HYBRID ESTABLISHMENT OF CALANTHE GROUP AND SCAR MARKER DEVELOPMENT FOR HYBRID IDENTIFICATION

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ABSTRACT

Morphological study for the selection of desirable characteristics, patterns of hybrid construction, *in vitro* culture of putative hybrids and some parental species and hybrid determination using molecular SCAR marker technique of the orchid *Calanthe* group were studied, investigated and interpreted. The results on morphological study indicated that several species in the *Calanthe* and *Phaius* genera and seven species from both *Calanthe* and *Phaius* genus were picked up for breeding experiment because of their attractive and distinguish morphological characteristics for commercialization. For hybrid improvement, the breeding systems via intergeneric, interspecific cross pollination and intraspecific self-pollination among seven species were conducted by hand pollination method. The results revealed that three types of intraspecific self-pollination; autogamous, geitonogamous and xenogamous, had no effect on the percentage of pod setting in both *Calanthe* and *Phaius* genus. Interspecific hybrids originated from *P. mishmensis* × *P. tankervilliae* and their reciprocal cross as well as a hybrid initiated from *P. mishmensis* × *P. tankervilliae* var. *alba* was successfully created. Other intergeneric hybrids originated from *P. mishmensis* × *C. masuca* and *P. mishmensis* × *C. cardioglossa* were also achieved. However, no embryo formation in *P. mishmensis* × *C. cardioglossa* hybrids was observed. The mature seeds derived from two interspecific hybrids; *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba* were *in vitro* cultured

on modified Vacin and Went (VW) medium supplemented with different concentrations of coconut water and potato extract. The cultures were incubated under light and dark condition for 4 months. The results found that the highest percentage of seed germination (75.5%) in *P. mishmensis* × *P. tankervilliae* was observed on the VW medium added with 150 ml/L CW and cultured under dark condition whereas the highest percentage of hybrid seed germination (100%) in *P. mishmensis* × *P. tankervilliae* var. *alba* could receive on the VW medium augmented with 50 g/L PE. In comparison to the developmental efficiency, the results indicated that adding 150 ml/L CW into the VW medium could stimulate the highest developmental stage (17.5%) in *P. mishmensis* × *P. tankervilliae* while adding 50 g/L of PE into the VW medium tended to stimulate better developmental stage (31.0%) in *P. mishmensis* × *P. tankervilliae* var. *alba*. The influence of organic supplements on growth and multiplication efficiency of twelve week-old self-pollinated seedlings from two species; *P. mishmensis* and *P. tankervilliae* and their hybrid (*P. mishmensis* × *P. tankervilliae*) was studied. Seedlings were cultured on ½ Murashige and Skoog (MS) medium supplemented with various concentrations of CW combined with PE. The results indicated that *P. mishmensis* presented the highest number of shoots (3.03 shoots/explant) when cultured on the medium added with 50 ml/L CW and 50 g/L PE whereas *P. tankervilliae* and putative hybrid of *P. mishmensis* × *P. tankervilliae* improved the highest shoot number (3.93 shoots/explant) and (2.43 shoots/explant), respectively when cultured on the medium added with 25 ml/L CW and 50 g/PE. Additionally, plantlets of *P. mishmensis* were selected and transferred to grow on seven different planting substrates and the results revealed that acclimatized plantslets showed 100 % of survivals when mixing soil, sand, and coconut husk chip were used as planting substance. Determination of the hybrids received from *Calanthe* group using molecular SCAR marker technique was developed and identified using a random RAPD primer. The results showed that four out of twenty random primers were initially screened as specific primers. Subsequently, specific RAPD fragments and sequences were designed as SCAR primers. Thirteen SCAR primer pairs were developed. The results showed that five primer pairs could be specific to identify these orchids particularly in the genus level. Meanwhile, three primer pairs could be applied for the *Calanthe* orchid identification within the species level. This suggested that

three designed SCAR markers including Pmis524, Pmis364 and Ptan285 were successfully developed as specific primers which could be used for identification of these putative hybrids of *Calanthe* group. From the overall study, an investigation on hybrid improvement and *in vitro* culture of the *Calanthe* group was successfully established and determination of hybrids using molecular SCAR marker is an efficient technique for hybrid indication and identification.

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CHAPTER I

INTRODUCTION

Background

Orchidaceae is one of the largest families of flowering plants. Roughly 25,000 species comprising up to 10 % of all angiosperms, including five subfamilies and about 870 genera were recognized. The *Calanthe* group, a member of Orchidaceae family in Epidendroideae subfamily and Collabienae tribe, is currently identified into 200 known species. Recently, around 260 species of *Calanthe* group have been identified from all over the world. In Thailand, three different genera of the *Calanthe* group such as *Calanthe* R.Br., *Phaius* Lour. And *Cephalantheropsis* Guillemin was reviewed and 31 described species were discovered (Kurzweil, 2010). However, this group is somewhat complicated and difficult to identify because of their morphological similarity. Consequently, they are considered as complex group so far. The *Calanthe* group are generally found in several geographical ranges for instance, rainforest, evergreen forest and shrub forest that habit as epiphytic orchids ranging from 150 to 1500 *meters above sea level* (MASL). Some species are grown on the rock as terrestrial and lithophytic orchids (Santisuk et al., 2011). Most of *Calanthe* species can be commonly found in evergreen forest and rainforest of Thailand. Herein, several Thai *Calanthe* species including *C. cardioglossa*, *C. ciliciae* and *P. mishmensis* (Santisuk et al., 2011) *C. simplex* are now listed as endemic and rare species (Kurzweil, 2010). Recently, *C. herbacea* is considered as a new record for Thailand. Furthermore, *Cephalantheropsis longipes*, one of *Calanthe* members, is also recognized as new locality in Thailand (Kurzweil, 2010). Because some orchid species in *Calanthe* group display their distinctive characters of flowers e.g. colorful with a long duration of blooming, many of them are deliberated as elusive species. Besides, the vulnerable *Calanthe* species are now being over-collected from natural habitats for commercial purposes, resulting in rapidly decreasing number and ultimately risk of extinction.

Conserving genetic resources of orchids via *in situ* and *ex situ* methods seems to be important in order to maintain a natural parental species for further sustainable usage. In this case, the problems of smuggling orchids from the forests could be diminished. Orchid breeding is one of an alternative way for improving new varieties from wild orchids. This alternative choice has been successfully improved for many new orchid hybrids for commercialization. *Several Thai wild orchids exhibit special characteristic morphological features* such as color, shape, scent and longevity that are required for breeder. Nowadays, plant tissue culture, a new tool, is adopted for assisting plant propagation and breeding. This technology is not only an efficient technique for *ex situ* conservation but can also be applied for *in vitro* mass propagation of several orchid species. Additionally, tissue culture is a potential method to support artificial hybridization for creating new plant types. Many Thai orchids have been studied and reported in tissue culture but only a few numbers of Thai *Calanthe* group are somehow being studied for *in vitro* culture. As prominent characteristics, the *Calanthe* from foreign countries is now being improved and developed for a new variety via intraspecific, interspecific, and intergeneric breeding program through the biotechnological process for commercialization whereas Thai *Calanthe* orchids are not being studied and improved to new varieties at all. Therefore breeding program of Thai *Calanthe* orchids is very interesting. Generally, conventional orchid breeding programs have been conducted to improve the horticultural traits and genetic diversity within a species or a range of closely related orchid species. The conventional breeding for reciprocal cross improvement and the morphological identification of hybrids is relatively time-consuming. In addition, the morphological appearances of hybrids are sometimes complex and hard to identify especially in the *Calanthe* group. Determination of hybridity via molecular markers seems to be practicable and usable by amplifying polymorphic bands specifically present in parental species and hybrids. Several molecular marker techniques have been developed for specific purposes. At present, optimal and specific molecular marker techniques are useful for identification and characterization in several orchid hybrid species. However, each molecular marker techniques encounter the different limitation of application. Therefore, the selection of suitable and rapid reliable molecular marker techniques for hybrid and species identification is needed. Hence, an efficient *in vitro* propagation for the conservation

of some Thai *Calanthe* group was developed. Establishment of intergeneric and interspecific hybrids within the *Calanthe* group was performed and the development of molecular markers for rapid confirmation of *Calanthe* hybrids using simple and reliable biotechnological tools was initiated and applied.

Objectives

The aim of this project was to study on *in vitro* propagation some orchids in *Calanthe* group. Development of interspecific and intergeneric hybrids within the *Calanthe* group and identification of those orchid hybrids using SCAR marker were also performed and investigated.

Scope and limitation

Different self and cross pollination within and between the genus as described by intraspecific, interspecific and intergeneric hybridization were conducted and investigated. Successful self and cross pollination of their species or hybrids were continuously performed by *in vitro* culture methods started from asymbiotic seed germination, some factors affecting growth and development of seedlings and transplantation to the greenhouse. In addition, identification of some hybrids using SCAR marker was also conducted.

Expected benefits and application

The *Calanthe* group are now cultivated as ornamental potted plants. Successfully, the development of some Thai *Calanthe* hybrids receive from this study could lead to increasing demand for commercialization. An efficient *in vitro* culture technique used for rapid propagation and conservation of some *Calanthe* species would also be developed for further sustainable use. Moreover, noticeable characteristics of the *Calanthe* group are also very interesting for the breeding program. New hybrids from this varietal improvement supposed to be desirable for the future market demand.

CHAPTER II

EFFECT OF POLLINATION TYPE ON FRUIT SETTING IN THE CALANTHE GROUP AND THEIR HYBRIDS

Summary

Effect of breeding systems; intergeneric, interspecific cross-pollination and intraspecific self-pollination among 7 species in the *Calanthe* group *Calanthe cardioglossa*, *C. masuca*, *C. triplicata*, *C. lyoglossa*, *P. mishmensis*, *P. tankervilliae* and *P. tankervilliae* var. *alba* were performed by hand pollination method. The results found that three types of intraspecific self-pollination; autogamous, geitonogamous and xenogamous, had no effect on the percentage of pod setting. However, xenogamous type had an effect on pod size and weight as well as the percentage of embryo formation. Interspecific hybrids originated from *P. mishmensis* × *P. tankervilliae*, reciprocal cross and *P. mishmensis* × *P. tankervilliae* var. *alba* were successfully created. Two other intergeneric hybrids of *P. mishmensis* × *P. masuca* and *P. mishmensis* × *C. cardioglossa* were also achieved. However, no embryo formation was found on *P. mishmensis* × *C. cardioglossa* hybrids.

Introduction

Orchids are one of the most important groups which have been continuously improved into new hybrids for commercialization. Because of a rich diversity in orchid species, several native species distributed in Asian region have potential for hybrid improvement. For instance, *Phalaenopsis*, an originated species in East Asian region, has successfully mass produced as potted plant in the Netherlands (Griesbach, 2002). Moreover, *Dendrobium* has also been developed as famous Thai orchid hybrids for commercialized and exported to several countries all over the world. Because of a rich in orchid diversity, other Thai orchid's species have also potential for breeding and could be improve to a new commercialized varieties. This includes the *Calanthe* group. Nowadays, 33 species of Thai *Calanthe* group have been discovered and identified (Kurzweil, 2010). Many species included in this group are very interesting

for breeding into new hybrids for commercialization. However, breeding system for hybrid improvement program need to be studied and developed.

Plant breeding, the production of new crop varieties, is expected to be superior to their parents. New crops are evolved by means of selection, introduction, hybridization, polyploidy induction, mutation and tissue culture. In general, intraspecific hybridization means the mating between members of the same species while interspecific hybridization means the mating occurs between members of different species. Intergeneric hybridization refers to mating between the members of different genera. Generally, types of breeding system in plant are also diverse such as autogamy, xenogamy by self- incompatibility and mixed mating system. Study on breeding system for hybrid improvement have been reported in a number of species i.e. interspecific hybrids among cultivars of hardy *Hibiscus* species and interspecific hybrids of *Alstroemeria* (Aros, 2019). In orchid breeding system, interspecific and intergeneric hybridization have also been continuously studied and successfully developed. Several reports on orchid hybridization study have been performed and investigated for example in *Cypripedium candidum* and *C. pubescens* (Klier, Leoschke, & Wendel ,1991), *Bletilla striata* (Chung, & Chung, 2005), *Haemaria discolor* (Ker) Lindl. var. *dawsoniana* (Shiau et al., 2005), *Ascocentrum ampullaceum* var. *auranticum* × *Vanda coerulea* (Kishor et al., 2006), *Chloraea* (Humaña, Cisternas, & Valdivia, 2008), *Aerides vandarum* and *Vanda stangeana* (Kishor et al., 2008), intergeneic hybridization; *Renanthera imschootiana* Rolfe × *Vanda coerulea* Griff.ex L. (Kishor, & Sharma, 2009), *Epidendrum fulgens* × *E. puniceoluteum* (Pinheiro et al., 2010), *Oncidium sarcodes* x *Oncidium Aloha* (Faria, Colombo, & Hoshino, 2015), *Cattleya* orchid (*Cattleya forbesii* × *Cattleya bowringiana*) (Colombo et al., 2017), etc.

