The lip had three main lobes, with the two lateral lobes being slightly fringed and the center lobe had two slightly ovate-oblong lobes, the same as the male parent (RCO) (Fig. 6D, 6E).The apomixtic progeny WXY1 produced flowers resembling *H. radiata* (Fig. 6F). One progeny derived from the cross combination of HRA × RCY (WXY2), confirmed as a true hybrid through PCR-RFLP analysis, showed an intermediate plant morphology between the parents (data not shown) but had not flowered because only one year had passed after acclimatization.

Fig. 4 - Plant morphology of hybrids. (A) WXO1, (B) WXO2, (C) WXY1.

Fig. 5 - Leaves of the parents and hybrids. (A) HRA, (B) WXO1, (C) WXO2, (D) RCO.

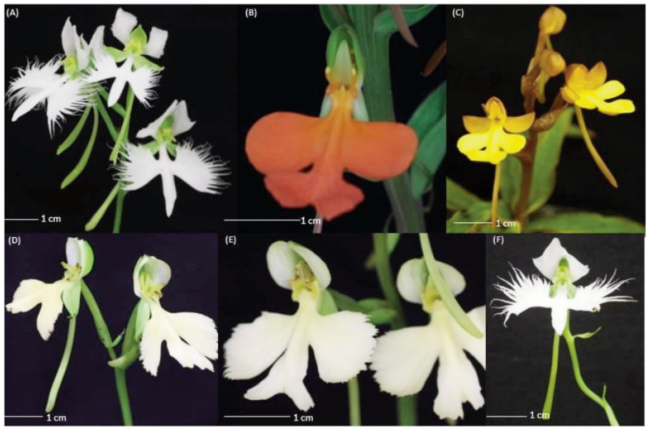

Fig. 6 - Floral morphology of the parents and hybrids. (A) HRA, (B) RCO, (C) RCY, (D) WXO1, (E) WXO2, (F) WXY1.

Plants in the genus *Habenaria* produce a storage organ having species-specific morphology. HRA produced stolons during the growing season, and new tubers formed at the top of the stolon. The HRA tuber was oval with a smooth surface (Fig. 7A). RCO produced a long oval tuber at the bottom end of the stem. The RCO tuber was bigger than that of HRA. The tuber had a rough surface and was densely covered with hair (Fig. 7C). Both WXO1 and WXO2 had

tubers with intermediate morphological characteristics of the parents (Fig. 7B) (Table 2).

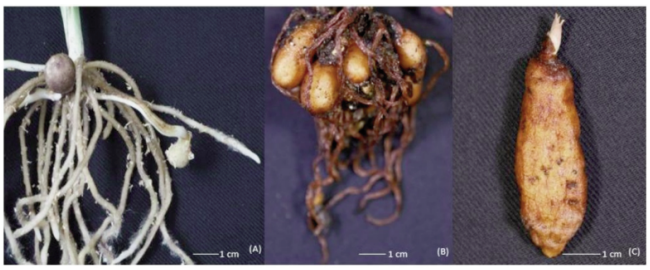

Fig. 7 - Tubers of the parents and hybrids. (A) HRA, (B) WXO2, (C) RC.

4. Discussion and Conclusions

Interspecific hybridization is a powerful breeding method that can produce new traits in ornamental plants including orchids. This study aimed to produce new *Habenaria* hybrids by using two different ecotype species (*H. radiata* and *H. rhodocheila* complex). The two species chosen in this study are different in not only plant morphology but also flowering physiology. Because the flowering time of *H. radiata* is during June to August under natural conditions, *H. rhodocheila* complexes were grown in a growth chamber controlled at 20°C to match the flowering time.

