

## Development and morphological characteristics of interspecific hybridization of *Vanda liouvillei* Finet (1921) and *Vanda curvifolia* (Lindl.) L.M. Gardiner (2012) and the second-generation hybridization

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### Abstract

The *Vanda* genus is a beautiful Thai orchid that has been bred for use as cut flowers and ornamental plants. The development and morphological characteristics of the first-generation hybridization of *Vanda liouvillei* Finet as the female plant and *Vanda curvifolia* (Lindl.) L.M. Gardiner as the male plant (F<sub>1</sub>) and the hybridization of the second-generation hybrid plants (F<sub>2</sub>) on the ability of pod formation and seed germination in vitro were investigated. The result showed that 60% pod formation, the age and size of mature pods was 149 days with 1.15 cm in width and 3.50 cm in length after hand-pollination of the first-generation hybridization (F<sub>1</sub>). The hybrid seeds contained embryos and germinated after 23 days of sowing on a modified Vacin and Went (1949) agar medium. The first flowering occurred in vibrant orange-red petals with a red to yellow color graduated, erect inflorescence, and a fishtail-shaped lip similar to those of its male and female parent plants after three years and eleven months in the greenhouse. In the hybridization of the second-generation hybrid plants (F<sub>2</sub>), pod formation was 100%; the age and size of mature pods was 219 days with 0.77 cm in width and 2.33 cm in length after hand-pollination, smaller than those of the first-generation hybrid pods (F<sub>1</sub>). Seeds of the second-generation hybrid plants (F<sub>2</sub>) contained embryos and germinated after 28 days of sowing. The hybridization of the second-generation hybrids (F<sub>2</sub>) is for the development and production of small potted orchids with bright flower colors.

**Keywords:** Pollinia, Pollination, Pod, Protocorm, Hybrid

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## Introduction

The *Vanda* genus belongs to the Orchidaceae family of about 73 species (Gardiner and Cribb, 2013) distributed in Southeast Asia (Govaerts, 2012). Thailand has 9 species of *Vanda*, known for their beautiful, brightly colored flowers and fragrant scents, used as cut flowers and ornamental plants. Many *Vanda* hybrids have been created for use as cut flowers and ornamental plants (Thammasiri, 2016). One of these Thai orchids is *Vanda liouvillei* Finet, a medium-sized orchid native to Thailand, Myanmar, and Laos. Its lip is often fish-tail shaped, and it is protected by CITES Appendix II (De, 2022). In Thailand, *Vanda liouvillei* can also be intergeneric hybridized with other Thai orchids, such as *Aerides houlettiana*, *Aerides multiflora*, and *Rhynchostylis coelestis* (Jitsopakul et al., 2022), and also with *Seidenfadenia mitrata* (Rchb.f.) Garay (Jitsopakul et al., 2023). The potential of using *Vanda curvifolia* as the female plant and *Vanda liouvillei* as the male plant for hybridization was first registered as *Vanda* Red Rocket by Krull-Smith in 2021. The factors for success in orchid breeding depend on the quality of the pollinia (Indraloka et al., 2019), the flowering time, the compatibility of parent plants (Luo et al., 2012), the fertility of parents, the formation of pods (Vale et al., 2011), the maturity of pods and seeds, germination of seed, and development of plantlets (Utami and Hariyanto, 2019). Seeds of orchids are very small lack endosperm and are difficult to germinate in nature (Li et al., 2021). In vitro culture was employed for the germination of hybrid orchid seeds on a modified Vacin and Went (1949) medium supplemented with sucrose and coconut water (Utami et al., 2017; Utami and Hariyanto, 2019; Jitsopakul et al., 2022, 2023, 2025). Studying morphological characteristics of plants is necessary for comparing hybrid plants to their parents, such as plant size, leaf shape, inflorescences and flower appearance (Jangyukala and Hemant, 2021). Observing the morphological characteristics of new hybrid orchids is crucial for identifying and classifying plant species due to their diverse and observable nature (Hartati et al., 2021; Wu et al., 2022). The characteristics for distinguishing between plant species and their hybrids are plant size, leaves, flowers, and lips (Purwantoro et al., 2005; Santos et al., 2011; Hartati and Samanhudi, 2024) that are linked to pollination and seed production (Aros et al., 2019). In addition, the environment also affects the morphology of hybrid plants because they must adapt