Orchid hybrid improvement of the *Calanthe* group has been formerly studied and developed in Japan (Shin et al., 2011). About 10 natural species of the Japanese *Calanthe* group have been brought for breeding and they were able to produce the new hybrids of more than 3,000 characters, which are remarkable in size, shape as well as beautiful color and could be developed for cut flower and ornamental plants. Hybridization of the *Calanthe* group has then been continuously developed i.e. interspecific *Calanthe* hybrids (Baque et al., 2011), intergeneric hybrids, *Gastrophaius*

'Dan Rosenberg' which was originated from *P.tankervilliae* × *Gastrorchis tuberculosa* and *Phaiocalanthe* 'Kryptonite' which was improved from *Calanthe* 'Rozel' × *P. tankervilliae* .Thai *Calanthe* group are also containing many different attractive and charming characteristics which can be improved and developed into new commercialized hybrids. Therefore, breeding system of Thai *Calanthe* group is being of interested for studied and investigated.

Materials and Methods

Plant materials

All 7 species of *Calanthe* group used as initial materials in this study consisted of *Calanthe cardioglossa*, *C. masuca*, *C. triplicata*, *C. lyoglossa*, *Phaius mishmensis*, *P. tankervilliae* and *P. tankervilliae* var. *alba*. They were all planted and conserved at Ban Romklao Botanical Garden under the royal initiative project of Phitsanulok Province and Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The topographic features of Queen Sirikit Botanic Garden and Ban Romklao Botanical Garden are presented in the Table 1 and the flowers and flowering period of each species are showed in the Figure 1 and Table 2.

Table 1 Topographic features of the *Calanthe* collection and cultivation areas

Places	Latitude	Longitude	Altitude (meters above see levels)
Queen Sirikit Botanic Garden, Chiang Mai	16:45:22.1800000000004	100:12:9.96999999997211	800
Ban Romklao Botanical Garden, Phitsanulok	17:36:37.6600000000039	100:54:17.7500000000000	1100

Table 2 The flowering period of 7 species of *Calanthe* group used as initial materials for breeding system study

The *Calanthe* group	Flowering period
C. cardio glossa Schlrt.	September - April
C. masuca (D. Don) Lindl	September - April
C. triplicata (Willemet) Ames	March - October
C. lyoglossa Rchb.f.	July-February
P. mishmensis (Lindl. & Paxton)Rchb. F.,	October- November
P. tankervilleae (Bank Blume), Mus.	Different time of the years
P. tankervilliae (Bank Blume), Mus.var. *alba*	Different time of the years

Hybridization study using self- and cross pollination methods

Hybridization study was carried out at Ban Romklao Botanical Garden under the royal initiative project of Phitsanulok Province and Queen Sirikit Botanic Garden, Chiang Mai, Thailand. Hybridization was independently performed depending upon the period of blooming of each species. The mating couples were flowering synchronously as expressed in Table 3 - 5. Hybridization process was conducted via self- and/or cross pollination methods in the morning between 8 and 10 am. The flowers that bloomed for 72 hours were selected (recently opened flower 72 hours after anthesis) for pollination by transferring pollinia on to the top of the female pollen to the recently opened flowers stigma. Hand pollination was performed by self-pollination in the same flower called autogamous and from different flower on the same plant called geitonogamous for cross-pollination. Xenogamous, cross pollination across flower of another plant was also performed. The total self-, cross- and their reciprocals were twenty eight mating couples. The hand pollinated flowers were labelled individually and tagging the date and the time of pollination. After hybridization, the fully physiological maturity pods were harvested and tested for seed qualification and germination efficiency. Observing data; fruit setting, pod size (width and length), pod weight were noticed and recorded as shown in the Table 5-8 (result session). Successfully F1 hybrid seeds derived from each achieved crossing species were used for morphological study, viability test and germination efficiency.

Table 3 Intraspecific hybridization of orchids in the *Calanthe* group

Genus	Species	Pollination period	Period of fully physiological maturity for pod harvesting (days)
Calanthe	*C. cardioglossa*	November	40-50
	C. lyoglossa	November	40-50
	C. masuca	August	40-50
Phaius	*P. mishmensis*	November	46-50
	P. tankervilliae var. *alba*	November	58-65
	P. tankervilliae	March	58-65

Table 4 Interspecific hybridization of orchids in the *Calanthe* group

Genus	Interspecific hybridization		Pollination period	Period of fully physiological maturity for pod harvesting (days)
	Female	Male		
Calanthe	*C. cardioglossa*	*C. lyoglossa*	November	-
	C. cardioglossa	*C. masuca*	November	-
	C. masuca	*C. lyoglossa*	November	45-50
Phaius	*P. mishmensis*	*P. tankervilliae* var. *alba*	November	58-65
	P. mishmensis	*P. tankervilliae*	November	58-65
	P. tankervilliae	*P. mishmensis*	November	58-65

Table 5 Intergeneric hybridization of orchids in the *Calanthe* group

Genus	Intergeneric hybridization		Pollination period	Period of fully physiological maturity for pod harvesting (days)
	Female	Male		
Phaius × *Calanthe*	*P. mishmensis*	*C. cardioglossa*	November	46-50
	P. mishmensis	*C. masuca*	November	46-50
Calanthe × *Phaius*	*C. cardioglossa*	*P. mishmensis*	November	-
	C. masuca	*P. mishmensis*	November	-

Viability Test

Seeds received from pods were tested for viability using Tetrazolium chloride (TZ) assay. Viability test of seeds obtained from hybridization process was assessed with three replicates per sample. A solution of 1 % TZ was added and the seeds were incubated in the dark at 40 °C for 24 h (Hosomi et al., 2012). Treated seeds defined as viable seed were counted and photographed under compound light source microscope (Olympus BX43) and camera adaptor Olympus (DP21) using digital imaging. Embryos containing viable seeds received from intraspecific, interspecific and intergeneric hybridization methods were observed and measured by width; length and diameter were recorded and indicated as embryo formation efficiency and embryo size.

Seed germination efficiency test

The matured hybrid seeds derived from successful mating couple pods were examined for seed germination efficiency. Hybrid seeds were germinated under dark condition. Matured hybrid seeds species were cultured on modified VW (1962) medium supplemented with 150 mL/L of coconut water, 50 g/L of potato extract and added with 8 g/L agar. The pH of the medium was adjusted to 5.2. Seed germination was observed and recorded after 16 weeks of culture under light condition at 20

$\mu mol.m^{-2}.s^{-1}$ for a period of 12 hours per day. Observation and recording data for seed germination were performed at 16 weeks.

Results

Morphological characteristics of the *Calanthe* group use for breeding system program

In order to find out the distinguish morphological characteristics for further desirable selection of hybrids, the process of morphological selection study for breeding program was conducted through the different types of orchid breeding in some genera and species of Thai *Calanthe* group (Figure 1). From an investigation the general morphological characteristics of *Calanthe* group are observed, recorded and demonstrated in Table 1. Dominant characteristics of some studying orchids in *Calanthe* group including flower color, flower size and length of inflorescence are being of interest and desirable for hybrid breeding program. The morphological data indicated various morphological characteristic depending upon ecological range as shown in the Table 1. In the breeding system experiment, *C. cardioglossa* (Figure 1 B), *C. masuca* (Figure 1C) and *C. lyoglossa* (Figure 1 H), members of *Calanthe* group were used for hand-self and cross pollination because these three *Calanthe* species displayed interested characteristics for instance the long inflorescence with colorful flower and distinguished labellum shape. In addition, the *Phaius* orchids, members of *Calanthe* group, were also used to study in the breeding system by hand-self and cross pollination. Three *Phaius* species of *P. mishmensis* (Figure 2 D), *P. tankervilliae* and *P. tankervilliae* var. *alba* (Figure. 2 E, F) were selected because of a dominant morphologies i.e. the long inflorescence with diverse colorful flower, large flower size, long blooming period and flowering throughout the year.

Effect of hybridization type on fruit setting and pod quality of *Calanthe* group

Intraspecific hybridization between two different species of the *Phaius* genus; *P.mishmensis*, *P. tankervilliae* and *P. tankervilliae* var. *alba* via autogamous, geitonogamous and xenogamous self-pollination type was observed and noticed. The results showed that different types of intraspecific hybridization had no significantly affected on the percentage of pod setting and pod size. On the contrary, *P.*

tankervilliae var. *alba* has a relatively low percentage of pod setting. However, the period of pod maturity before dehiscence in *P. mishmensis* seemed to be quicker than *P. tankervilliae*. In consideration to the same pollination type, the results indicated that the percentage of pod setting varied depend upon species. The highest percentage of pod setting (77.5 %) was observed and recorded when geitonogamous was performed for intraspecific pollination in *P. tankervilliae* (Figure 6). The result was showed in Table 5. As well as in the *Calanthe* genus, the results found that different types of intraspecific self-pollination had no significantly affected on the percentage of pod setting and pod size. On the contrary, *Calanthe masuca* had a relatively low percentage of pod setting (Figure 4). However, the period of pod maturity before dehiscence in this genus showed no different (Figure 8). In consideration to the same pollination type, the results indicated that the percentage of pod setting varied depend upon species. The highest percentage of pod setting (100%) was observed and recorded when autogamous was performed for intraspecific hybridization in *C. lyoglossa*. The mentioned results were presented in Table 5.

Interspecific hybridization between two different species with one variety of the genus *Phaius* was performed via cross-pollination and investigated. The results showed that all interspecific hybridization could produce interspecific hybrids with relatively high the percentage of pod setting. The highest percentage of pod setting (80 %) was obtained when cross-pollination between *P. mishmensis* and *P. tankervilliae* was done while their reciprocal cross could also produce the higher percentage of pod setting (70%). The relative results was also observed in cross pollination between *P. mishmensis* and *P. tankervilliae* var. *alba* (Figure 7), which could also give the higher percentage of pod setting (70%). The result also indicated that the period of pod maturity before dehiscence as well as pod size as described by length, width and weight of pods received from cross pollination of this genus showed no significantly different. In comparison to the size and weight of pod between hybrids and parental type, the results showed that the pods obtained from the hybrid cross between *P. tankervilliae* (♀) and *P. mishmensis* (♂) showed larger and gain more weight than the self-pollinated *P. mishmensis*. On the contrary, the hybrid pods obtained from the cross pollination between *P. tankervilliae* (♀) and *P. mishmensis* (♂) seemed to be relatively smaller than self-pollinated *P. tankervilliae* (Figure 9). Interspecific

hybridization in the *Calanthe* genus was also cross-pollinated and studied. The results found that only one cross pollination between *C. masuca* (♀) and *C. lyoglossa* (♂) could produce hybrids with the low percentage of pod setting (20%) while other cross pollination between *C. cardioglossa* (♀) *C. lyoglossa* (♂) and *C. cardioglossa* (♀) × *C. masuca* (♂) could not produce hybrids at all (Table 7).

Intergeneric hybridization between the *Phaius* and *Calanthe* genus was also cross-pollinated and investigated. The results showed that successful pod setting in the intergeneric cross-pollination could observe when *P. mishmensis* was used as female plant whereas no pod setting was observed when *P. mishmensis* was used as male parent. The highest percentage of pod setting (52%) could receive from the intergeneric hybrid between *P. mishmensis* (♀) *C. cardioglossa* (♂) whereas only 30% of pod setting was found in the intergeneric hybrid between *P. mishmensis* (♀) *C. masuca* (♂) (Table 9). However, hybrid seeds initiated from *P. mishmensis* (♀) *C. cardioglossa* (♂) showed no embryo formation. Furthermore, the reciprocal of these two crosses hybrids produced no pods anyhow (Table 10).