The fruit settings in *H. radiata* × *H. rhodocheila* complex were higher than in the opposite crosses. The germination of the obtained seeds was also different depending on the cross combination (Table 1). The seeds obtained from *H. radiata* × *H. rhodocheila* complex showed higher seed germination than those in the opposite crosses. Unilateral cross incompatibility is often observed in interspecific crossings including orchids (Johansen, 1990; Borba *et al*., 1999). The hybridity of the obtained progenies was investigated by using RFLP analysis. The results showed that all the tested progenies of HRA × RCO were true hybrids, whereas all the tested progenies of HRA × RCP and RCY × HRA were apomicts. Both true hybrids and apomicts were found in the progenies of HRA × RCY. The results of RFLP analysis were consistent with the observed plant morphological characteristics of the obtained progenies. The production of apomixis, including oblique and facultative, is known in some genera including *Habenaria* in the family Orchidaceae (Batygina *et al*., 2003). Zhang and Gao (2018) reported obligate apomixis in *H. malintana*. The present study showed that both true hybrids and apomicts appeared in *H. radiata* and *H. rhodocheila*. Successful crossing of different flower types of *H. radiata* has been reported (Kim *et al*., 2010). Adthalungrong *et al*. (2015) reported successful reciprocal crossing between RCP and RCY. These findings indicate that both *H. radiata* and *H. rhodocheila* do not have obligate apomixis. We consider that the facultative apomixis observed in this study is induced by interspecific crossing between distantly related species. In many plants, pollination is necessary for induction of apomixis (den Nijs and van Dijk, 1993). When HRA was used as a female parent, the frequency of apomict production varied depending on the pollen parents. The results suggest that the growth of the pollen tube in the ovary differed depending on the *H. rhodocheila* complex genotype. In HRA × RCY, only one pod was harvested, and both an apomict and true hybrid were obtained from the pod. This finding suggests that different embryogenesis, sexual and asexual, occurred at the same time. Further detailed morphological and genetic investigation of embryo development in interspecific hybridization is required to explain these phenomena. If the occurrence of apomixis is unpredictable in a practical breeding program, efficient selection of true hybrids is essential. The present study selected true hybrids at the early developmental stage of progenies through PCR-RFLP analysis. Selecting plants in the *in vitro* stage will be useful in a practical breeding program.

Table 2 - Morphology characteristics of two *Habenaria* species and interspecific hybrids

Name	Plant form (cm)		Leaves (cm)		Number of leaves	Flower (cm)		Stigma (cm)	Number of flowers	Inflorescences (cm)	Spur (cm)	Tuber (cm)		Storage organ characteristics
	Height	Spread	Length	Width		Length	Width					Length	width	
H. radiata (n=5)	13.8±2.4	15.1±0.4	6.9±0.7	0.5±0.1	5.0±3.0	3.2±0.1	2.2±0.1	0.3±0.1	2.8±0.4	18.8±2.0	3.3±0.8	1.5±0.1	1.1±0.2	Round-oval with many long stolons
H. rhodocheila (orange) (n=5)	22.3±3.1	23.4±0.6	9.5±1.9	2.2±0.4	6.4±0.2	3.1±0.0	2.3±0.1	0.4±0.1	9.4±1.2	25.8±2.4	3.5±0.5	3.6±1.5	1.5±0.2	Long oval
WXO1 (n=2)	10.8±2.8	9.3±1.0	3.2±0.7	0.8±0.1	4.0±1.0	2.4±0.2	1.6±0.2	0.4±0.1	1.5±0.5	12.9±3.4	3.1±0.1	1.4±0.1	1.0±0.1	Round-oval
WXO2 (n=2)	16.0±2.5	20.4±1.6	7.4±0.1	2.4±0.1	6.0±1.0	2.7±0.1	1.8±0.1	0.3±0.0	8.5±1.5	22.0±1.8	3.3±0.0	1.7±0.2	0.9±0.2	Round-long oval with many short stolons

