Maejo Int. J. Sci. Technol. **2018,** *12*(03), 241-250

Maejo International Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Intraspecific and interspecific crossability of some *Eulophia* species

Pongnatee Pintajam [*]**, Weenun Bundithya and Nuttha Potapohn**

Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand

* Corresponding author, e-mail: cloud.l.mirror@gmail.com

Received: 6 June 2017 / Accepted: 6 November 2018 / Published: 13 November 2018

Abstract: The intraspecific and interspecific crossability of some *Eulophia* spp. was assessed. It was found that the selfing percentages of *E. andamanensis*, *E. flava*, *E. graminea*, *E. macrobulbon* and *E. spectabilis* were 100.00, 100.00, 86.36, 86.67 and 92.30% respectively. The fruit set percentages of *E. flava* × *E. spectabilis* and *E. spectabilis* × *E. flava* were both 100.00%, while those of *E. spectabilis* × *E. andamanensis* and *E. spectabilis* × *E. macrobulbon* were 75.00 and 100.00% respectively. However, the fruit set of reciprocal crosses did not occur. The interspecific hybridisation between *E. spectabilis* and *E. graminea* was not successful. The genetic relationship of a number of *Eulophia* species was determined by random amplification of polymorphic DNA (RAPD) analysis. A total of twelve *Eulophia* samples –eight from *E. spectabilis* and one each from *E. andamanensis*, *E. flava*, *E. graminea* and *E. macrobulbon*–from five species could be separated into two groups by a combination of three primers. The first group consisted of *E. andamanensis* and *E. graminea*, and the others comprised *E. flava*, *E. macrobulbon* and all *E. spectabilis* specimens. The genetic data in this study correspond with the morphologically based classification at the species level.

Keywords: terrestrial orchid, genetic relationship, RAPD, *Eulophia* spp., crossability

---

INTRODUCTION

Orchid is one of the most economically important ornamental plants in Thailand, valued at more than 3,000 million Baht annually. The majority of exported orchids are cut flowers and potted plants of epiphytic orchids. Terrestrial orchids are not very well studied although many species have potential as commercial orchids in Thailand, for example *Calanthe*, *Cymbidium*, *Eulophia*, *Geodorum*, *Paphiopedilum* and *Spathoglottis* [1].

Eulophia belongs to the subfamily Epidendroideae and tribe Cymbidieae. The underground stem of this genus is a corm. The inflorescence is a raceme and usually arises from the base of the pseudobulb. The peduncle is erect and 30-100 cm long. *Eulophia* has various flower shapes and colours [2]. Thus, it has the potential for potted plants and its flower for a cut flower, especially *E. spectabilis*, which produces a good flower size, shape and number with an adequate vase life. It can grow in acidic media and low mineral conditions and does not require low temperature for growth and flowering. Therefore, the cultivation of this terrestrial orchid is possible all around Thailand. At Huai Hong Khrai Royal Development Centre, several types of *E. spectabilis* have been found with flowers of different colours. Therefore, improving *E. spectabilis* by interspecific hybridisation with other *Eulophia* spp. to introduce additional characters such as colour and flower shape to meet market demand might assist marketing strategies. In order to create novel hybrids with this species, some basic information on its crossability with other related species, as well as the genetic relationship of these species, should be obtained.

Some studies on breeding of terrestrial orchids, for examples *Habenaria*, *Pecteilis* [3] and *Paphiopedilum* [4], have been done but little information on this topic is available for *Eulophia*. The aim of this research is to study the crossability of *E. spectabilis* and the genetic relationship of this plant and a number of other *Eulophia* species using random amplification of polymorphic DNA (RAPD) technique for analysis to provide basic information for breeding programmes.

MATERIALS AND METHODS

Plant materials

Eulophia andamanensis Rchb. f., *E. graminea* Lindl., *E. macrobulbon* (Par. & Rchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. were used as parental plants (Figure 1). The plants were obtained from Huai Hong Khrai Royal Development Centre, Doi Saket district, Chiang Mai province. *E. flava* (Lindl.) Hook. f. was also employed. It was cultivated in an orchid nursery at Mae Hia Agricultural Centre for Research, Demonstration and Training, Mueang district, Chiang Mai province.