Effect of pollination type on seed formation and seed quality of *Calanthe* group

Intraspecific hybridization via 3 different pollination types; autogamous, geitonogamous and xenogamous, of two different species and one variety of the *Phaius* genus (*P. mishmensis*, *P. tankervilliae* and *P. tankervilliae* var. *alba*) was studied and observed. The result found that different types of intraspecific hybridization had no significantly affected on embryo formation, seed viability and seed quality in each *Phaius* species. The relative highest percentage of embryo formation (99.5-100%) as well as seed viability (99.5-100%) could be observed in all intraspecific hybridization of *P. tankervilliae* followed by 92.0-93.3% of embryo formation and 82.5-85.6% of seed viability in *P. tankervilliae* var. *alba*. The lowest percentage of embryo formation (23.0-32.8%) as well as seed viability (22.0-32.2%) was found in *P. mishmensis* (Table 6). In consideration to embryo size of each *Phaius* species, the results found that no significant difference in both width and length of embryo in all 3 different pollination types of *P. tankervilliae* was noticed. In contrast to *P. tankervilliae* var. *alba,* where the width and length of embryo size showed significantly different when compared to each pollination types. In case of *P.*

mishmensis, the result relatively showed little significant difference in width and length of embryo size.

Effect of different types of intraspecific hybridization in the *Calanthe* genus was also conducted and recorded. The results showed that the relative highest percentage of embryo formation (92.0-99.5%) as well as seed viability (92.0-99.5%) could obtain in all intraspecific self-pollination types of *Calanthe lyroglossa* followed by 79.9-84.9% of embryo formation and 78.8-84.9% of seed viability in *C. cardioglossa* whereas the lowest percentage of embryo formation (56.0-76.2%) as well as seed viability (53.6-76.2%) was found in *C. masuca*. In comparison to the embryo size of each *Calanthe* species, the results indicated that no significant difference in both width and length of embryo in all 3 different pollination types of *C. lyroglossa* was investigated. In contrast to *C. cardioglossa* (Figure 3), where the width and length of embryo size showed significantly different when compared to each pollination types. In case of *C. masuca*, the result relatively showed no significant difference in width of embryo size but the length of embryo was somehow exhibited significantly different especially in autogamous type (Table 6).

Interspecific hybridization between two different species of the same genus of *Phaius* and *Calanthe* was also studied and investigated. In the genus *Phaius*, *P. mishmensis* was selected to use as female parent while *P. tankervilliae* and *P. tankervilliae* var. *alba* were used as male parent for cross-pollination study. The results showed that the highest percentage of embryo formation as well as seed viability of hybrids originated from both *P. mishmensis* (♀) and *P. tankervilliae* var. *alba* (♂) hybrids and *P. mishmensis* (♀) and *P. tankervilliae* (♂) hybrids were 50.6% and 49.6%, respectively. However, the reciprocal cross of *P. tankervilliae* (♀) × *P. mishmensis* (♂) indicated less percentage of embryo formation (5.3%) and seed viability (5.3%). In comparison to the growth characteristic of seed and seed component, the results indicated that the width and length of embryo in seeds derived from both *P. mishmensis* (♀) × *P. tankervilliae* var. *alba* (♂) and *P. mishmensis* (♀) × *P. tankervilliae* (♂) relatively showed no significant different. In contrast to the embryo size of hybrid seeds derived from *P. tankervilliae* (♀) × *P. mishmensis* (♂), the results clearly showed that width and length of embryo were larger than that received from *P. mishmensis* (♀) × *P. tankervilliae* var. *alba* (♂) and *P. mishmensis*

(♀) × *P. tankervilliae* (♂) (Table 2.6 and Figure 2.8). In the *Calanthe* species, interspecific hybridization was performed in 3 different species; *C. cardioglossa*, *C. lyoglossa* and *C. masuca*. The results found that interspecific cross-pollination in all hybrids of the *Calanthe* genus was not succeeded to produce pod. Therefore, no hybrid formation could receive from this genus (Table 8).

Intergeneric hybridization between the *Phaius* and *Calanthe* genus was also conducted and recorded. The mating couples consisted of *P. mishmensis* (♀) × *C. cardioglossa* (♂) and *P. mishmensis* (♀) × *C. masuca* (♂) and their reciprocal cross. The results found that successful hybrid production could receive from the intergeneric hybrid between *P. mishmensis* (♀) × *C. masuca* (♂). However, the percentage of embryo formation (6%) and embryo viability (6%) showed very low. Other intergeneric hybrids initiated from either *P. mishmensis* (♀) × *C. cardioglossa* (♂) (Figure 10) or from *P. mishmensis* (♀) × *C. masuca* (♂) produced no embryo in seeds (Figure 9). The results showed the low percentage of seed viability (6.0 %) However, the embryo size of the putative hybrids showed no difference when compared to parental plants (Table 10).

Discussion

Effect of breeding systems; intergeneric, interspecific cross pollination and intraspecific self-pollination among 7 species in the *Calanthe* group were studied for hybridization ability and performed by hand pollination method. The results found that three types of intraspecific self-pollination; autogamous, geitonogamous and xenogamous, might effect on the percentage of pod setting depending upon *Phaius* and *Calanthe* species. The highest percentage of pod setting was observed and recorded when geitonogamous was performed for intraspecific hybridization in *P. tankervilliae* whereas the highest percentage of pod setting in *Calanthe* species could be noticed when autogamous type was self-pollinated. In general, autogamous pollination type is widespread in Orchidaceae and this pollination type can especially be found in terrestrial orchids (Dressler, 1993). In our study, the results indicated that *Calanthe lyroglossa* of which having smallest flower gave the highest percentage of pod setting via autogamous followed by *C. masuca* and *C. cardioglossa*, respectively. It was feasible that the highest pod setting success in *Calanthe lyroglossa* is

determined by flower size and floral morphology especially the distance between rostellum and stigmatic fluid. It is easier for self-pollination and autopollination of this species because rostellum is very short and allowing contact between the stigmatic secretions and the pollinia. The relative previous study has also been reported in *Eulophia* sp. Other relative studies on autopollination were also reported in *Nervillia* (Gale, 2007) and *Epipactis* sp. The effect of pollination types on successive pod setting in the genus *Phaius* indicated different results apart from the genus *Calanthe*. The results found that the geitonogamous type of pollination in *P. tankervilliae* gave the highest percentage of pod setting when compared to other pollination types and other species. Similar results have been investigated and reported in *P. delavayi* (Li et al., 2011) and *P. tankervilliae* (Lee et al., 2016) The assumption of successful geitonogamous pollination type in the *Phaius* species might also be related to the flower structure and reproductive organs.

Interspecific hybridization in the genus *Phaius* and *Calanthe* were also conducted and recorded. The results found that interspecific cross between three different species of *Phaius*; *P. tankervilliae* and *P. mishmensis* and *P. tankervilliae* var. *alba* was successfully developed and fruit setting could be observed and produced new hybrids. However, fruit setting was not entirely observed in interspecific cross pollination between 3 different species of *Calanthe*; *C. cardioglossa*, *C. masuca* and *C. lyroglossa*. Therefore, no new hybrids derived from interspecific hybridization in the *Calanthe* genus could be produced and developed. In case of the *Phaius* genus, successful hybrid production in the selected species of *Phaius* might be due to the close genetic relationship as previously studied in sequence analysis of ITS gene and reported in phylogenetic analysis of sheds new light on the relationship in *Calanthe* and alliance in China (Zhai et al., 2014). In our results, interspecific hybridization between *P. mishmensis* × *P. tankervilliae* as parental mating could produce new hybrids. The new hybrids could also develop into seedlings. This might be due to the adjacent chromosome number in *P. mishmensis* (2n = 44) (Teoh, 1980) and *P. tankervilliae* (2n = 48) (Jones, 1980). On the contrary, interspecific hybridization in the *Calanthe* genus was unsuccessful. This might be due to the fact that each studying species of the *Calanthe* genus is completely separated into different section which has a long genetic distance as indicated by phylogenetic study in the *Calanthe* group (Jun

et al., 2000). Therefore, the crossability within the different *Calanthe* species seems to be relatively difficult.

Intergeneric hybridization between the *Phaius* and *Calanthe* genus; *P. mishmensis* (♀) × *C. cardioglossa* (♂), *P. mishmensis* (♀) × *C. masuca* (♂), *C. cardioglossa* (♀) × *P. mishmensis* (♂) and *C. masuca* (♀) × *P. mishmensis* (♂) indicated a relative low fruit setting percentage only 52% in *P. mishmensis* (♀) × *C. cardioglossa* (♂) and 30% in *P. mishmensis* (♀) × *C. masuca* (♂), respectively. No fruit setting was observed in *C. cardioglossa* (♀) × *P. mishmensis* (♂) and *C. masuca* (♀) × *P. mishmensis* (♂). However, only intergeneric cross pollination between *P. mishmensis* (♀) × *C. masuca* (♂) could produce embryo (6%) and develop into seedlings. This might be due to the close genetic relationship between *P. mishmensis* and *C. masuca* (Jun et al., 2014) whereas other intergeneric crosses produced no embryo inside pods. This could be assumed that no fertilization between pollinia and egg was occurred during pollination and fertilization process.

Conclusions

Effect of breeding systems via intraspecific, interspecific and intergeneric hybridization among 7 species in the *Calanthe* group containing *C. cardioglossa*, *C. masuca*, *C. triplicata*, *C. lyoglossa*, *P. mishmensis*, *P. tankervilliae* and *P. tankervilliae* var. *alba* were performed by hand pollination method. Three types of intraspecific self-pollination; autogamous, geitonogamous and xenogamous, had no effect on the percentage of pod setting. However, xenogamous type had an influence on pod size and weight as well as the percentage of embryo formation. Interspecific hybrids originated from *P. mishmensis* × *P. tankervilliae*, reciprocal cross and *P. mishmensis* × *P.tankervilliae* var. *alba* were successfully created. Two other intergeneric hybrids of *P. mishmensis* × *P. masuca* and *P. mishmensis* × *C. cardioglossa* were also achieved. However, no embryo formation was found on *P. mishmensis* × *C. cardioglossa* hybrids.

Figure 1 Flower morphology of *Calanthe* used in this study, *Calanthe rosea* (A), *C. cardioglossa* (B), *C. masuca* (C), *C. vestita* (D), *C. triplicata* (E), *C. rubens* (F), *C. densiflora* (G), *C. lyoglossa* (H), and *C. clavata* (I)

Figure 2 Flower morphology of *Phaius* and *Chephalantheropsis* used in this study ***Phaius indochinensis*** **(A),** ***P. takeoi*** **(B),** ***P. flava*** **(C),** ***P. mishmensis*** **(D),** ***P. tankervilliae*** **(E),** ***P. tankervilliae*** **var.** ***alba*** **(F),** ***Cephalantheropsis obcordata*** **(G), and** ***Ce. longipes*** **(H)**

Table 6 Morphological characteristics of the *Calanthe* group used for breeding program in this study

Genus	Species	Flower Characteristics					Ecology		
		Size (mm)	Stalk length (cm)	Color	Flowering time	Flowering period (Day)	Habit	Altitude	Location collecting
Calanthe	*C. masuca*	20-45	50	Purple	September - April	30	Terrestrial	150-1,500	RK
	C. rubens	15-20	46	Pink	December - February	30	Terestrial	800	PR
	C. triplicata	28-30	44-68	White	March - October	45	Terestrial	200-1,400	PH
	C. rosea	19-30	46	Pink	November - February	30	Terestrial	200-900	PR
	C. densiflora	10-20	45	Yellow	August - December	30	Terestrial	1,350-1,530	PR
	C. cardioglossa	14-18	21-53	Pink to lilac White red	September - April	30	Terestrial Lithophytic	300-1,640	RK
	C. lyroglossa	10-12	24-46	Yellow	July-February	30	Terestrial	725-1,400	RK
Phaius	*P. tankervilliae*	40-58	50	Reddish - Brown	DT	60	Terestrial	50-2,000	MR
	P. tankervilliae var. *alba*	40-58	50	White	DT	50	Terestrial	50-2,000	RK
	P. mishmensis	50-60	17-32	Rose - coloured Dark pink	October- November	45	Terestrial	900-1,300	RK
	P. flava	25-45	35	Yellow / Green	DT	30	Terestrial	700-1,650	PH
	P. indochinensis	20-30	30-50	Brownish -Yellow	November - September	30	Terestrial	300-1,300	RK
	P. takeoi	40-50	30-50	With pale green	July - September	30	Terestrial	500-1,400	RK
Cephalan theropsis)	*Ce. longipes*	15-18	10-20	Yellow or Orange-Yellow	July	30	Terestrial	1200	PH
	Ce. obcordata	20-30	30-66	Yellow or Orange yellow	September -March	30	Terestrial	1,100-1,400	PR