We obtained new interspecific *Habenaria* hybrids, WXO1, WXO2, and WXY2. Both WXO1 and WXO2 showed intermediate morphological characteristics of the parents (Table 2). It is well-known in Orchidaceae that interspecific hybrids exhibit an intermediate morphological characteristic of parent. When the intermediate characteristics is observed in detail, the characteristics of either the female parent or the male parent often appear strongly in each organ as shown in this study. In case of *Ascocentrum ampullaceum* var. *aurantícum* × *Vanda coerulea*, the progenies showed flowers having pink petal color and spur which come from female parent and orange mottles on the petals come from male parent (Kishor *et al*., 2006). Interspecific hybrids between *Vanilla planifilia* and *V. aphylla* showed two types, light green plants without leaf resembling male parent and green plants with leaves resembling female parent (Divakaran *et al*., 2006). The flowers of WXO1 and WXO2 were pale yellow. The major flower pigment of *H. rhodocheila* complex is considered to be carotenoids (Sinumporn *et al*., 2015). In flowers that contain carotenoids as a main flower pigment, the flowers are orange when the amount of carotenoid is high and yellow when it is low (Kishimoto *et al*., 2007). It is assumed that the carotenoid content of the WXO1 and WXO2 petals is very low because one parent HRA has white petals, resulting in pale yellow petals in the hybrids. The interspecific hybrid of HRA × RCY named WXY2 showed intermediate morphological characteristics in leaves between parents (data not shown). Because that the hybrid was not large enough to give flowering, the floral characteristics was not determine yet. Interspecific hybrids often have flower color intermediate between the parents as reported in the reciprocal crossing progenies of RCP and RCY, which produced pale orange and pink flowers (Adthalungrong *et al*., 2015). The hybrids obtained in this study are new, but the ornamental value is not high enough. Further breeding steps such as backcrossing and self-pollination are required for flower pigment accumulation and flower form improvement. Furthermore, the temperature response of these hybrids is not yet clear because the resulting hybrids were grown under constant temperature conditions. It is necessary to clarify the characteristics of cultivation to evaluate the hybrids.

We obtained new interspecific hybrids between *H. radiata* and *H. rhodocheila* complex. The interspecific hybridization produced both true hybrids and apomicts. The apomicts were distinguished at the early developmental stage of the progenies through PCR-RFLP analysis. The obtained hybrids showed an interesting flower shape and color, which were intermediate between the parents. Further breeding processes, especially back crossing, is required to improve the ornamental value of the hybrids.

References

ABBAS S.R., GARDAZI S.D.A., SABIR S.M., SHAH A.H., ABBAS M.R., BATOOL A., 2013 - *"ABBAS" DNA extraction method from plants*. - Int. J. Sci. Eng. Res., 4(7): 989-994.

ADTHALUNGRONG A., SACHATI S., SARUNA M., 2015 - *Hybridization between* Habenaria rhodocheila *and* H. xanthocheila *and inheritance of flower color*. - Acta Horticulturae, 1087: 351-356.

BATISTA J.A.N., BORGES K.S., DE FARIA M.W.F., PROITE K., RAMALHO A.J., SALAZAR G.A., VAN DEN BERG C., 2013 - *Molecular phylogenetics of the species-rich genus* Habenaria *(Orchidaceae) in the New World based on nuclear and plastid DNA sequences*. - Mol. Phylogenet. Evol., 67: 95-109.

BATYGINA T.B., BRAGINA E.A., VASILYEVA V.E., 2003 - *The reproductive system and germination in orchids*. - Acta Biol. Cracov. Ser. Bot., 45(2): 21-34.

BORBA E.L., SHEPHERD G.J., SEMIR J., 1999 - *Reproductive systems and crossing potential in three species of* Bulbophyllum *(Orchidaceae) occurring in Brazilian 'campo rupestre' vegetation*. - Plant Syst. Evol., 217: 205-214.

CULLEN J., KNEES S.G., CUBEY H.S., SHAW J.M.H., 2011 - *The European garden flora flowering plants. Vol. I*. - Cambridge University Press, Cambridge, UK, pp. 472.

DEN NIJS A.P.M., VAN DIJK G.E., 1993 - *Apomixis*, pp. 229-245. - In: HAYWARD M.D., N.O.BOSEMARK, and I. ROMAGOSA (eds.) *Plant breeding: Principles and prospects*. Chapman & Hall, London, UK, pp. 550.

DIVAKARAN M., NIRMAL BABU K., RAVINDRAN P.N., PETER K.V., 2006 - *Interspecific hybridization in vanilla and molecular characterization of hybrids and selfed progenies using RAPD and AFLP markers*. - Sci. Hortic., 108(4): 414-422.

HARUKI K., HOSOKI T., NAKO Y., OHTA K., 1997 - *Possibility of classification in some species of* Lilium *by PCR-RFLP of ribulose-1, 5-bisphosphate carboxylase large subunit (rbcL) gene and ribosomal RNA gene*. - J. Japan. Soc. Hort., 66(1): 189-192.