Hybridisation and Crossability

Intraspecific hybridisation

All *Eulophia*, i.e. *E. andamanensis*, *E. flava*, *E. graminea*, *E. macrobulbon* and *E. spectabilis* (only white flower and white lip with yellow stripe), were employed for selfing. Pollination was done during 8:00-10:00 a.m. The anther cap was removed to collect pollinia at the top of the column of the male parent flower using clean toothpicks. The pollinia were then put on the stigma under the male part of female parent flower. The fruit set (number of seed pods per hybridised flowers) was recorded using five flowers for each species. Seeds were obtained from mature fruit after about 5 months for all *Eulophia*. They were germinated using tissue culture technique on Vacin and Went medium [5]. Seed germination was recorded at 6 months by measuring the seedling coverage in germination containers (100% when entire area was covered with seedlings and 0% when no germination occurred).

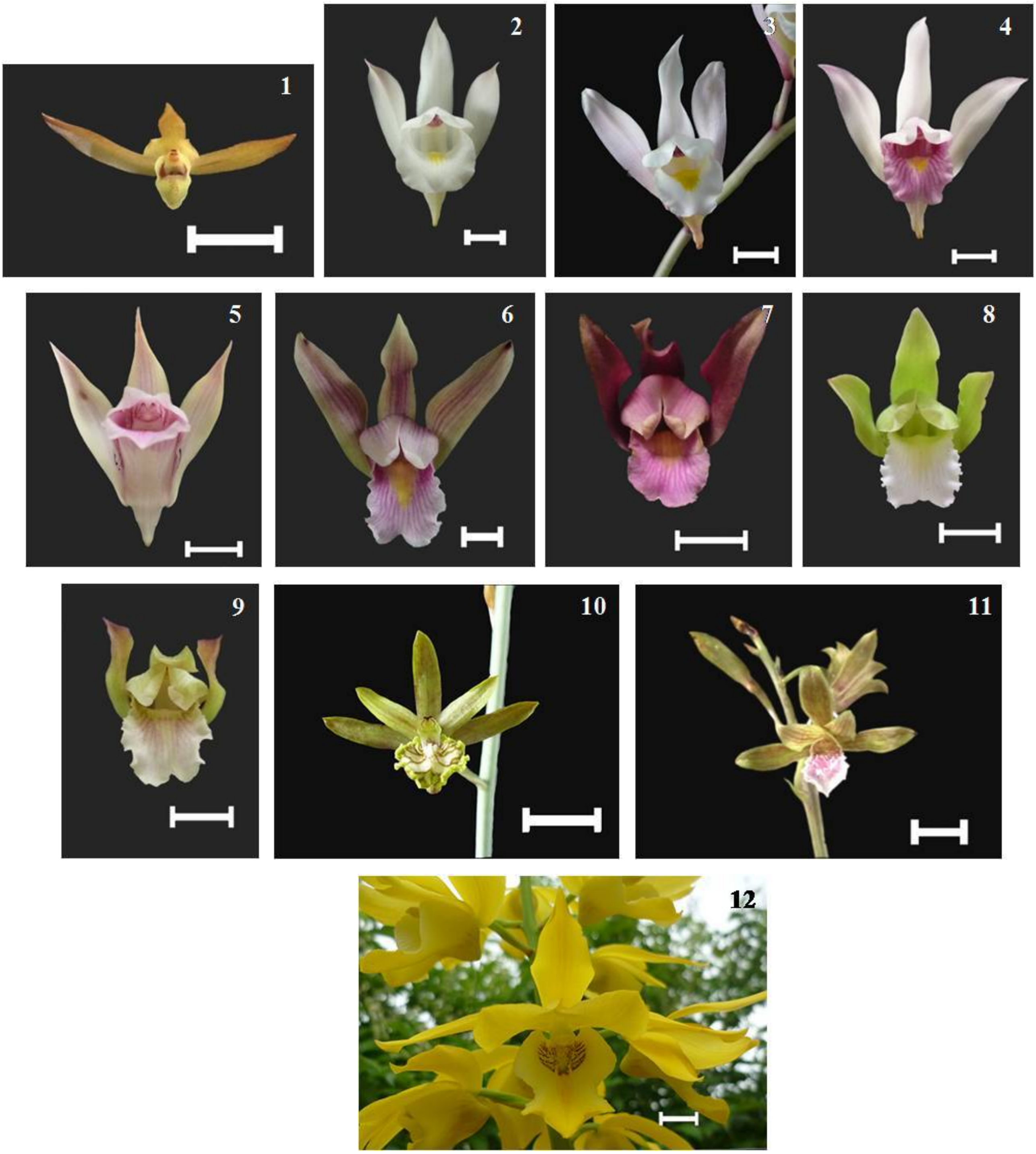

Figure 1. *Eulophia* used in hybridisation and genetic relationship analysis: (1) *E. macrobulbon*; (2) *E. spectabilis* (white flower, white lip with yellow stripe); (3) *E. spectabilis* (pink-white flower, white lip with yellow stripe); (4) *E. spectabilis* (pink-white flower, pink lip with yellow stripe); (5) *E. spectabilis* (pink flower, pink lip with yellow stripe); (6) *E. spectabilis* (green-pink flower, pink lip with yellow stripe); (7) *E. spectabilis* (dark pink to red flower, pink lip); (8) *E. spectabilis* (yellow-green flower, white lip); (9) *E. spectabilis* (pink-green flower, pink lip); (10) *E. andamanensis*; (11) *E. graminea*; and (12) *E. flava* (bar = 1 cm)

Maejo Int. J. Sci. Technol. **2018,** *12*(03), 241-250

Interspecific hybridisation

Hybridisation as well as reciprocal crosses between *E. spectabilis* (only white flower and white lip with yellow stripe) and other *Eulophia* (*E. andamanensis*, *E. flava*, *E. graminea* and *E. macrobulbon*) was done. The fruit set and seed germination were recorded.

Genetic Relationship

DNA extraction

Twelve samples from five *Eulophia* species, viz. *E. andamanensis*, *E. flava*, *E. graminea*, *E. macrobulbon* and *E. spectabilis*, were employed. Genomic DNA was extracted from leaves using a modified cetyltrimethyl ammonium bromide method [6].