Remark: RK = Ban Romklao Botanical Garden, MR = Queen Sirikit Botanical Garden, PR = Lung Wut Phurua Garden, PL = Phu Luang Wildlife Sanctuary, PH = Phu Hin Rong Kla National Park, DT = Different time of the years

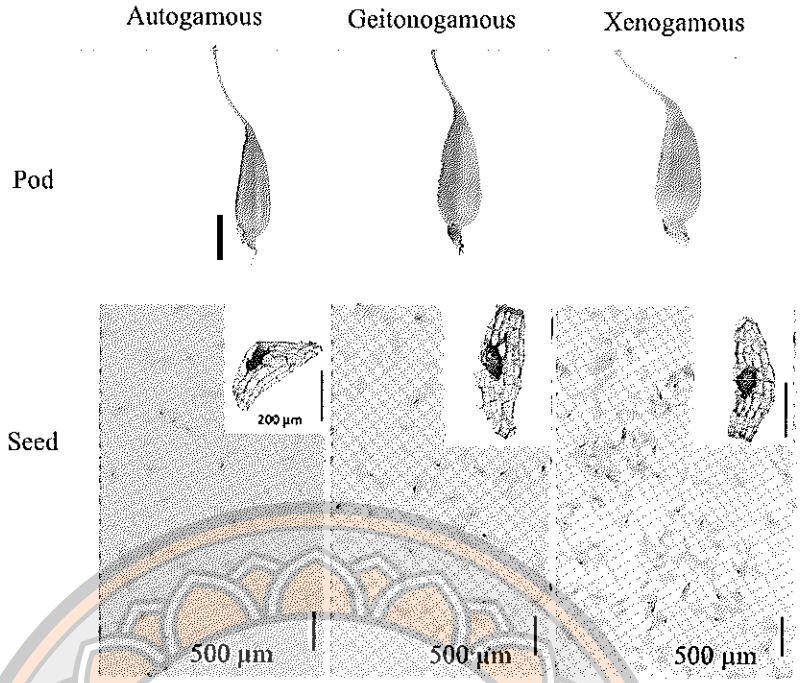


Figure 3 The characteristic of matured pod (Upper) and seed stained with tetrazolium Solution for viability test (Lower) of *C. cardioglossa*

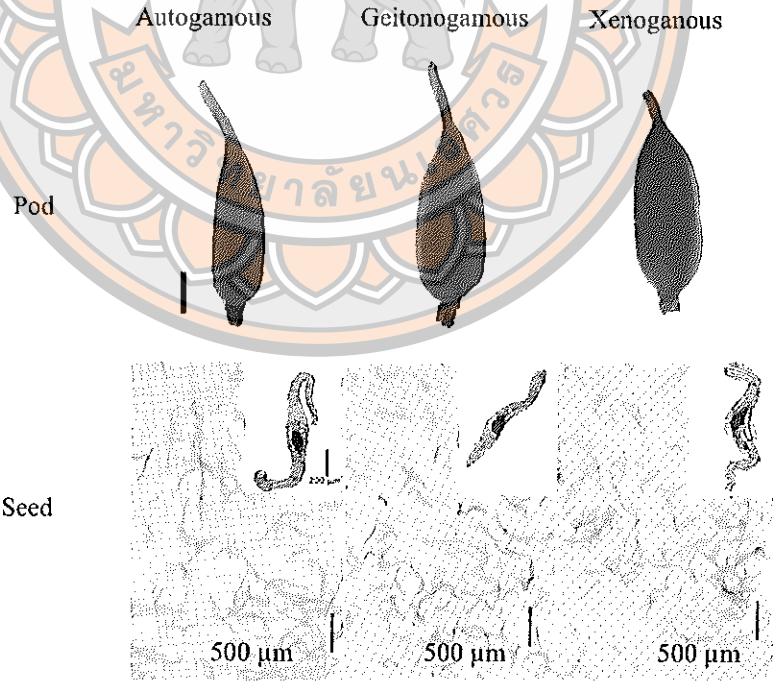


Figure 4 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of *C. masuca*

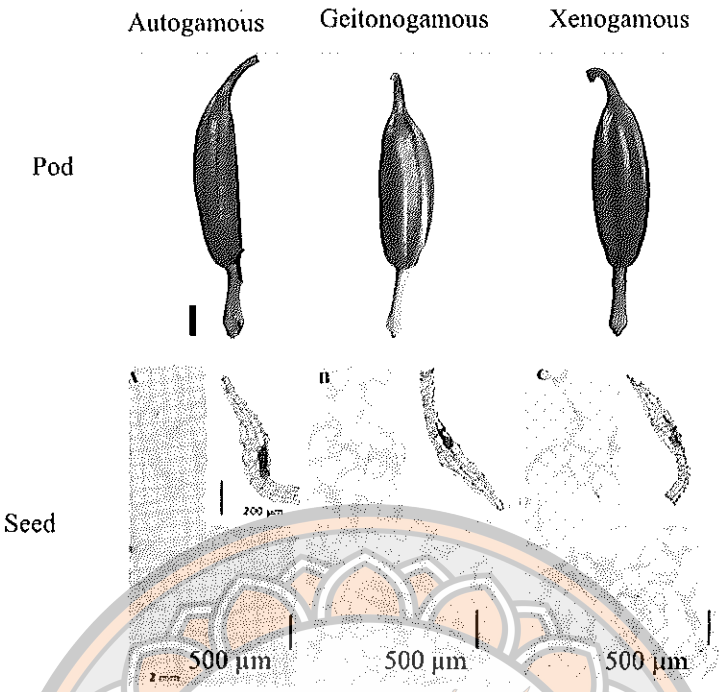


Figure 5 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of *P. mishmensis*

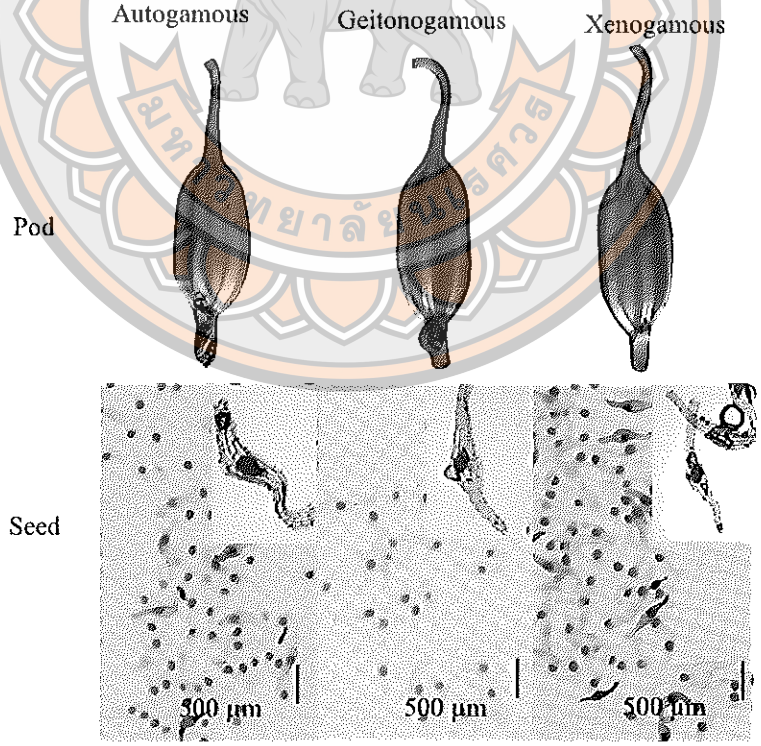


Figure 6 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of *Phaius tankervilliae*

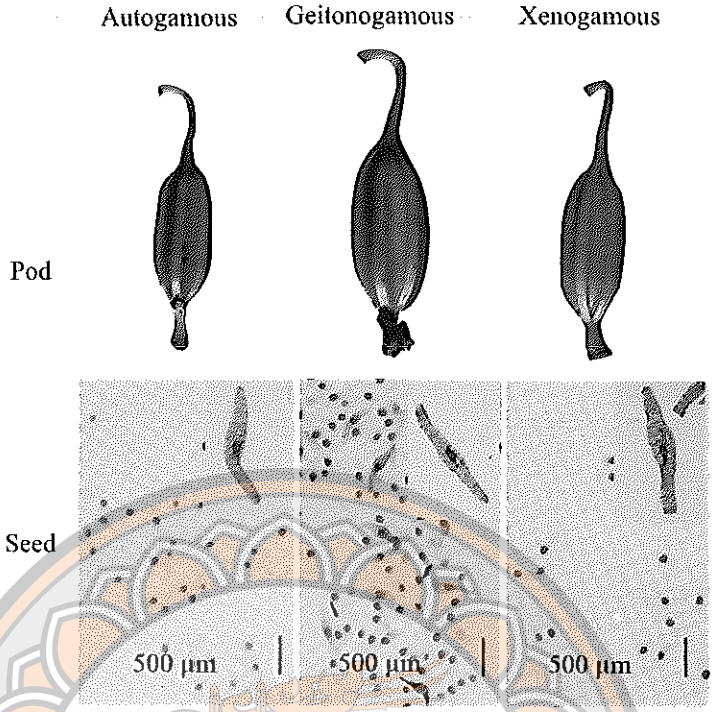


Figure 7 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of *Phaius tankervilliae* var. *alba*

Table 7 Pod setting, pod size and pod weight of interspecific pollination of the *Calanthe* and *Phaius* genus

Genus	Species	Type of intraspecific	Total of pollinated flower	No. of pod setting	Pod setting (%)	Day to pod maturity	Pod size (cm) [1]		Pod [1] weight (g)
							Width	Length	
Calanthe	*C. cardioglossa*	Autogamous	40	11	27.5	40- 50	0.6 ± 0.4 ns	1.8 ± 0.6 ns	0.3 ± 0.4 ns
		Geitonogamous	40	7	17.5	40- 50	0.9 ± 0.1	2.3 ± 0.2	0.5 ± 0.2
		Xenogamous	40	20	50.0	40- 50	0.7 ± 0.2	2.5 ± 0.1	0.6 ± 0.2
	C. lyoglossa	Autogamous	10	10	100.0	40- 50	0.6 ± 0.1 ns	1.4 ± 0.1 ns	0.3 ± 0.1 b
		Geitonogamous	10	5	50.0	40- 50	0.6 ± 0.1	1.4 ± 0.1	0.3 ± 0.3 a
		Xenogamous	10	6	60.0	40- 50	0.7 ± 0.1	1.8 ± 0.1	0.4 ± 0.1 ab
	C. masuca	Autogamous	10	3	30.0	40- 50	0.4 ± 0.3 ns	3.9 ± 0.3 ns	3.0 ± 1.8 ns
		Geitonogamous	10	4	40.0	40- 50	1.7 ± 0.6	3.2 ± 0.3	3.2 ± 0.3
		Xenogamous	10	2	20.0	40- 50	2.5 ± 0.6	3.9 ± 0.4	3.9 ± 0.4
Phaius	*P. mishmensis*	Autogamous	40	26	65.0	46 - 50	1.6 ± 0.2 ns	4.6 ± 0.2 ns	3.5 ± 0.1ns
		Geitonogamous	40	17	42.5	46- 50	1.6 ± 0.1	4.6 ± 0.1	3.9 ± 0.4
		Xenogamous	40	25	62.5	46-50	1.7 ± 0.4	4.7 ± 0.3	4.0 ± 0.4
	P. tankervilliae var. *alba*	Autogamous	10	4	40.0	58-65	2.1 ± 0.2 ns	4.7 ± 0.3 ns	6.0 ± 1.9 ns
		Geitonogamous	10	4	40.0	58-65	2.3 ± 2.3	5.0 ± 0.5	7.7 ± 1.2
		Xenogamous	10	5	50.0	58-65	2.3 ± 0.2	5.0 ± 0.4	7.7 ± 2.1
	P. tankervilliae	Autogamous	40	12	30.0	58- 65	2.4 ± 0.3 ns	4.7 ± 0.3 ns	9.0 ± 2.2 ns
		Geitonogamous	40	31	77.5	58- 65	2.4 ± 0.1	5.7 ± 0.1	15.7 ± 6.3
		Xenogamous	40	24	60.0	58- 65	2.4 ± 0.2	5.3 ± 0.2	10.2 ± 3.2

Values are means ± SD of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant [1]

Table 8 Embryo characteristics and seed germination ability of intraspecific hybrids in the *Calanthe* group