to various conditions (Pablo et al., 2018). The flowering of hybrid orchids is considered a success in breeding, and it usually takes 3-10 years after pollination (Devi et al., 2023). The aim of this research focused on the intergeneric hybridization of *Vanda liouvillei* as the female plant and *Vanda curvifolia* as the male plant on the formation of pods, the germination of seed, the development and morphological characteristics of the first-generation hybrid plants ( $F_1$ ), and the ability of hybridization in the second-generation hybrid plants ( $F_2$ ) on pod formation and seed germination on the medium in vitro for producing smaller potted plants with bright colors of flowers.

## Material and Methods

### Plant material

Interspecific hybridization of *Vanda liouvillei* Finet (1921) as the female plant (Plate 1A-1B) and *Vanda curvifolia* (Lindl.) L.M. Gardiner (2012) as the male plant (Plate 1C-1D) was studied in the greenhouse of the Rajamangala University of Technology Isan, Surin Campus, Surin Province, Thailand. The flowers of both plant species appeared during March and April 2019.

### Interspecific hybridization of *Vanda liouvillei* and *Vanda curvifolia* on pod formation and seed germination in vitro

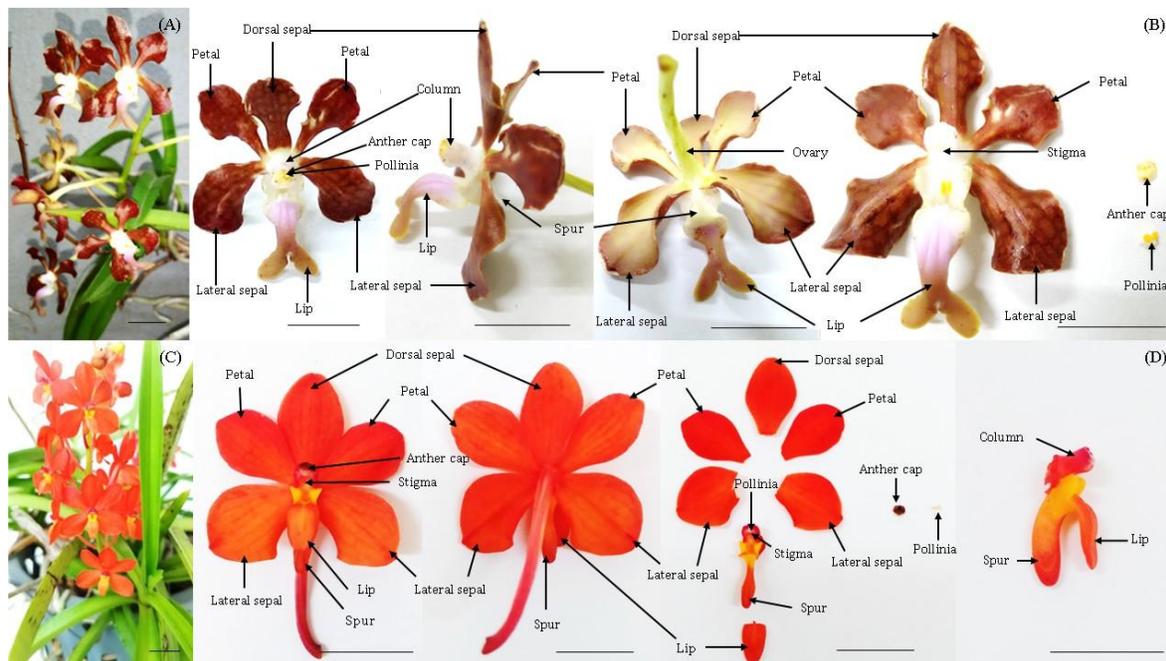
Pollinia from blooming flowers of *Vanda curvifolia* were collected and transferred to 20 flowers of *Vanda liouvillei*, after removing the pollinia of *Vanda liouvillei* to prevent self-pollination. After 30 days of hand-pollination, the fertilizing ability of the pollinia was determined by recording the pod formation, age, and size of mature pods. The mature pods of the first-generation hybrid ( $F_1$ ) were collected when their color changed from green to yellow. To prepare the mature hybrid pods for further study, they were cleaned with a detergent solution, rinsed with tap water for 5 minutes, immersed in 95% ethyl alcohol for 1 minute, and then flamed with a lamp inside a laminar air-flow cabinet until the flame went out. The pods were cut to remove the seeds, and then the seeds were sown on a modified VW (1949) agar medium supplemented with 1% sucrose, 15% coconut water, 0.7% agar, and a pH 5.2. After seeds germinated into protocorms, they were transferred to a modified VW (1949) agar medium supplemented with 2% sucrose, 15% coconut