DNA amplification

RAPD was performed in a total volume of 20 µL containing 10 ng template DNA, 100 µM dNTP mix, 100 ng OPA or OPF primer, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.8 unit *Taq* DNA polymerase and sterilised water. The amplification was performed in a thermal cycler (Perkin Elmer Gene Amp PCR System 2400). The PCR programme was set as indicated by Sinumporn [7]. The amplified products were examined by 1.8%-agarose gel electrophoresis. The gel was stained with ethidium bromide and observed with a UV box.

Genetic analysis

The RAPD bands were recorded as present (1) or absent (0) and assembled into matrix data. Genetic distances were calculated using the unweighted pair group method with arithmetic mean (UPGMA) for constructing a dendrogram.

RESULTS AND DISCUSSION

A study on the intraspecific and interspecific crossability of *E. spectabilis* was conducted. It was found that the selfing of this terrestrial orchid as well as other species in the same genus (*E. andamanensis*, *E. flava*, *E. graminea* and *E. macrobulbon*) was very successful and occurred at the same frequency as in other terrestrial orchids such as *Habenaria erichmichaelii* Hance, *H. rhodocheila* Hance, *H. xanthochelia* Ridl. [3], *Cymbidium insigne*, *C. lowianum*, *C. sinense* and *C. tracyanum* [8]. In this study each species exhibited *in vitro* germination at 77.00-95.50% (Table 1). In nature autogamy occurs in some terrestrial orchid species which produce numerous small fruits, for examples *Corallorhiza odontorhiza* and *Lister corduts*. Although self-pollination is prevented by ecological or structural barriers, in particular the rostellum tissue in most orchid species, it is possible with the availability of pollinators [9].

The interspecific hybridisation between *E. spectabilis* and other *Eulophia* reveals that *E. spectabilis* × *E. flava* as well as the reciprocal cross is 100% successful (Table 1). The results are similar to crosses of *H. erichmichaelii* × *H. rhodocheila*, *H. rhodocheila* × *H. lindleyana* [3], *Paphiopedilium bellatulum* × *P. callosum* and *P. bellatulum* × *P. primulium* [4]. Seed germination of *E. spectabilis* × *E. flava* and *E. flava* × *E. spectabilis* is at 86.00% for both crosses (Table 1).