Genus	Intraspecific		Seed		Embryo Size (N=30)		Asymbiotic seed germination test
	Species	Intraspecific pollination type	Embryo(%)	Viability (%)	Width ± SE	Length ± SE	
Calanthe	*C. cardioglossa*	Autogamous	84.6	84.6	71.5 ± 2.3 a	119.4 ± 2.9 a	+
		Geitonogamous	79.9	78.8	62.3 ± 2.3 b	104.9 ± 3.5 b	+
		Xenogamous	84.9	84.9	73.5 ± 2.2 a	120.5 ± 2.7 a	+
	C. lyoglosa	Autogamous	95.5	95.5	55.1 ± 1.6 ns	84.0 ± 1.9 ns	+
		Geitonogamous	92.0	92.0	54.7 ± 1.8	76.7 ± 2.8	+
		Xenogamous	95.3	95.3	56.2 ± 1.9	82.2 ± 2.7	+
	C. masuca	Autogamous	76.2	76.2	65.0 ± 2.9 ns	112.3 ± 3.8 a	++
		Geitonogamous	63.7	62.7	62.9 ± 2.8	95.5 ± 2.4 b	++
		Xenogamous	56.0	53.6	64.0 ± 2.1	92.3 ± 3.8 b	++
Phaius	*P. mishmensis*	Autogamous	23.0	22.0	60.3 ± 3.5 ns	106.6 ± 4.4 a	++
		Geitonogamous	32.2	32.2	48.8 ± 1.7	91.8 ± 6.0 b	++
		Xenogamous	32.8	30.9	54.6 ± 2.3	97.3 ± 4.5 ab	++
	P. tankervilliae var. *alba*	Autogamous	92.0	85.6	97.6 ± 4.9 a	141.3 ± 4.9 b	+++
		Geitonogamous	89.9	83.5	82.9 ± 3.0 b	157.6 ± 5.8 a	+++
		Xenogamous	93.3	82.5	89.9 ± 3.4 ab	144.6 ± 2.1 b	+++
	P. tankervilliae	Autogamous	100.0	100.0	85.0 ± 2.8 ns	150.3 ± 2.8 ns	+++
		Geitonogamous	99.5	99.5	85.6 ± 2.4	149.8 ± 3.0	+++
		Xenogamous	100.0	100.0	91.0 ± 1.7	146.2 ± 2.1	+++

Values are means ± SE of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] Data were obtained from the 3rd leaf of each plant. Quality of seed germination + = poor, ++ = good, +++ = very good

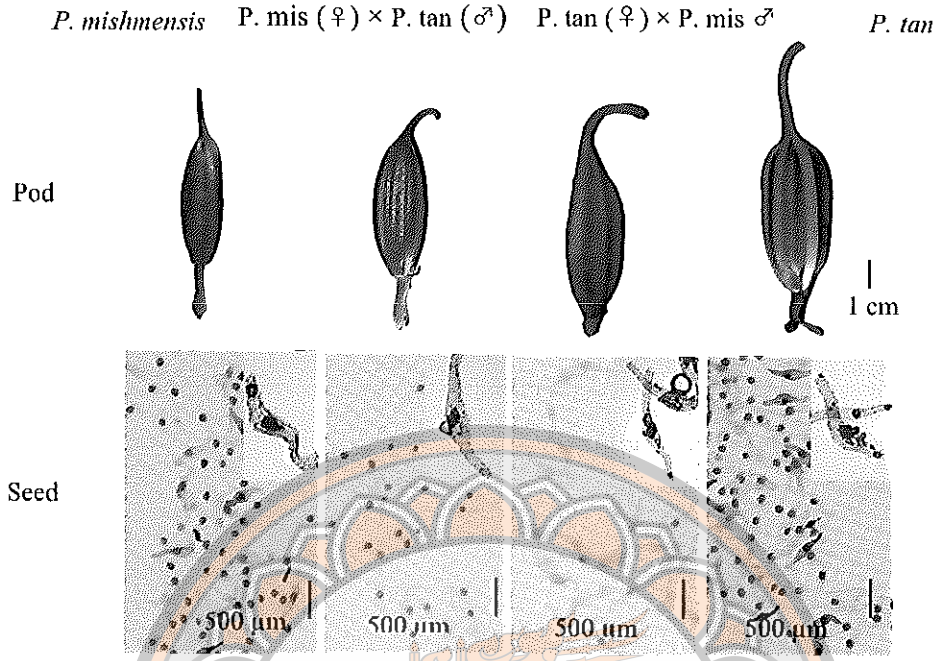


Figure 8 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of *P. mishmensis*, *P. tankervilliae* and their hybrids

Table 9 Morphological characteristics of pods of interspecific hybrids in the *Calanthe* group

Interspecific crosses		Total of pollinated flower	No. of pod setting flower	Pod setting (%)	Day to pod maturity	Pod size (cm) [1]		Pod weight [1] (g)
Female (♀)	Male (♂)					Width	Length	
C. cardioglossa	*C. lyoglossa*	10	-	-	-	-	-	-
C. cardioglossa	*C. masuca*	5	-	-	-	-	-	-
C. masuca	*C. lyroglossa*	5	1	20	40- 50	ND	ND	ND
P. mishmensis	*P. tankervilliae* var. *alba*	10	7	70	58-65	2.3 ± 0.1	4.8 ± 0.3	6.6 ± 1.0
P. mishmensis	*P. tankervilliae*	10	8	80	58-65	1.9 ± 0.3	5.5 ± 0.2	8.1 ± 2.0
P. tankervilliae	*P. mishmensis*	10	7	70	58-65	1.6 ± 0.5	5.4 ± 0.2	6.2 ± 1.8

Values are means ± SD of pods. Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] ND = Not determined

Table 10 Embryo characteristics and seed germination ability of interspecific hybrids in the *Calanthe* group

Interspecific cross		Embryo Size (N=30)				Asymbiotic seed germination test
Female (♀)	Male (♂)	Size				
		Embryo (%)	Viability (%)	Width ± SE	Length ± SE	
C. cardioglossa	*C. lyoglossa*	-	-	-	-	-
C. cardioglossa	*C. masuca*	-	-	-	-	-
C. masuca	*C. lyoglossa*	0	0	-	-	-
P. mishmensis	*P. tankervilliae* var. *alba*	50.6	49.6	60.3 ± 3.5	69.5 ± 8.7	+++
P. mishmensis	*P. tankervilliae*	50.6	49.6	45.6 ± 2.7	69.1 ± 3.8	+++
P. tankervilliae	*P. mishmensis*	5.3	5.3	85.6 ± 4.2	116.3 ± 8.1	++

Values are means ± SE of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] Data were obtained from the 3rd leaf of each plant

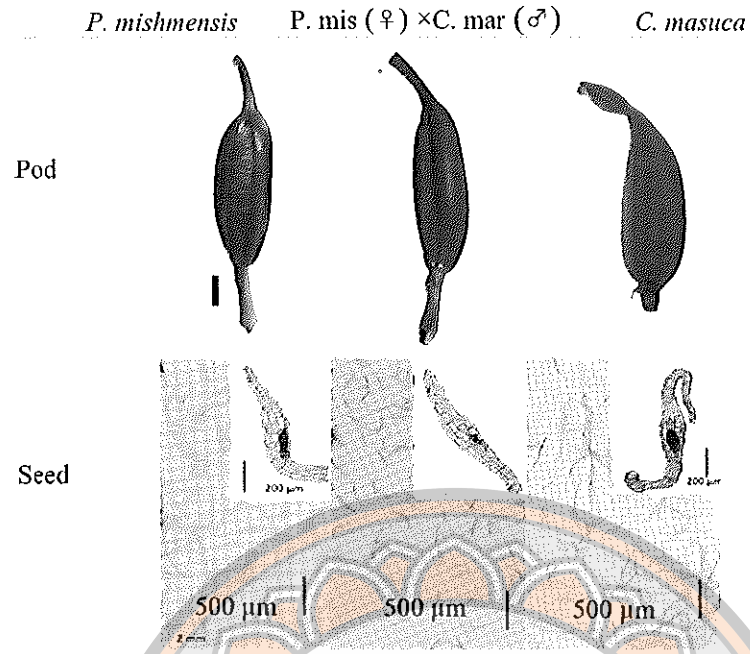


Figure 9 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of intergeneric hybrids of *P. mishmensis* and *C. masuca* and their parents

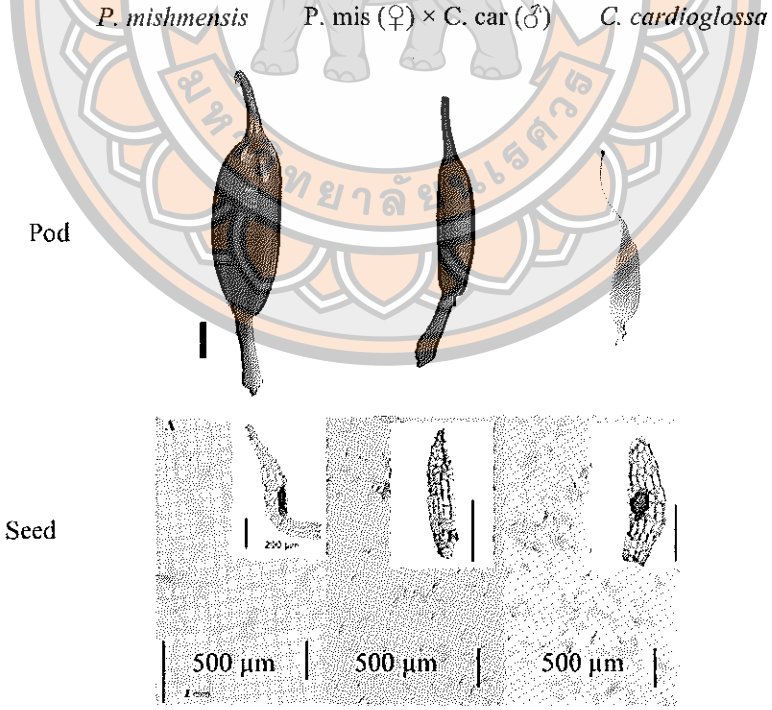


Figure 10 The characteristic of matured pod (Upper) and seed stained with tetrazolium solution for viability test (Lower) of intergeneric hybrids of *P. mishmensis* and *C. cardioglossa* and their parents

Table 11 Morphological characteristics of pods from intergeneric hybrids the *Calanthe* group

Intergeneric		Total of pollinated flower	No. of pod setting flower	Pod setting (%)	Day to pod maturity	Pod size (cm) [1]		Pod weight [1] (g)
Female (♀)	Male (♂)					Width	Length	
P. mishmensis	*C. cardioglossa*	25	13	52	46 - 50	1.3 ± 0.1	3.8 ± 0.4	2.6 ± 0.3
P. mishmensis	*C. masuca*	10	3	30	46 - 50	4.9 ± 0.3	14.0 ± 0.1	11.6 ± 0.1
C. cardioglossa	*P. mishmensis*	10	0	-	-	-	-	-
C. masuca	*P. mishmensis*	10	0	-	-	-	-	-

Values are means ± SE of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] Data were obtained from the 3rd leaf of each plant

Table 12 Embryo characteristics and seed germination ability of intergeneric hybrids in the *Calanthe* group

Intergeneric		Size		Embryo Size (N=30)		*In vitro* seed germination test
Female (♀)	Male (♂)	Embryo (%)	Viability (%)	Width ± SE	Length ± SE	
C. cardioglossa	*P. mishmensis*	-	-	-	-	-
C. masuca	*P. mishmensis*	10	0	-	-	-
P. mishmensis	*C. cardioglossa*	0	0	-	-	-
P. mishmensis	*C. masuca*	6.0	6.0	62.2 ± 9.6	92.2 ± 5.2	+

Values are means ± SE of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] Data were obtained from the 3rd leaf of each plant

CHAPTER III

EFFECT OF LIGHT AND ORGANIC SUPPLEMENTS ON ASYMBIOTIC SEED GERMINATION OF INTERSPECIFIC CROSSES-DERIVED SEEDS

Summary

The matured of two interspecific hybrids; namely *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba* seeds were cultured on modified VW (1962) medium supplemented with different concentrations of coconut water and potato extract. The cultures were incubated under light and dark condition and seed germination efficiency were observed and recorded after 4 months. In case of *P. mishmensis* × *P. tankervilliae* hybrids, the results showed that the highest percentage of seed germination (75.5%) was investigated on the VW medium added with 150 mL/L CW and cultured under dark condition whereas the highest percentage of germination (100%) in *P. mishmensis* × *P. tankervilliae* var. *alba* hybrid seeds could receive on the VW medium augmented with 50 g/L PE. In comparison to the developmental efficiency, the results indicated that adding 150 mL/L CW to the VW medium could stimulate highest developmental stage (17.5%) while adding 50 g/L of PE to the VW medium tended to accelerate better developmental stage (31.0%) when compared to other medium.