JIN W.T., JIN X.H., SCHUITEMAN A., LI D.Z., XIANG X.G., HUANG W.C., LI J.W., HUANG L.Q., 2014 - *Molecular systematics of subtribe Orchidinae and Asian taxa of Habenariinae (Orchideae, Orchidaceae) based on plastid matK, rbcL and nuclear ITS*. - Mol. Phylogenet. Evol., 77: 41-53.

JOHANSEN B., 1990 - *Incompatibility in* Dendrobium *(Orchidaceae)*. - Bot. J. Linn. Soc., 103: 165-196.

KIM S.Y., ENDO M., YUN P.Y., KANNO A., 2010 - *Production*

of intraspecific hybrids between wild-type and petaloid-sepal cultivars in Habenaria radiata. - Sci. Hortic., 124: 415-418.

KIM S.Y., YUN P.Y., FUKUDA T., OCHIAI T., YOKOYAMA J., KAMEYA T., KANNO A., 2007 - *Expression of a DEFICIENS-like gene correlates with the differentiation between sepal and petal in the orchid,* Habenaria radiata *(Orchidaceae)*. - Plant Sci., 172: 319-326.

KISHIMOTO S., SUMITOMO K., YAGI M., NAGAYAMA M., OHMIYA A., 2007 - *Three routes to orange petal color via carotenoid components in 9 Compositae species*. - J. Japan. Soc. Hort., 76(3): 250-257.

KISHOR R., SHA VALLI KHAN P.S., SHARMA G.J., 2006 - *Hybridization and in vitro culture of an orchid hybrid Ascocenda 'Kangla'*. - Sci. Hortic., 108: 66-73.

KURZWEIL H., 2009 - *The genus* Habenaria *(Orchidaceae) in Thailand*. - Thai For. Bull. (Bot.), Special Issue: 7-105.

MALMGREN S., 1996 - *Orchid propagation; Theory and practice,* pp. 63-72. - In: ALLEN C. (ed.) *North American native terrestrial orchids. Propagation and production*. *Conference Proceedings, March 16-17*. The North American Native Terrestrial Orchid Conference, Germantown, MD, USA, pp. 116.

MITOMA M., KAJINO Y., HAYASHI R., ENDO M., KUBOTA S., KANNO A., 2019 - *Molecular mechanism underlying pseudopeloria in* Habenaria radiata *(Orchidaceae).* - Plant J., 99: 439-451.

MITOMA M., KANNO A., 2018 - *The greenish flower phenotype of* Habenaria radiata *(Orchidaceae) is caused by a mutation in the SEPALLATA-like MADS-box geneHrSEP-1*. - Front. Plant Sci., 9: 1-12.

MITSUKURI K., ARITA T., JOHKAN M., YAMASAKI S., MISHIBA K., ODA M., 2009 - *Effects of type of explant and dark preconditioning on bud formation in* Habenaria radiata *(Thunb.)* in vitro. - HortScience, 44(2): 523-525.

PEDRON M., BUZATTO C.R., SINGER R.B., BATISTA J.A.N., MOSER A., 2012 - *Pollination biology of four sympatric species of* Habenaria *(Orchidaceae: Orchidinae) from southern Brazil*. - Bot. J. Linn. Soc., 170: 141-156.

PRIDGEON A., 1992 - *The illustrated encyclopedia of orchids*. - Timber Press, Portland, Oregon, USA, pp. 138.

SINUMPORN P., FUKAI S., NARUMI T., POTAPOHN N., 2015 - *New usage of* Habenaria radiata *as a cut flower*. - Acta Horticulturae, 1087: 193-200.

STEWART S.L., KANE M.E., 2006 - *Asymbiotic seed germination and* in vitro *seedling development of* Habenaria macroceratitis *(Orchidaceae), a rare Florida terrestrial orchid*. - Plant Cell Tissue Organ Cult., 86: 147-158.

TANAKA N., YUKAWA T., HTWE K.M., MURATA J., 2015 - *An orchid checklist of Mt. Popa, Central Myanmar*. - Bull. Natl. Mus. Nat. Sci., Ser. B., 41(2): 69-89.

ZHANG W., GAO J., 2018 - *High fruit sets in a rewardless orchid: a case study of obligate agamospermy in* Habenaria. - Aust. J. Bot., 66: 144-151.