In this study *E. flava* plants which were cultivated in a nursery at Mae Hia Agricultural Centre for Research, Demonstration and Training (340 MSL) could not be used as successfully as the female parent. However, those cultivated at the Huai Hong Khrai Royal Development Study

Table 1. Percentages of fruit set and seed germination from intraspecific and interspecific hybridisation of *E. spectabilis* and other *Eulophia* spp.

Female plant	Male plant	Number of pollinated flowers	Number of seed pods	Fruit set (%)	Seed germination (%)
E. spectabilis	*E. spectabilis*	13	12	92.30	95.50±7.07
E. flava	*E. flava*	5	5	100.00	91.40±3.50
E. macrobulbon	*E. macrobulbon*	15	13	86.67	83.33±15.27
E. andamanensis	*E. andamanensis*	10	10	100.00	85.00±3.53
E. graminea	*E. graminea*	22	19	86.36	77.00±5.70
E. spectabilis	*E. flava*	5	5	100.00	86.00±6.52
E. flava	*E. spectabilis*	5	5	100.00	86.00±4.18
E. spectabilis	*E. macrobulbon*	13	13	100.00	86.25±13.78
E. macrobulbon	*E. spectabilis*	20	0	0.00	-[1]
E. spectabilis	*E. andamanensis*	4	3	75.00	80.00±6.12
E. andamanensis	*E. spectabilis*	5	0	0.00	-[1]
E. spectabilis	*E. graminea*	5	0	0.00	-[1]
E. graminea	*E. spectabilis*	9	1	11.11	0.00±0.00

Note: *E. spectabilis* in this study is in the form of white flower and white lip with yellow stripes.
[1]Data unavailable due to no fruit set

Centre (800 MSL) could be used when hybridised with *E. spectabilis*. This could be due to *E. flava* being a low temperate orchid normally found at 800 m above mean sea level or higher. In order to hybridise this species successfully, crosses should be made on the plant that grows in cool temperatures.

Other hybridisations are successful when using *E. spectabilis* as the female parent. For *E. spectabils* × *E. andamanensis* and *E. spectabilis* × *E. macrobulbon*, the percentages of fruit set are 100% and 75% respectively (Table 1). Seeds of *E. spectabils* × *E. andamanensis* and *E. spectabilis* × *E. macrobulbon* that are cultivated *in vitro* can germinate at 80.00% and 86.25% respectively (Table 1).

The fruit set of *E. graminea* × *E. spectabilis* is only 11.11% and none of the seeds germinate and the reciprocal cross also fails (Table 1).

According to Rakpaibulsombat [10], there are five main reasons for unsuccessful hybridisation. The first two are sterility of one of the parents and the different chromosome numbers of the parents. The third is an unsuitable parent plant; if the female flower is smaller than the male one, the ovary tube of the former is relatively too narrow for penetration by the pollen tube of the male parent. The fourth is the unsuitable environment for growth and development of the female plant. For example, if the female parent is a temperate plant, it should be grown at a low enough temperature to enable interspecific hybridisation. The last reason is the genetic relationship as the hybridisation of interspecific parents will not be successful if the parents are distantly related.

This study shows that *E. andamanensis*, *E. flava*, *E. graminea*, *E.macrobulbon* and *E. spectabilis* are self-fertile because they can set seeds after selfing. However, the interspecific hybridisation of *E. andamanensis* × *E. spectabilis* and *E. macrobulbon* × *E. spectabilis* is not successful.

Interestingly, when *E. spectabilis* is used as female parent, seed pods develop successfully. The hybridisation of *E. spectabilis* as female parent is likely to be possible when the male parent has a smaller chromosome number, considering 2n=54 for *E. spectabilis* [11], 2n=42 for *E. andamenensis* [12] and 2n=48 for *E. macrobulbon* [11], despite a much bigger flower size of *E. spectabilis* compared to those of *E. andamanensis* and *E. macrobulbon*. As found in several trials of hybridisation of epiphytic and terrestrial orchids having different flower sizes, for example *Ascocentrum ampullaceam* (Roxb.) Schltr var. *auranticum* × *Vanda coelurea* Griff. [13], *Paphiopedilum jakii* × *P. villosum* and *P. philippinense* × *P. villosum* [4], it is difficult to get seed pods. The results of this study indicate that *E. spectabilis* can be used as female parent better than other species.