Introduction

The orchid seed germination study was earlier attempts to initiate germination involved placing seeds onto organic substances such as sphagnum moss, bark, or leaf mold, but this often proved unsuccessful (Arditti, 1967). However, the first to recognize the role of fungi in orchid seed germination by co-culturing fungi with orchid seeds symbiotic germination was discovered since last 100 years ago. (Bernard 1899; Burgeff, 1909). Orchid seeds in the natural site were germinated only the following infection by

mycorrhizal fungi that provide biochemical changes in mycorrhizal synthesis then stimulate embryo development (Energy, 2007). Therefore symbiotic germination of orchid seeds is still limited by specificity of mycorrhizal species and orchid host specification (Kauth et al., 2008).

In vitro asymbiotic seed germination was firstly examined by Lewis Knudson by using nutrient solutions supplemented with 1% sucrose (Knudson, 1922). This medium was successfully used to germinate seeds of several epiphytic orchid genera. However, the germination rate of orchid seeds was low. Generally, many mineral nutrition factors were affected on asymbiotic seed germination for instance water, carbohydrates, minerals, and vitamins. Because of a minimal carbohydrate reserves in orchid seeds, an exogenous source of carbohydrate is then required for *in vitro* asymbiotic seed germination of orchid.

Potato extract (PE) the extract of potato tubers was contains carbohydrate amino acid, important vitamins (C, B_1, and B6) and mineral elements (potassium, iron, magnesium). There was a sharp increase in the number of pollen plants produced from wheat there were cultured on an agar solidified medium containing only an extract of boiled potatoes and growth regulators. Potatoes extract alone or combined with components of conventional culture medium has since be found to provide a useful medium for the anther culture of wheat and some cereal plant. Potato extract being added to media for orchid micro propagation media has beneficial effects on some species (*Phalaenopsis*, *Doritaeanopsis*) coconut water (CW) used in tissue culture media. The liquid endosperm contains a number of amino acid, nucleic acids, several vitamins, sugar alcohols, plant hormone (auxins, cytokinins), minerals and other unidentified substances many factors affecting in vitro orchid seed germination have been studied and reported. Organic supplements are one of the main factors affecting growth, development of several plants (Molnar et al., 2011) and also improved orchid seed germination in several species (Arditti, & Ernst, 1993; Arditti, 2008; Park, & Yeung, 2018). Successful re searches on using organic additives to improve *in vitro* seed germination and growth of orchid seedlings have been reported in several orchid species i.e. *Dendrobium lituiflorum* Lindl.(Vyas et al., 2009), *Phalaenopsis violacea* (Gnasekaran et al., 2010), *Vanda* Kasem's Delight (Gnasekaran et al., 2012), *Vanda roxburgii* (Islam et al., 2011),

Vanda helvola Blume. (David et al., 2015), *Dendrobium* sp. (Islam et al., 2016), *Bulbophyllum dhaninivatii* Seidenf. (Kongbangkerd et al., 2016) and *Cypripedium macranthos* Sw. (Huh et al., 2016) etc.

In addition, light is also an important factor affecting in vitro orchid seed germination Light is often considered more important than temperature in orchid seed germination; although research into non-orchid seed germination indicates that temperature seem to be more important in controlling germination (Arditti, 1967; Leon, & Owen, 2003; Walck, & Hidayati, 2005). Effects of light on asymbiotic seed germination of some orchid species has been reported i.e. *Goodyera pubescence* (McKinley, & Camper, 1997); *Phragmipedium longifolium*, *P. pearcei* and *P. humboldtii* (Muñoz, & Jiménez, 2008); *Calanthe* hybrids (Baque et al., 2011); *Bletia purpurea* (Johnson, & Kane, 2012) and *Paphiopedilum spicerianum* (Chen et al., 2015). Up to date, effect of light combined with organic supplements on asymbiotic seed germination of two hybrids improved from *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba* have not yet been studied and elucidated. Therefore, this study was to compare seed germination performance under light and dark condition, the effect of organic supplements on in vitro seed germination of two hybrid seeds of *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba*

Materials and Methods

Plant materials and asymbiotic seed germination

The matured capsules (xx days after pollination) derived from two interspecific crosses, *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba* were disinfected with 15% (v/v) Clorox® solution for 20 minutes. Then, the seeds were cultured on semi-solid VW (Vacin and Went, 1962) medium supplemented with 150 mL/L CW or 50 mg/L PE or both 150 mL/L CW and 50 mg/L PE. The media was adjusted pH to 5.7 and added 8 g/L agar before autoclaving at 121 °C for 15 minutes. The seeds were cultured under dark and light (cool-white LED, 20 µmol/m^2/s, 12 h/day) conditions at 25 ± 2 °C for 4 months. The seed germinations were observed and recorded

under a stereomicroscope every month. The experiment was performed in triplicated (100 individual seeds per replicate) for each treatment. The statistical differences of data were analyzed by One-way ANOVA and Duncan's multiple range tests (DMRT).

Results

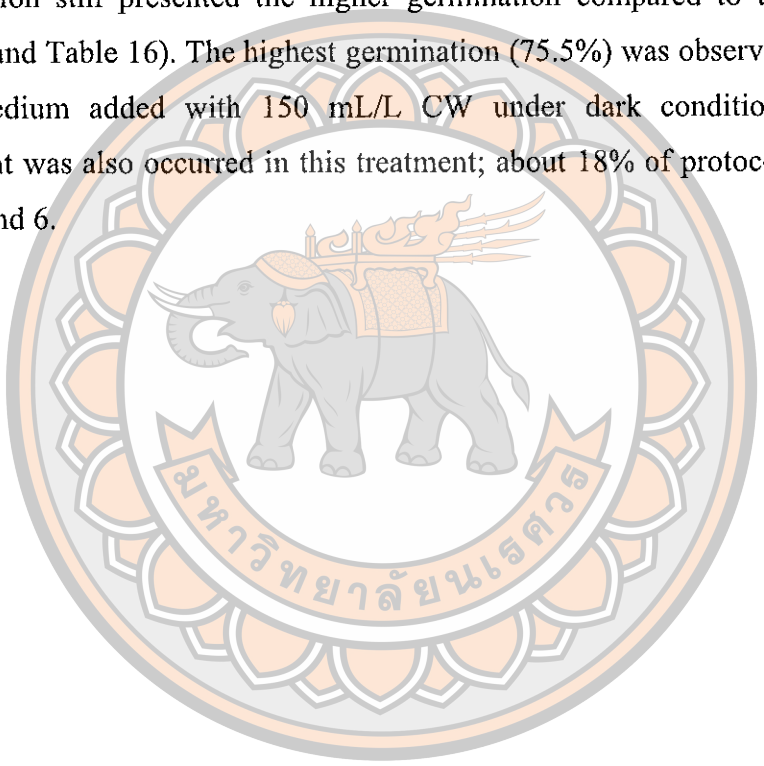
Protocorm development of interspecific crosses-derived seeds

Seed germination and protocorm development of seeds derived from both interspecific crosses under in vitro culture could be classified by main morphology transition to 6 developmental stages (Figure 11). After within 2- or 3-weeks culture, embryo inside the seed was enlarged and ruptured from testa (stage 2), this transition was considered as seed germination. Then, the rhizoid was formed (stage 3) and following apical meristem production (stage 4). Next, the first leaf was developed from apical meristem (stage 5) and the root was produced (stage 6) which is the final stage where mature seedling is formed.

Effect of light and organic supplements on seed germination and protocorm development of *P. mishmensis* × *P. tankervilliae* seeds

The results showed that seeds became swollen rapidly after inoculation onto culture media, and germination was occurred within 1 month of culture (Table 13). The seeds seem to better germinate under dark condition, especially in VW and VW added with both PE and CW media which has germination in dark condition higher than light condition up to 2-3 folds. When an organic supplement was concerned, under light condition, VW added with PE promoted better germination. However, under dark condition the supplementation of both PE and CW to the media promoted better germination which is the best germination (25.5%) at 1 month after culture. After 2 months of culture, the seed germination under dark condition was increased rapidly up to 2-3 folds of those at 1 month after culture while slightly increase in light condition (Table 14). The seeds cultured in all medium under dark condition presented better germination rate than the light condition. The seed cultured with the media containing PE or PE and CW under dark condition showed quicker development, some protocorm reached stage 5 after 2 months of culture. The highest seed germination (60.5%) was found in the medium

added with PE, dark condition At 3 months after culture, the seeds incubated under dark condition also give higher germination percentage than the light condition (Table 15). However, in VW added with PE and CW, light could promote better development, some protocorm developed to seedling stage (stage 6) while no seedling was observed in dark condition. Notably, some died protocorm was observed in the media containing CW or PE and cultured under light condition. At the end of observation (4 months after culture), the dark condition still presented the higher germination compared to the light condition (Figure 14 and Table 16). The highest germination (75.5%) was observed in seed cultured in VW medium added with 150 mL/L CW under dark condition. The best seed development was also occurred in this treatment; about 18% of protocorm could develop to stage 5 and 6.



Effect of light and organic supplements on seed germination and protocorm development of *P. mishmensis* × *P. tankervilliae* var. *alba* seeds

The results showed that seeds became swollen rapidly after inoculation onto culture media. The seeds were germinated after cultured for 1 month. The semi-solid VW medium without adding organic supplements and cultured under light condition gave the lowest percentage of germination (10%). On the contrary, seeds showed the highest percentage of germination when cultured under dark condition. In particular, the medium containing 50 g/L PE , 150 mL/L CW and a combination between 50 g/L PE with 150 mL/L CW showed the percentage of germination up to 28.5%, 28.5% and 28.0%, respectively (Table 17). In addition, developmental stage of seeds under both light and dark condition had no clearly signed for the development (Figure 11).

After 2 months of culture, seeds germinated under dark condition tended to improve better germination and development than under light condition. The results showed that seeds cultured under dark condition on the medium without organic supplements increased the highest percentage of developmental stage (3^{rd} stage: rhizoids stage and 4^{th} stage: Protomeristem stage) at 28.0% and 6.0%, respectively. Nonetheless, the medium without organic supplements showed the percentage of germination up to 78.5% (Table 18). However the medium containing 150 mL/ L CW and cultured under dark condition showed the percentage of seed development up to 8.5 % (Table 18).

When the seeds were continuously cultured until 3 months, the results showed that seeds cultured under dark condition tended to give higher germination percentage than under light condition. The medium added no organic supplements could increase the highest percentage of seed development until 3^{rd} stage (Rhizoids stage) at 31 %. Similarly, the medium containing 50 g/L PE could increase the highest percentage of seed development until 4^{th} stage (Protomeristem stage) at 21.0 %. The medium without organic supplements showed could improve the highest percentage of germination up to 96.0% (Table 19). However, the medium adding with 50 g/L PE and cultured under dark condition gave the percentage of seed development up to 14.0% (Table 19).

An improve germination and developmental stage of hybrid seeds investigated after 4 months of culture showed that there were significantly different when compared to the light and dark condition. The results showed that dark condition tended to stimulate the highest percentage of germination, especially in the VW medium added with 50 g/L PE of which induce the highest percentage germination up to 100%. Moreover, better developmental stage of seeds could obtain when seeds were cultured on the VW medium without organic supplements. On this medium, seeds could develop into the 3rd stages (rhizoid stage) at 31.5 % and the VW medium added with 150 mL/L CW could stimulate the seed to develop until reached at the 4th stages (Proto meristem stage) at 22.5 %. Higher growth and development of seeds into complete seedling plants was improved by PE. The results indicated that the VW medium supplemented with 50 g/L PE could induce the highest percentage germination until reached at 5th stage (first leaf) and 6th stage (shoot and root) at 14.5% and 16.5 %, respectively. From the results, it was suggest that cultivation under the dark condition on the medium added with 50 g/L PE could induce the highest percentage of germination and developmental stage of the embryo at 100 % and 31.0 %, respectively (Figure 14 and Table 20).

Discussion

The morphological development of two cross interspecific putative hybrids was namely *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba,* were culture on different medium supplement with organic compound as CW and PE.