water, 10% banana, 0.2% activated charcoal, 0.7% agar and pH 5.2 to develop shoots and roots. The culture was maintained under a 10 hour per day photoperiod by a fluorescent lamp at  $25\pm 2^{\circ}\text{C}$ .

### Transplantation of the first-generation hybrid plantlets ( $F_1$ )

The height of the first-generation hybrid plantlets ( $F_1$ ), about 3-4 cm after culture on a modified VW (1949)

agar medium, was removed from the bottles. They were washed with water to remove a medium and then transferred into plastic pots without planting materials. They were maintained in the greenhouse and were watered once every morning. The experiment was conducted with 5 replicates, each containing 100 plants. After 60 days, the survival of the hybrid plantlets was observed.



**Plate-1.** Plants and flower structure of (A-B) *Vanda liouvillei* Finet; (C-D) *Vanda curvifolia* (Lindl.) L.M. Gardiner (Scale: 1 cm.).

### Morphological characterizations of the first-generation hybrid plants ( $F_1$ )

Descriptive morphological characterizations were then conducted on *Vanda liouvillei* plants, *Vanda curvifolia* plants, and the first-generation hybrid plants of *Vanda liouvillei* with *Vanda curvifolia* ( $F_1$ ) when the first-generation hybrid plants ( $F_1$ ) first flowered. These characterizations were based on direct observation and documentation in the greenhouse. A total of 8 quantitative parameters and 52 characters were recorded for the hybrid plants (3 characters), leaves (7 characters), inflorescences (5 characters), flowers (6 characters), dorsal sepals (7 characters), lateral sepals (7 characters), petals (7 characters), and lips (10 characters). Each sample was repeated five times.

### Hybridization of the second-generation hybrid plants ( $F_2$ )

The second-generation hybridization ( $F_2$ ) was performed by hand-pollinating fully open flowers of hybrid orchids with pollinia from the same flower. The mature pods were then observed and recorded for age, size, and embryo development. Embryos were observed in hybrid seeds of  $F_2$  taken from mature pods at 40x magnification under a compound light source microscope. Hybrid pods of the second-generation hybridization ( $F_2$ ) were cleaned and then seeds were sown on the same medium and in vitro culture conditions as the first-generation hybrid plants ( $F_1$ ). The same process was followed for the second-generation hybrid ( $F_2$ ), and the resulting seed germinated into green protocorms, which were recorded.

### Statistical analysis

Data collections were analyzed using ANOVA for a completely randomized design (CRD) and significant differences were evaluated by the Duncan's Multiple Range Test (DMRT).