The hybridisation between *E. spectabilis* and *E. graminea* fails even when a reciprocal cross is done. This could have resulted from the difference in flower morphology, chromosome number and genetic relationship of both the terrestrial orchids: *E. spectabilis* 2n=54 [11] and *E. graminea* 2n=56 [14].

The analysis of the genetic relationship of *Eulophia* species was conducted by the RAPD technique. It was found that five primers, namely OPA4, OPA10, OPA20, OPF1 and OPF13, yield polymorphic bands for all terrestrial orchids in this study (Figure 2). Single primers cannot distinguish these orchids in correspondence with their morphological characteristics. However, a combination of OPA4, OPA10 and OPF13 is appropriate for grouping *Eulophia*.

The genetic similarity was used to construct a similarity matrix [15] (Table 2). The similarity values were calculated by scoring the total number and the polymorphic number of bands obtained from a combination of OPA4, OPA10 and OPF13 primers. Similarity values obtained range from 0.382 between *E. flava* (sample 12) and *E. graminea* (sample 11) to 0.971 between *E. spectabilis* (pink-white flower and white lip with yellow stripe: sample 3) and *E. spectabilis* (pink-white flower, pink lip with yellow stripe: sample 4). The genetic similarity of *E. spectabilis* of various colours (lanes 2 - 9) ranges between 0.618 - 0.971 (Table 2).

Data scored from the same primer combinations were used to generate a UPGMA dendrogram, which shows that these orchids can be grouped into two major clusters (Figure 3). The first cluster (I) includes three species: all of *E. spectabilis*, *E. macrobulbon* and *E. flava*. The second cluster (II) comprises two species: *E. andamanensis* and *E. graminea*. The variation of *E. spectabilis* can also be distinguished using this dendrogram, except for those with white flower with yellow stripe (sample 3) and pink flower with yellow stripe (sample 4).

These findings indicate that *E. andamanensis* and *E. graminea* are related. These two species share quite a number of similar morphological characteristics, e.g. stem, tuber, leaf and inflorescence. The only differences are flower colour and flower shape. From the dendrogram, *E. spectabilis*, *E. flava* and *E. macrobulbon* in all colours are grouped together (Figure 3). This may be due to their similarity in terms of underground stem and leaf. However, their flower shape, number of flowers per inflorescence and flower colour are different.

The genetic relationship study reveals that *E. spectabilis* is more closely related to *E. flava* than other *Eulophia*. This could explain why the crossability between *E. spectabilis* and *E. flava* is more successful than in other pairs while the hybridisation of *E. spectabilis* × *E. graminea* fails.