In generally for both symbiotic and asymbiotic orchid seed germination to be effective, many conditions must be addressed such as photoperiod, temperature, and mineral nutrition. (Kauth et al., 2008). In this study the main factor was light and dark conditions the results was showed the seeds were rapid germination, after cultured for one month, all of medium without organic compose of supplement it was found that cultivation in dark conditions tended to germinate more than cultivation in light conditions. Lightless conditions tended to give a high germination percentage and found that the mechanism of seed germination water, it is showed that when water passes through the membrane (Testa) causes germination (Kauth et al., 2008). For seed germination van Waes & Debergh (1986) reported that even small increases in light intensity from complete darkness to 1.2 μmol m^{-2} s^{-1} reduced germination of several European terrestrial orchids. Asymbiotic germination of *Cypripedium acaule*, a North American terrestrial orchid, was lower when seeds were incubated in a 16/8 h photoperiod (6.7% germination) compared to complete darkness (96.7%) (St-Arnaud et al., 1992).

From the results after 4 months. In case of *P. mishmensis* × *P.tankervilliae* hybrids, the results showed that the highest percentage of seed germination (75.5%) was investigated on the VW medium added with 150 mL/L CW and cultured under dark condition whereas the highest percentage of germination (100%) in *P. mishmensis* × *P. tankervilliae* var. *alba* hybrid seeds could receive on the VW medium augmented with 50 g/L PE. In comparison to the developmental efficiency, the results indicated that adding 150 mL/L CW to the VW medium could stimulate highest developmental stage (17.5%) while adding 50 g/L of PE to the VW medium tended to accelerate better developmental stage (31.0%) when compared to other medium.

The results was showed the rhizoid stage protocorm cultured in complete darkness often produce more rhizoids than those in light related the study asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Stewart, &

Kane 2006). Several researchers reported that germination of orchids increases with brief periods of illumination. Rasmussen et al., (1990) reported 75% germination of *Dactylorhiza majalis* when seeds were illuminated after imbibition for 10 days prior to dark incubation. This was a significant increase from 45% germination under continual darkness. Zettler, & McInnis (1994) reported similar results with symbiotic germination of *Platanthera integrilabia*. Germination increased from 20% under complete darkness to 44% when seeds were exposed to 7 days of a 16/8 h photoperiod prior to dark incubation. While the exact function of light pretreatment is not understood, mycorrhizal fungi may benefit from brief periods of illumination (Zettler, & McInnis, 1994).

For organic compounds in this study we showed seed were culture on VW medium addition of PE 50 g / L combine with CW 150 mL /L that could induce seed germination higher than other formulas compared to medium without organic compounds. Because organic supplement were pound components, such as mineral, vitamin, and carbohydrate.

For potato extract contains carbohydrate amino acid, important vitamins (C, B_1 and B6) and mineral elements (potassium, iron, magnesium). of amino acids have also been used as a substitute nitrogen source. (Raghavan, 1964) reported that only certain amino acids increase seed germination of *Cattleya*. Glycine, the simplest amino acid, decreased overall germination of *Cattleya* seeds from 53% to 41%. However, germination in the presence of arginine, proline, and glutamine was similar to that with ammonium nitrate (Raghavan, 1964). Nitrogen has long been considered as an important role in the germination of orchid seeds. Recent reports have shown that while one asymbiotic culture media may support initial germination, another medium may better support subsequent development. Stenberg, & Kane, (1998) & Kauth et al., (2006)

Coconut water or liquid endosperm contains a number of amino acid, nucleic acids, several vitamins, sugar alcohols, plant hormone (auxins, cytokinins), minerals and other unidentified substances many factors affecting in vitro orchid seed germination have been studied and reported. In addition it was organic nucleic sugar, sugar alcohol and more in CW. There are plant growth regulators such as Auxin and Cytokinin and more than that it is reported that adding ammonium to a large quantity can inhibit germination

of seeds. Many researches were successfully seed germination such as many orchids genera namely *Vanda* hybrids, *Habenaria*, *Cattleya* and *Cymbidium* hybrids was greater when grown on a medium with a high ratio of ammonium to nitrate (Curtis, & Spoerl, 1948). The regenerative capacity of *P. mishmensis* to form protocorm in vitro provides an opportunity for propagate development of a commercially viable production system, which facilitates storage. After cultuer two month we found that rhizoid was development form germination stage and found that without organic supplement rhizoid are well develop. In contrast the seed s was developed to first leave stage and the orgasmic need to us for stimulate protocorm.

From the results can suggest that. The medium that compose of potato extract the main of potato extract was starch and amino acid Organic supplement for seed germination such as Nitrogen has long been considered as an important role in the germination of orchid seeds. Various orchid species respond differently to various amino acids during germination, and therefore further investigation should be carried out. Since not all amino acids are beneficial for seed germination, combinations of amino acids may increase germination (Spoerl, & Curtis, 1948).When inorganic nitrogen, such as ammonium, is utilized by germinating seeds, the nitrogen is converted to amino acids (Majerowicz et al., 2000). Using amino acids as the sole nitrogen source in orchid seed germination may lead to more efficient nitrogen assimilation by bypassing certain nitrogen conversion steps; however, this may be species specific and should be further investigated. Moreover it have been many the factor that effect to orchids seed germination for instant s carbohydrate, temperature, plant growth regulator, dormancy, seed viability, seed age, cold stratification seed coat treatment and hormone inhibitor.

In practice, researchers have largely ignored the importance of temperature during orchid seed germination and seedling development. The use of PGRs in asymbiotic orchid seed germination has not been clarified.

Conclusions

The matured of two interspecific hybrids; namely *P. mishmensis* × *P. tankervilliae* and *P. mishmensis* × *P. tankervilliae* var. *alba* seeds were cultured on modified VW (1962) medium supplemented with different concentrations of coconut water and potato extract. The cultures were incubated under light and dark condition and seed germination efficiency were observed and recorded after 4 months. In case of *P. mishmensis* × *P. tankervilliae* hybrids, the results showed that the highest percentage of seed germination (75.5%) was investigated on the VW medium added with 150 mL/L CW and cultured under dark condition whereas the highest percentage of germination (100%) in *P. mishmensis* × *P. tankervilliae* var. *alba* hybrid seeds could receive on the VW medium augmented with 50 g/L PE. In comparison to the developmental efficiency, the results indicated that adding 150 mL/L CW to the VW medium could stimulate highest developmental stage (17.5%) while adding 50 g/L of PE to the VW medium tended to accelerate better developmental stage (31.0%) when compared to other medium.

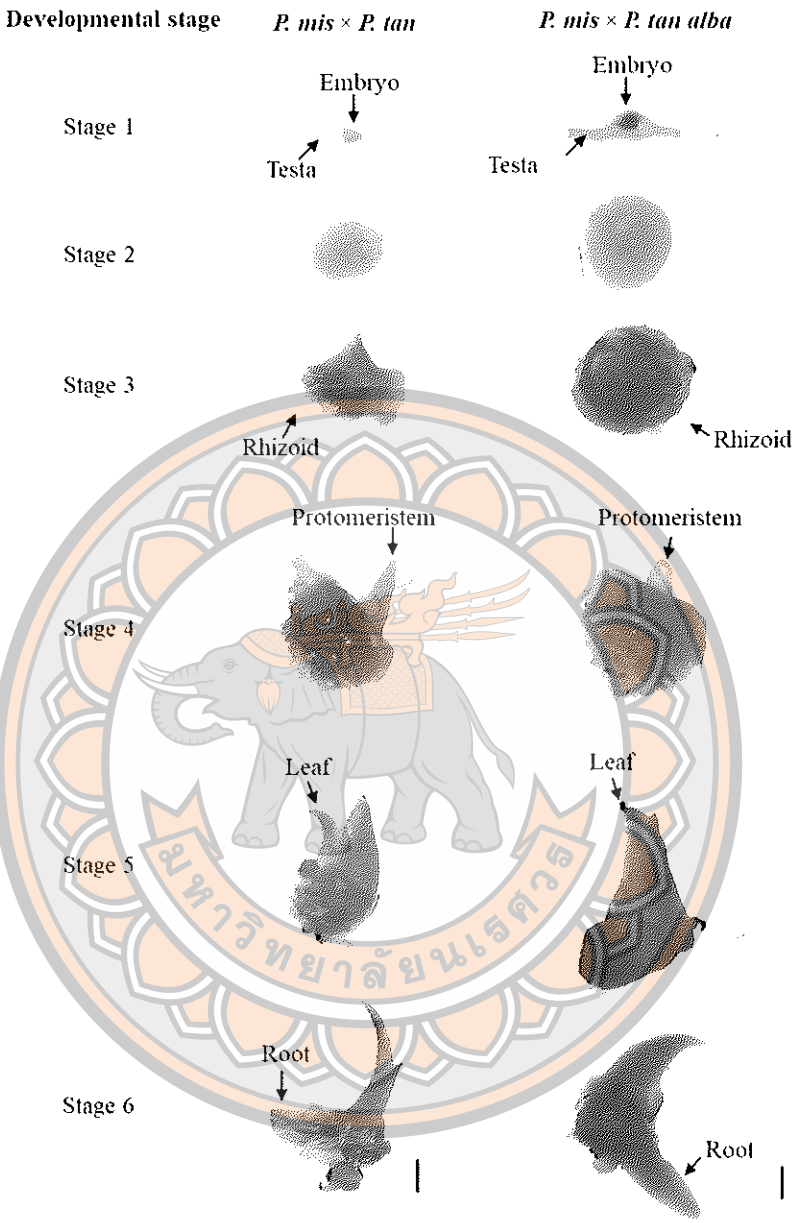


Figure 11 Protocorm developmental stage after asymbiotic germination of *P. mishmensis* × *P. tankervilliae* (left) and *P. mishmensis* × *P. tankervilliae* var *alba* (right) seeds: Stage 1, no development of seed; Stage 2, embryo enlargement and rupture from the testa (germination); Stage 3, appearance of rhizoids; Stage4, apical meristem production; Stage 5, emergence of the leaf; Stage 6, emergence of the root

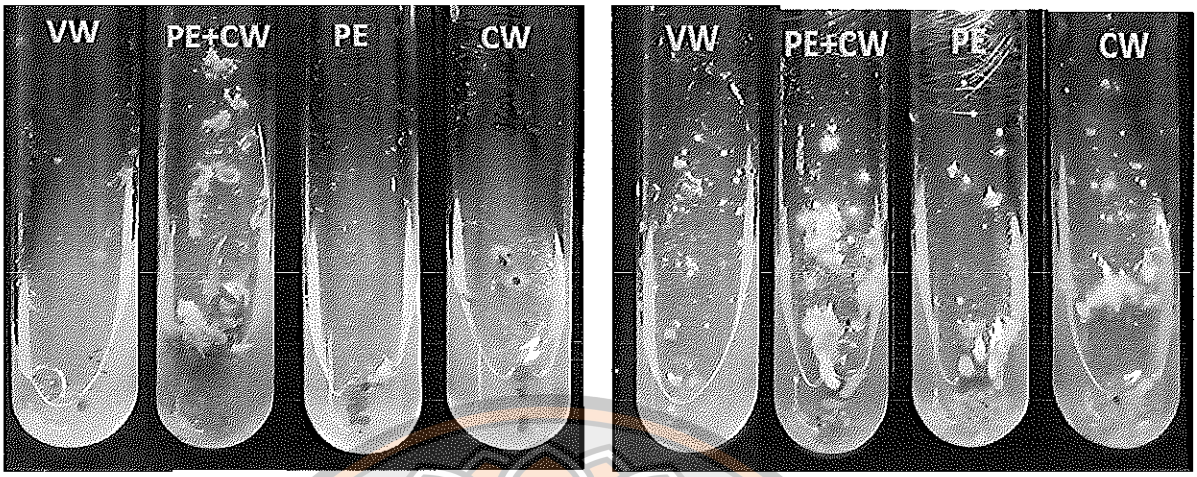


Figure 12 Asymbiotic germination of *P. mishmensis* × *P. tankervilliae* seeds under light (left) and dark (right) condition after 4 months of culture

Table 13 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* after cultured for 1 month

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	94.0 ± 0.8 a	6.0 ± 0.8 c	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	6.0 ± 0.8 c
	Dark	80.5 ± 0.5 c	19.5 ± 0.5 b	0.0	0.0	0.0	0.0	0.0	19.5 ± 0.5 b
VW + CW	Light	88.0 ± 2.0 b	12.0 ± 2.0 c	0.0	0.0	0.0	0.0	0.0	12.0 ± 2.0 c
	Dark	85.3 ± 2.4 b	14.6 ± 2.4 c	0.0	0.0	0.0	0.0	0.0	14.6 ± 2.4 c
VW + PE	Light	80.0 ± 0.8 c	20.0 ± 1.6 b	0.0	0.0	0.0	0.0	0.0	20.0 ± 1.6 b
	Dark	78.0 ± 0.8 cd	22.0 ± 1.6 ab	0.0	0.0	0.0	0.0	0.0	22.0 ± 1.6 ab
VW + PE + CW	Light	88.5 ± 0.5 b	11.5 ± 1.0 c	0.0	0.0	0.0	0.0	0.0	11.5 ± 1.0 c
	Dark	74.5 ± 0.9 c	25.5 ± 0.9 a	0.0	0.0	0.0	0.0	0.0	25.5 ± 0.9 a