## Results and Discussion

### Interspecific hybridization on hybrid pod formation and hybrid seed germination in vitro

The interspecific hybridization between *Vanda liouvillei* and *Vanda curvifolia* was successful. The flowers of *Vanda liouvillei* turned dark and withered within 2-3 days after hand-pollination, resulting in pod formation. Approximately 60% of  $F_1$  hybrid pods were formed after 30 days of hand-pollination (Table 1). The  $F_1$  mature hybrid pods were harvested after 149.75 days of hand-pollination and the size was 1.15 cm in width and 3.50 cm in length (Plate 2A-2B). This is longer than the pods of *Vanda curvifolia* (0.58 cm in width and 2.44 cm in length) (Jitsopakul et al., 2022), but shorter than the pods of *Vanda liouvillei* (1.03 cm in width and 4.00 cm in length). After 23 days, the seeds of  $F_1$  were germinated on the modified VW (1949) agar medium. The addition of 15% coconut water to the medium promoted orchid seed germination, while the addition of 0.2% activated charcoal enhanced root formation (Nisha, 2017). The resulting protocorms were then transferred to the same modified VW (1949) agar medium for 30 days, where they developed into shoots and roots (Plate 2C). The results were consistent with what was reported in the previous studies, which have shown that supplementing the medium with coconut water is beneficial for orchid seed germination (Utami et al., 2017; Nisha, 2017; Mercadóa and Delgado, 2020). Additionally, immature pods from Vandaceous orchids have been found to germinate after 130 days of hand-pollination, much earlier than the mature pods (210-240 days) (Kishor et al., 2006; Kishor and Sharma, 2009).

### Transplantation of the first-generation hybrid plantlets ( $F_1$ )

Hybrid plantlets of the first-generation hybridization ( $F_1$ ) between *Vanda liouvillei* and *Vanda curvifolia* with well-developed shoots and roots and a height of approximately 3-4 cm, were removed after 120 days

of culture and transplanted into plastic pots without planting materials. The plantlets showed a survival rate of 92.20% after 60 days under greenhouse conditions (Plate 2D).

### Morphological characterization of the first-generation hybrid plants ( $F_1$ )

In this study,  $F_1$  has the first successful flowering of about 5% in February 2025 after being maintained in a greenhouse for three years and eleven months (Plate 3A-3B). The morphological characteristics of the first-generation hybrid plants ( $F_1$ ) in a total of 8 quantitative parameters and 52 characters were observed and recorded (Table 2) in plant size, leaves, flowers, and lips (Hartati et al., 2021; Wu et al., 2022). The first-generation hybrid plants ( $F_1$ ) are shorter than their parents. The leaves of the hybrid plants are smaller, with 1.10 cm x 13.88 cm (Plate 2E-2F). The racemes are composed of 6.50 flowers arranged in an alternate pattern (Plate 3C), with 6.2 cm x 5.85 cm of inflorescence (Plate 3D-3E). The hybrid flowers are a vibrant orange-red color with a graduated, and consist of three sepals and two petals; the sepals and petals have the same color, referred to as the calyx, where the sepals are arranged in one row with the petals. The shapes of sepals and petals of hybrid flowers are obovate. The dorsal sepals (0.72 cm x 1.66 cm) and lateral sepals (0.94 cm x 1.16 cm) have an obtuse apex and no undulate or waving at the edges (Plate 3F). The size of petals (0.92 cm x 1.18 cm) is larger than those of the male plant (*Vanda curvifolia*) but smaller than those of the female plant (*Vanda liouvillei*). The front of the orange-red lip is fishtail-shaped, with 0.42 cm x 1.10 cm, which is similar to that of the female plant, but it is different in size, and the fishtail of the female flower has a deeper groove. The lip is orange-red along the outer two-thirds, with the inner one-third exhibiting a gradual yellow coloration, while the side lobe has a yellow ground color, similar to that of the male plant (*Vanda curvifolia*). Overall, the hybrid flower displays a combination of characteristics from both parents. The success of the hybridization was verified through morphological analysis of the hybrid flower, which exhibited inheritance of key traits from the parent species. Notably, the hybrid combined desirable characteristics from both parents, including erect inflorescence, flower coloration, and lip morphology.