Maejo Int. J. Sci. Technol. **2018,** *12*(03), 241-250

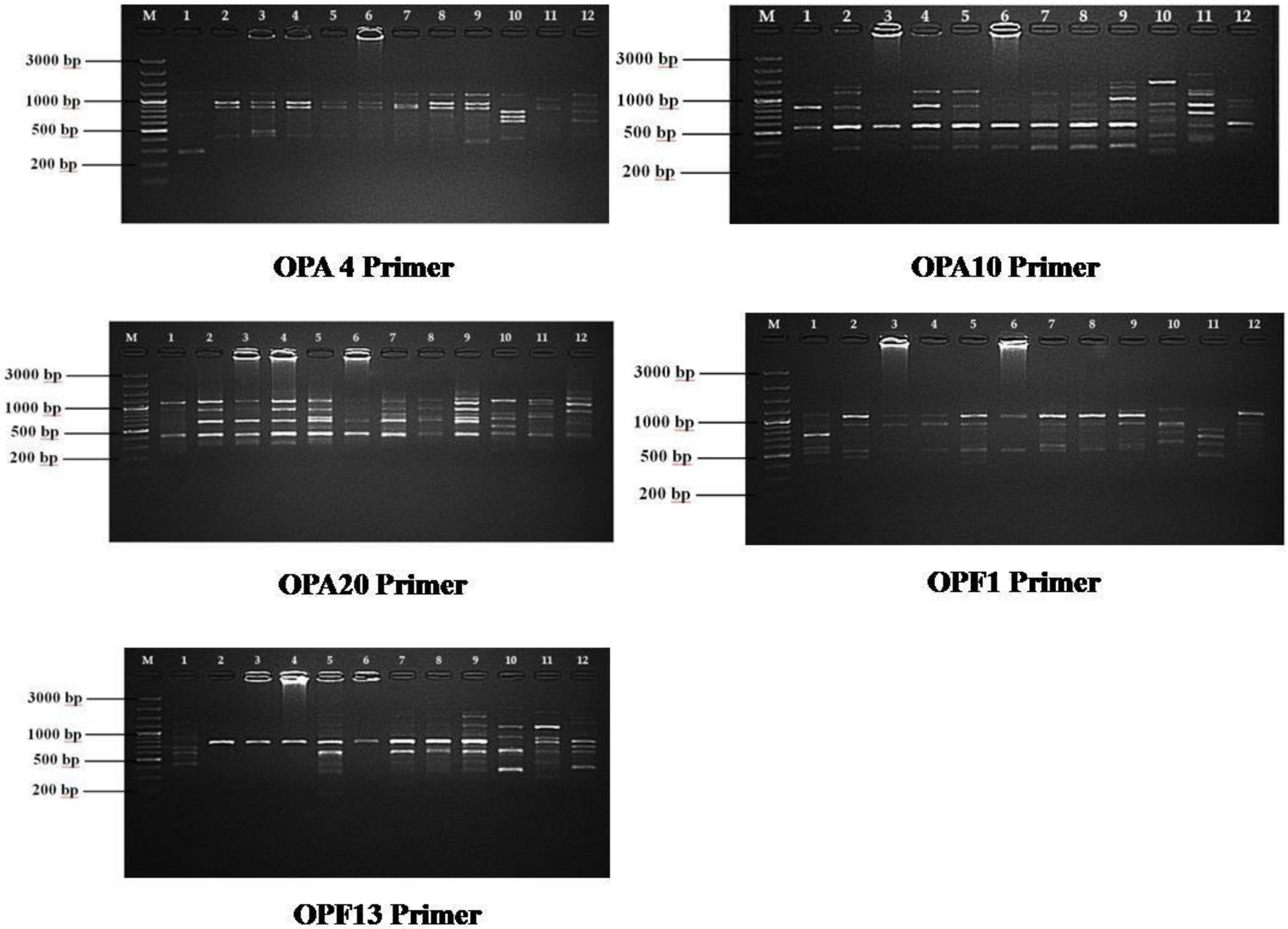

Figure 2. RAPD products of 12 samples from five *Eulophia* species using 10-nucleotide primers. [(M) 100 bps plus DNA ladder; (1) *E. macrobulbon*; (2) *E. spectabilis* (white flower, white lip with yellow stripe); (3) *E. spectabilis* (pink-white flower, white lip with yellow stripe); (4) *E. spectabilis* (pink-white flower, pink lip with yellow stripe); (5) *E. spectabilis* (pink flower, pink lip with yellow stripe); (6) *E. spectabilis* (green-pink flower, pink lip with yellow stripe); (7) *E. spectabilis* (dark pink to red flower, pink lip); (8) *E. spectabilis* (yellow-green flower, white lip); (9) *E. spectabilis* (pink-green flower, pink lip); (10) *E. andamanensis*; (11) *E. graminea*; and (12) *E. flava*].

The use of the UPGMA technique also explains the clustering of other terrestrial orchids, for example *Paphiopedilum* and *Pragmipedilum* based on leaf morphology but not flower colour [16], and *Habenaria* and *Pecteilis* based on flower colour [7].

Fruit development is not a good indicator of successful breeding. Even though a fruit set occurs, the seed may not germinate. The fruit can develop with stimulation by auxin from pollen when pollination occurs [17]. Evidently, the pollen extract can stimulate the ovary to develop fruit without fertilisation [18].

Maejo Int. J. Sci. Technol. **2018,** *12*(03), 241-250

Table 2. Similarity matrix of 12 *Eulophia* samples from five species

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.000											
2	0.618	1.000										
3	0.647	0.912	1.000									
4	0.676	0.941	0.971	1.000								
5	0.706	0.853	0.882	0.912	1.000							
6	0.676	0.882	0.912	0.941	0.853	1.000						
7	0.765	0.794	0.824	0.853	0.882	0.853	1.000					
8	0.735	0.882	0.912	0.941	0.912	0.941	0.912	1.000				
9	0.647	0.735	0.706	0.735	0.765	0.735	0.824	0.794	1.000			
10	0.441	0.471	0.500	0.471	0.500	0.471	0.500	0.529	0.441	1.000		
11	0.500	0.471	0.441	0.471	0.500	0.529	0.559	0.529	0.500	0.529	1.000	
12	0.588	0.618	0.647	0.676	0.706	0.676	0.706	0.733	0.588	0.500	0.382	1.000

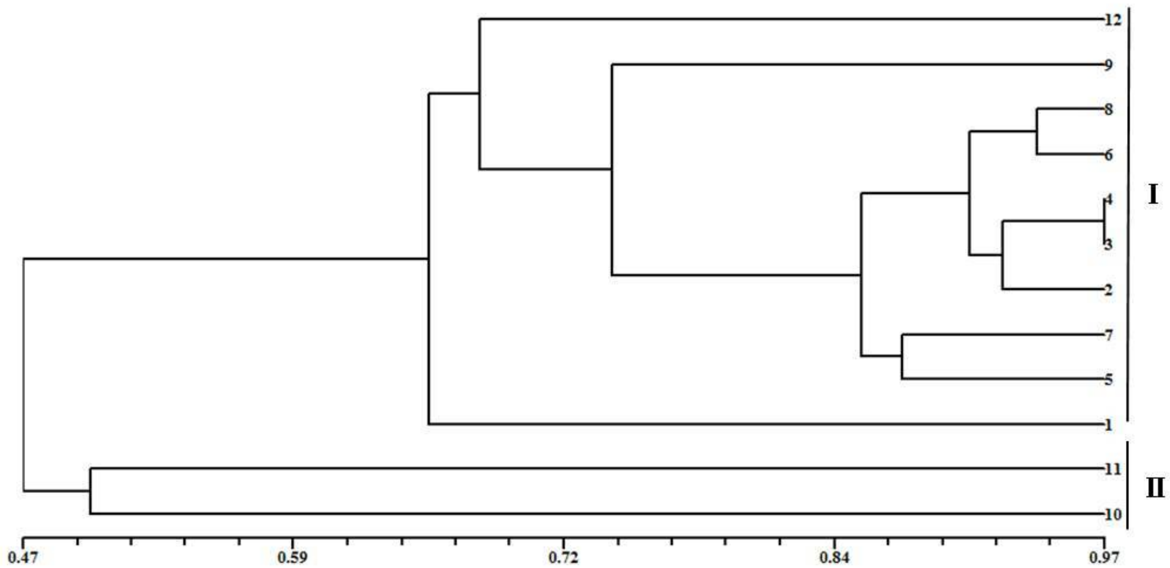

Figure 3. Dendrogram of *Eulophia* based on UPGMA cluster analysis and similarity index using three primers [(1) *E. macrobulbon*; (2) *E. spectabilis* (white flower, white lip with yellow stripe); (3) *E. spectabilis* (pink-white flower, white lip with yellow stripe); (4) *E. spectabilis* (pink-white flower, pink lip with yellow stripe); (5) *E. spectabilis* (pink flower, pink lip with yellow stripe); (6) *E. spectabilis* (green-pink flower, pink lip with yellow stripe); (7) *E. spectabilis* (dark pink to red flower, pink lip); (8) *E. spectabilis* (yellow-green flower, white lip); (9) *E. spectabilis* (pink-green flower, pink lip); (10) *E. andamanensis*; (11) *E. graminea*; and (12) *E. flava*].

CONCLUSIONS

This study on the intraspecific and interspecific crossability of *E. spectabilis* species shows that selfing is successful in all *Eulophia* spp. studied, i.e. *E. andamanensis*, *E. flava*, *E. graminea*, *E. macrobulbon* and *E. spectabilis*. The interspecific hybridisation between *E. spectabilis* and other

Eulophia reveals that the hybridisation between *E. flava* and *E. spectabilis* is achievable, whereas that between *E. spectabilis* and *E. andamanensis* or *E. macrobulbon* is possible when *E. spectabilis* is used as the female parent. The interspecific hybridisation between *E. graminea* and *E. spectabilis* is not successful. The genetic relationship indicates the possibility of crossability: the closer the genetic relationship, the greater the possibility of crossability.