### Hybridization of the second-generation hybrids (F<sub>2</sub>)

After 30 days of hand-pollination, the second-generation hybridization (F<sub>2</sub>) showed 100% success in pod formation. The age of mature pods was approximately 219 days and the size was 0.77 cm x 2.33 cm (Plate 2F-2G), which is smaller than the pods of the first-generation hybrid (F<sub>1</sub>). However, the age and size of the second-generation hybrid pods (F<sub>2</sub>)

were comparable to those of the male plant, with the pods being approximately 133 days and 2.44 cm x 0.58 cm (Jitsopakul et al., 2022). Hybrid seeds of the second-generation hybridization (F<sub>2</sub>) contained embryos (Plate 2H-2I) and germinated into green protocorms after sowing for 28 days, which germination time was longer than that of the first-generation hybrid seeds on the same medium and culture conditions (Plate 2J).

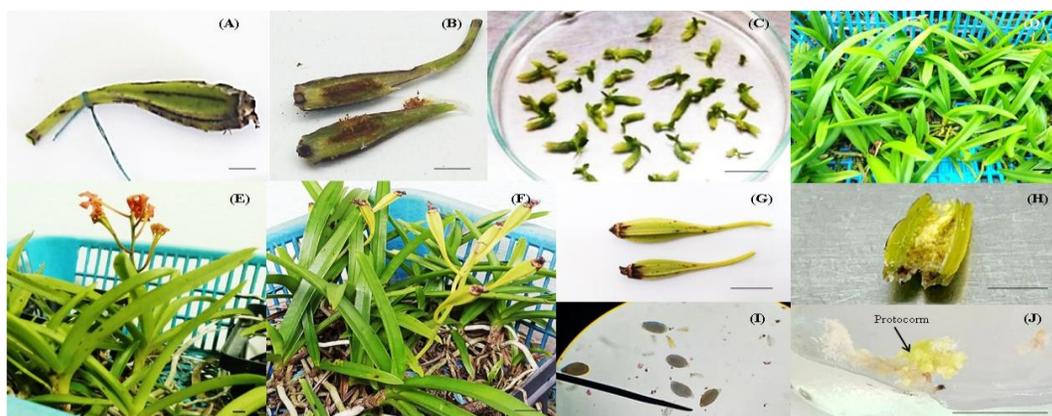
**Table-1.** Data collections of the first-generation hybrids (F<sub>1</sub>) and the second-generation hybrids (F<sub>2</sub>) of *Vanda liouvillei* Finet and *Vanda curvifolia* (Lindl.) L.M. Gardiner.

No.	Data collections	F <sub>1</sub> hybrids	F <sub>2</sub> hybrids
1	Number of flowers for hand-pollination	20 flowers	8 flowers
2	Number of hybrid pod formations	12 pods	8 pods
3	Hybrid pod formation	60% <sup>a</sup>	100% <sup>a</sup>
4	Days of hybrid mature pods	149.75±18.77 <sup>b</sup> days	219±7.76 <sup>a</sup> days
5	Width of hybrid pods	1.15±0.19 <sup>a</sup> cm	0.77±0.05 <sup>b</sup> cm
6	Length of hybrid pods	3.50±0.70 <sup>a</sup> cm	2.33±0.24 <sup>b</sup> cm
7	Days of hybrid seed germination after sowing on	23 <sup>b</sup> days	28 <sup>a</sup> days
8	Survival of hybrid plantlets in the greenhouse	92.20±2.04	-
9	The hybrid plants' first flowering	3 years and 11 months	-
10	Flowering time	February-March, 2025	-

Values represent means ± SD.

In each row, different letters (a, b) indicate significant difference at  $p < 0.05$  by the DMRT test.