ACKNOWLEDGEMENT

The Huai Hong Khrai Royal Development Study Centre is thanked for providing some plant materials used in this study.

REFERENCES

1. A. Thaithong, "Thailand Orchid", Amarin Printing and Publishing, Bangkok, **2002**, pp.10-11 (in Thai).
2. T. K. Bose and S. K. Bhattacharjee, "Orchid of India", Naya Prokash, Calcutta, **1989**, pp.272-280.
3. C. Kawchadee, W. Bundithya, C. Sotthikul and N. Potapohn, "Interspecific and intergeneric crossability of some *Habenaria* and *Pecteilis*, terrestrial orchids", *J. Agric.*, **2012**, *28*, 263-272 (in Thai).
4. W. Tongkham, N. Potapohn and C. Tiyayon, "Intersubgeneric crossability of some *Paphiopedilum* species", *J. Agric.*, **2015**, *31*, 241-249 (in Thai).
5. E. F. Vacin and F. W. Went, "Some pH changes in nutrient solutions", *Bot. Gaz.*, **1949**, *110*, 605-613.
6. P. Taywiya, "Analysis of genetic relationship and marker of genus *Phalaenopsis* and hybrids by molecular technique", *PhD Thesis*, **2010**, Chiang Mai University, Thailand.
7. P. Sinumporn, "Genetic relationship analysis of *Habenaria* and *Pecteilis* by RAPD technique", *MSc Thesis*, **2014**, Chiang Mai University, Thailand.
8. O. Wongnan, "Intersectional crossability of some *Cymbidium* species", *MSc Thesis*, **2010**, Chiang Mai University, Thailand (in Thai).
9. A. Dafni and P. Bernhardt, "Pollination of terrestrial orchids of southern Australia and the Mediterranean region: Systematic, ecological, and evolutionary implications", *Evol. Biol.*, **1990**, *24*, 193-252.
10. S. Rakpaibulsombat, "Orchid pod is hard to achieve", in "Orchid Cultivation from Experience" (Ed. S. Rakpaibulsombat), Dharmasarn Printing, Bangkok, **1997**, Ch.18 (in Thai).
11. P. Pintajam, C. Tiyayon and N. Potapohn, "Interspecific and intergeneric crossabilites of *Eulophia macrobulbon* (Par.& Pchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh.", *J. Agric.*, **2016**, *32*, 299-308 (in Thai).
12. S. Bunnag and P. Theerakulpisut, "Cytogenetics of some orchid species in plant genetics conservation at Khok Phu Ta Ka, Amphoe Phu Wiang, Khon Kaen", *KKU Res. J.*, **2007**, *12*, 393-401 (in Thai).
13. K. Rajkumar, P. S. S. V. Khan and G. J. Sharma, "Hybridization and *in vitro* culture of an orchid hybrid *Ascocenda* 'Kangla'", *Sci. Hortic.-Amsterdam*, **2006**, *108*, 66-73.
14. J. Prarasi, "Characterization of *Eulophia graminea* Lindl. at the Huai Hong Khrai Royal Development Study Centre", *MSc Thesis*, **2006**, Chiang Mai University, Thailand (in Thai).

Maejo Int. J. Sci. Technol. **2018,** *12*(03), 241-250

15. M. Nei and W. H. Li, "Mathematical model for studying genetic variation in terms of restriction endonucleases", *Proc. Natl. Acad. Sci. USA.*, **1979**, *76*, 5269-5273.
16. S. Y. Chung, S. H. Choi, M. J. Kim, K. E. Yoon, G. P. Lee, J. S. Lee and K. H. Ryu, "Genetic relationship and differentiation of *Paphiopedilum* and *Phragmepedium* based on RAPD analysis", *Sci. Hortic.-Amsterdam*, **2006**, *109*, 153-159.
17. S. Techapinyawat, "Plant Physiology", Kasetsart University Press, Bangkok, **2001**, p.199 (in Thai).
18. J. Arditti and R. L. Knauft, "The effects of auxin, actinomycin D, ethionine, and puromycin on post-pollination behavior by *Cymbidium* (Orchidaceae) flowers", *Am. J. Bot.*, **1969**, *56*, 620-628.