The symbol "-" means that there is no data available because plantlets of F<sub>2</sub> haven't been transferred to the greenhouse.



**Plate-2.** Hybrid pods and plantlets of *Vanda liouvillei* Finet x *Vanda curvifolia* (Lindl.) L.M. Gardiner. (F<sub>1</sub>) and the second-generation hybrid plants (F<sub>2</sub>). (A) F<sub>1</sub> pod; (B) F<sub>1</sub> seeds; (C) F<sub>1</sub> plantlets after culture on the modified VW (1949) agar medium for 30 days; (D) F<sub>1</sub> plantlets in greenhouse after 90 days; (E) F<sub>2</sub> pods formation after hand-pollination for 10 days; (F) 180 days; (G) 219 days of F<sub>2</sub> pods; (H) F<sub>2</sub> seeds; (I) F<sub>2</sub> embryos (40x); (J) F<sub>2</sub> seeds germinated into green protocorms after sowing on the modified VW (1949) agar medium under a 10 hour per day photoperiod by a fluorescent lamp at 25±2°C for 28 days.

**Table-2.** Comparison of morphology characteristics of *Vanda liouvillei* Finet, *Vanda curvifolia* (Lindl.) L.M.Gardiner and F<sub>1</sub> hybrids.

Plant parts	Characters	<i>V. liouvillei</i>	<i>V. curvifolia</i>	F <sub>1</sub> hybrid
Plant	Height of plant (cm)	10.07±1.04 <sup>a</sup>	8.00±0.30 <sup>b</sup>	3.70±0.57 <sup>c</sup>
	Height of canopy (cm)	27.67±2.52 <sup>a</sup>	23.33±2.06 <sup>ab</sup>	12.42±3.09 <sup>b</sup>
	Shape of canopy (cm)	28.33±3.47 <sup>b</sup>	47.67±2.55 <sup>a</sup>	21.33±225 <sup>b</sup>
Leaf	Shape of leaf	Flat	Flat	Flat
	Attitude of leaf	Semi-pendulous	Semi-pendulous	Horizontal
	Undulate of leaf	Present	Absent	Absent
	Leaf margin	Entire	Entire	Entire
	Width of leaf (cm)	1.93±0.19 <sup>a</sup>	1.23±0.04 <sup>b</sup>	1.10±0.00 <sup>b</sup>
	Length of leaf (cm)	28.30±1.99 <sup>a</sup>	20.23±2.37 <sup>b</sup>	13.88±1.87 <sup>c</sup>
	Color of surface	Green	Green	Green
Inflorescence	Type of inflorescence	Raceme	Raceme	Raceme
	Width of inflorescence (cm)	10.97±1.61 <sup>a</sup>	4.40±0.12 <sup>c</sup>	6.20±0.42 <sup>b</sup>
	Length of inflorescence (cm)	12.23±3.52 <sup>a</sup>	10.43±2.29 <sup>a</sup>	5.85±1.65 <sup>b</sup>
	Number of flowers	7.50±0.50 <sup>b</sup>	17.75±3.27 <sup>a</sup>	6.50±1.50 <sup>b</sup>
	Arranging of flower	Alternate	Alternate	Alternate
Flower	First flower position	Upper than tip	Upper than tip	Upper than tip
	Spur	Present	Present	Present
	Type of spur	Flat	Sac	Sac
	Attitude of spur	Pendulous	Pendulous	Pendulous
	Size of flower (width x length; cm)	3.73±0.05 <sup>a</sup> x 4.07±0.09 <sup>a</sup>	2.20±0.14 <sup>b</sup> x 2.30±0.14 <sup>c</sup>	2.28±0.19 <sup>b</sup> x 2.52±0.08 <sup>b</sup>
	Number of colors	Two tones	Solid	Solid
	Dorsal sepal	Shape from the front	Elongated obovate	Obovate
Shape of apex		Obtuse	Obtuse	Obtuse
Undulate		Present	Absent	Absent
Size of dorsal sepal (width x length; cm)		1.00±0.00 <sup>a</sup> x 1.75±0.05 <sup>a</sup>	0.66±0.05 <sup>b</sup> x 1.20±0.00 <sup>b</sup>	0.72±0.04 <sup>b</sup> x 1.66±0.08 <sup>a</sup>
Color pattern		Graduated	Absent	Graduated
Base color		Rust orange	Orange-red	Orange-red
Second color		Yellow	No	No
Lateral sepals	Shape from the front	Obovate	Ovate	Obovate
	Shape of apex	Obtuse	Acute	Obtuse
	Undulate	Present	Absent	Absent
	Size of lateral sepal (width x length; cm)	1.30±0.00 <sup>a</sup> x 1.70±0.00 <sup>a</sup>	0.88±0.04 <sup>b</sup> x 1.15±0.09 <sup>b</sup>	0.94±0.05 <sup>b</sup> x 1.16±0.05 <sup>b</sup>
	Color pattern	Graduated	Absent	Graduated
	Base color	Rust orange	Orange-red	Orange-red
	Second color	Yellow	Absent	Absent

Values represent means ± SD.

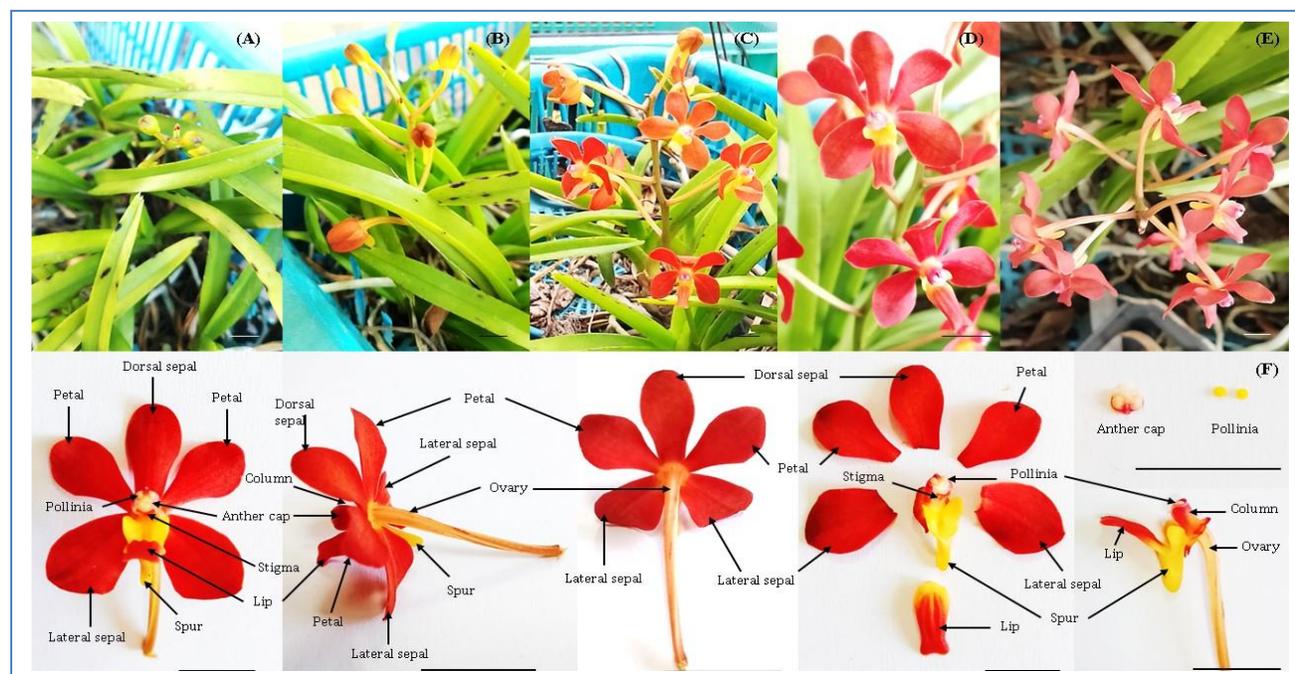
In each row, different letters (a, b) indicate significant difference at  $p < 0.05$  by DMRT test.

**Table-2.** Comparison of morphology characteristics of *Vanda liouvillei* Finet, *Vanda curvifolia* (Lindl.) L.M.Gardiner and F<sub>1</sub> hybrids.

Plant parts	Characters	<i>V. liouvillei</i>	<i>V. curvifolia</i>	F <sub>1</sub> hybrid
Petals	Shape from the front	Obovate	Obovate	Obovate
	Shape of apex	Obtuse	Obtuse	Obtuse
	Undulate	Present	Absent	Absent
	Size of petals (width x length; cm)	1.25±0.05 <sup>a</sup> x 1.70±0.10 <sup>a</sup>	0.67±0.05 <sup>c</sup> x 1.17±0.0 <sup>b</sup>	0.92±0.04 <sup>b</sup> x 1.18±0.04 <sup>b</sup>
	Color pattern	Graduated	Absent	Graduated
	Base color	Rust orange	Orange-red	Orange-red
	Second color	Yellow	Absent	Absent
	Lip	Shape from the front of lip	Fish tail	Rectangular
Bumps and ridges		Present	Present	Present
Undulate		Absent	Absent	Absent
Size of lip (width x length; cm)		1.03±0.11 <sup>a</sup> x 1.73±0.04 <sup>a</sup>	0.37±0.04 <sup>b</sup> x 0.53±0.09 <sup>c</sup>	0.42±0.04 <sup>b</sup> x 1.10±0.00 <sup>b</sup>
Variation of mid-lobe		Striped	Striped	Striped
Base color of lip		Rust orange	Orange-red	Red orange
Second color		Pinkish-purple	Orange-red	Yellow
Lower side color of lip		Brown	Yellow	Red
Ground color of side-lobe		Yellow-white	Yellow	Yellow
Shape of side-lobe		Flat	Flat	Flat

Values represent means ± SD.

In each row, different letters (a, b) indicate significant difference at  $p < 0.05$  by DMRT test.



**Plate-3.** Hybrid plants and flowers of *Vanda liouvillei* Finet x *Vanda curvifolia* (Lindl.) L.M. Gardiner (F<sub>1</sub>) after three years and eleven months of growth in the greenhouse. (A-B) the first flowering; (C-D) inflorescence; (E) alternate arrangement of flowers; (F) structure of hybrid flower (Scale: 1 cm.).

## Conclusions

The intergeneric hybridization between two Vandaceous orchids, *Vanda liouvillei* (female parent) and *Vanda curvifolia* (male parent), was successful as evidenced by pod formation, seed germination, and the development of hybrid plants. The morphological characteristics of the first-generation hybrid (F<sub>1</sub>) were evaluated using 8 quantitative parameters. A total of 52 were recorded, indicating small plants size, vibrant orange-red petals with a red- to- yellow color gradient, an erect inflorescence, and a fishtail-shaped lip, making the hybrids suitable for small potted orchids. The second-generation hybridization (F<sub>2</sub>) was also successful resulting in pod formation and in vitro seed germination.

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**Disclaimer:** None.

**Conflict of Interest:** None

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## Contribution of Authors

Jitsopakul N: Conceptualization of research, designing of the experiments, contribution of experimental materials, analysis of data and interpretation, and preparation of manuscript, validation and revised the manuscript.

Chunthaworn A & Jaidee K: Conducted experiments of field/laboratory experiments, data collection and analysis.

Pongket U: Conceptualization of research, conducted experiments, and preparation of manuscript.

Thammasiri K: Conceptualization of research, supervised the research, preparation of manuscript, validation and revised the manuscript.

All authors read and approved the final draft of the manuscript.

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