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 14 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* after cultured for 2 months

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	92.5 ± 2.75a	6.5 ± 2.0 d	1.0 ± 1.0 ns	0.0 ± 0.0 ns	0.0 ns	0.0 ns	0.0 ns	7.5 ± 2.7 c
	Dark	35.0 ± 12.5c	47.5 ± 3.5 ab	6.0 ± 2.4	0.5 ± 0.5	0.0	0.0	0.0	54.0 ± 4.6 ab
VW + CW	Light	79.0 ± 10.5ab	19.5 ± 9.7 cd	0.0 ± 0.0	1.5 ± 0.9	0.0	0.0	0.0	21.0 ± 10.5 cd
	Dark	41.0 ± 4.5 cd	55.0 ± 3.3 a	3.5 ± 1.2	0.5 ± 0.5	0.0	0.0	0.0	59.0 ± 4.5 a
VW + PE	Light	70.0 ± 9.7 abc	29.0 ± 9.9 abcd	0.0 ± 0.0	1.0 ± 1.1	0.0	0.0	0.0	30.0 ± 9.7 bcd
	Dark	39.5 ± 13.6 cd	49.0 ± 13.2 ab	8.5 ± 2.9	2.5 ± 1.9	0.5 ± 1.0	0.0	0.0	60.5 ± 13.6 a
VW + PE + CW	Light	75.5 ± 12.1 ab	23.0 ± 11.3 bcd	0.0 ± 0.0	1.5 ± 1.9	0.0	0.0	0.0	24.5 ± 12.1 cd
	Dark	56.5 ± 6.9 bcd	37.5 ± 3.5 abc	2.5 ± 1.5	2.0 ± 2.0	1.5 ± 1.5	0.0	0.0	43.5 ± 6.9 abc

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 15 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* after cultured for 3 months

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	84.5 ± 5.5 a	11.0 ± 5.2 c	1.0 ± 1.0 c	3.5 ± 1.7 ns	0.0 ± 0.0 ns	0.0 b	0.0 ns	15.5 ± 5.5 c
	Dark	49.0 ± 5.2 bc	28.5 ± 2.0 abc	6.0 ± 2.0 ab	15.5 ± 2.0	1.0 ± 1.0	0.0 b	0.0	51.0 ± 5.2 ab
VW + CW	Light	70.0 ± 16.3 ab	16.0 ± 6.3 bc	1.0 ± 1.0 c	3.5 ± 2.2	9.0 ± 7.1	0.0 b	0.5 ± 0.5	29.5 ± 16.3 bc
	Dark	33.0 ± 4.1 c	34.0 ± 4.3 ab	9.0 ± 0.57 a	14.0 ± 1.41	8.0 ± 1.4	2.0 ± 1.5 a	0.0	67.0 ± 4.1 a
VW + PE	Light	53.5 ± 12.2 abc	38.5 ± 12.0 a	3.0 ± 1.2 bc	2.5 ± 1.5	2.0 ± 1.4	0.0 b	0.5 ± 0.5	46.0 ± 12.2 abc
	Dark	45.0 ± 7.3 bc	36.0 ± 0.0 a	6.0 ± 2.4 ab	9.0 ± 3.3	4.0 ± 2.1	0.0 b	0.0	55.0 ± 7.3 ab
VW + PE + CW	Light	65.0 ± 12.1 abc	12.0 ± 2.7 c	0.5 ± 0.5 c	10.0 ± 4.1	11.0 ± 7.5	1.5 ± 0.9 ab	0.0	35.0 ± 12.1 abc
	Dark	52.0 ± 12.5abc	36.5 ± 6.7 a	2.5 ± 1.2 bc	7.0 ± 3.6	1.5 ± 1.5	0.5 ± 0.5	0.0	48.0 ± 12.5 abc

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 16 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* after cultured for 4 months.

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	78.5 ± 2.3 a	11.5 ± 0.9 c	4.5 ± 1.2 c	3.5 ± 0.9 b	2.0 ± 1.4 ns	0.0 b	0.0 ns	21.5 ± 2.3 c
	Dark	34.0 ± 6.7 c	31.5 ± 4.6 a	11.5 ± 1.5 abc	12.0± 2.7 a	9.0 ± 3.0	2.0 ± 1.4 ab	0.0	66.0 ± 6.7 a
VW + CW	Light	64.5 ± 12.5 ab	17.0 ± 2.0 bc	4.0 ± 1.41 c	3.5 ± 1.7 b	9.0 ± 3.0	2.0 ± 1.4 ab	0.5 ± 0.5	35.5 ± 12.5 ab
	Dark	25.0 ± 4.1 c	35.0 ± 4.7 a	9.0 ± 2.5 bc	13.5 ± 1.7 a	13.0 ± 3.1	4.5 ± 0.5 a	0.0	75.5 ± 4.1 a
VW + PE	Light	49.5 ± 10.17 bc	28.0 ± 5.0 ab	6.0 ± 2.9 bc	11.0 ± 3.0 a	5.0 ± 1.7	0.5 ± 0.5 b	0.5 ± 0.5	50.5 ± 10.17 ab
	Dark	29.0 ± 6.3 c	30.5 ± 2.2 a	18.5 ± 2.0 a	15.5 ± 1.7 a	6.5 ± 3.2	0.0 b	0.0	71.0 ± 6.3 a
VW + PE + CW	Light	53.5 ± 14.3 abc	11.5 ± 2.6 c	8.5 ± 3.4 bc	12.0 ± 3.5 a	12.0 ± 7.1	2.5 ± 1.2 ab	0.0	46.0 ± 14.3 abc
	Dark	38.5 ± 8.7 bc	24.0 ± 5.9 ab	14.5 ± 4.7 ab	11.0 ± 2.3 a	9.5 ± 3.5	2.0 ± 0.8 ab	0.0	61.5 ± 8.7 a

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 17 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* var. *alba* after cultured for 1 month

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	90.0 ± 0.8 a	10.0 ± 0.8 c	0.0	0.0	0.0	0.0	0.0	10.0 ± 0.8 c
	Dark	83.0 ± 1.0 b	17.0 ± 1.0 b	0.0	0.0	0.0	0.0	0.0	17.0 ± 1.0 b
VW + CW	Light	91.0 ± 0.5 a	9.0 ± 0.5 c	0.0	0.0	0.0	0.0	0.0	0.9 ± 0.5 c
	Dark	72.0 ± 0.8 c	28.0 ± 0.8 a	0.0	0.0	0.0	0.0	0.0	28.0 ± 0.8 a
VW + PE	Light	82.0 ± 0.8 b	18.0 ± 0.8 b	0.0	0.0	0.0	0.0	0.0	18.0 ± 0.8 b
	Dark	71.5 ± 0.5 c	28.5 ± 0.5 a	0.0	0.0	0.0	0.0	0.0	28.5 ± 1.0 a
VW + PE + CW	Light	88.0 ± 2.0 a	12.0 ± 2.0 c	0.0	0.0	0.0	0.0	0.0	12.0 ± 2.0 c
	Dark	81.0 ± 1.4 c	28.0 ± 1.4 a	0.0	0.0	0.0	0.0	0.0	28.0 ± 1.4 a

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 18 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* var. *alba* after cultured for 2 months

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	80.0 ± 6.0 a	16.0 ± 4.3 c	2.0 ± 2.0 b	2.0 ± 0.8 ns	0.0 c	0.0 ns	0.0 ns	20.0 ± 6.0 e
	Dark	21.0 ± 9.1 e	44.5 ± 11.7 a	28.0 ± 8.1 a	6.0 ± 3.5	0.0 c	0.0	0.0	78.5 ± 9.1 a
VW + CW	Light	79.5 ± 5.1 a	16.5 ± 4.1 c	0.0 b	4.0 ± 1.1	0.0 c	0.0	0.0	20.0 ± 5.1 e
	Dark	36.3 ± 3.4 de	41.5 ± 0.5 ab	9.0 ± 3.7 b	5.0 ± 3.0	8.5 ± 2.3 a	0.0	0.0	64.0 ± 3.4 ab
VW + PE	Light	62.0 ± 5.0 bc	34.5 ± 6.1 abc	1.1 ± 0.5 b	2.5 ± 1.5	0.0 c	0.0	0.0	38.0 ± 5.0 cd
	Dark	38.5 ± 2.0 de	44.5 ± 3.2 a	9.5 ± 2.5 b	2.5 ± 1.8	5.0 ± 5.7 b	0.0	0.0	61.5 ± 2.0 ab
VW + PE + CW	Light	72.0 ± 5.8 ab	24.0 ± 4.5 bc	3.0 ± 3.0 b	1.0 ± 0.5	0.0 c	0.0	0.0	28.0 ± 5.8 de
	Dark	49.0 ± 5.1 cd	42.5 ± 8.0 ab	3.0 ± 2.3 b	4.5 ± 1.5	1.0 ± 5.7 c	0.0	0.0	51.0 ± 5.1 bc

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 19 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* var. *alba* after cultured for 3 months

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	72.0 ± 3.3 a	22.5 ± 2.6 de	0.5 ± 0.5 b	3.5 ± 2.2 bc	1.0 ± 0.5 b	0.5 ± 0.5 d	0.0 ns	28.0 ± 3.3 d
	Dark	4.0 ± 4.0 d	38.5 ± 2.8 bc	31.0 ± 0.5 a	17.0 ± 4.1 ab	5.0 ± 2.8 ab	4.5 ± 2.3 a	0.0	96.0 ± 4.0 a
VW + CW	Light	65.0± 4.7 ab	18.0 ± 1.4 e	3.0 ± 1.2 b	1.5 ± 0.9 c	11.5 ± 3.0 ab	1.0 ± 0.5 cd	0.0	35.0 ± 4.7 cd
	Dark	14.0 ± 1.1 cd	52.5 ± 4.5 ab	9.0 ± 1.7 b	11.5 ± 2.6 ab	10.0 ± 2.7 ab	3.0 ± 1.0 ab	0.0	86.0 ± 1.1 ab
VW + PE	Light	48.5 ± 9.8 b	39.5 ± 7.3 bc	3.5 ± 2.3 b	5.0 ± 3.6 bc	3.0 ± 3.0 b	0.5 ± 0.5 d	0.0	51.5 ± 9.8 c
	Dark	10.0 ± 1.1 cd	45.0 ±1.9 d	10.0 ± 1.1 b	21.0 ± 3.3 a	10.5 ± 0.5 a	3.5 ± 0.9 a	0.0	90.0 ± 1.1 ab
VW + PE + CW	Light	50.0 ± 5.3 b	33.0 ± 2.6 cd	4.0 ± 2.7 b	9.0 ± 2.6 bc	4.0 ± 2.1 b	0.0 d	0.0	50.0 ± 5.3 c
	Dark	24.5± 10.6 c	59.0 ± 5.0 a	5.0 ± 2.3 b	4.0 ± 1.4 bc	5.0 ± 2.5 ab	2.5 ± 0.9 bcd	0.0	75.0 ± 10.6 b

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

Table 20 Germination and protocorm development of seed derived from *P. mishmensis* × *P. tankervilliae* var. *alba* after cultured for 4 months

Media	Light condition	Development stage						Death (%)	Germination (%)
		1	2	3	4	5	6		
VW	Light	55.5 ± 5.3 a	21.0 ± 2.8 de	12.0 ± 0.8 b	7.0 ± 2.5 bc	3.0 ± 1.7 b	1.5 ± 0.9 d	0.0 ns	44.5 ± 5.3 d
	Dark	3.0 ± 3.0 d	31.0 ± 3.0 bc	31.5 ± 3.2 a	15.0 ± 1.9 ab	8.0 ± 2.0 ab	11.5 ± 4.9 abc	0.0	97.0 ± 3.0 a
VW + CW	Light	49.5 ± 4.5 ab	18.5 ± 1.2 e	12.0 ± 1.6 b	3.5 ± 1.2 c	9.5 ± 2.8 ab	7.0 ± 2.3 cd	0.0	50.5 ± 4.5 cd
	Dark	3.0 ± 0.5 d	37.5 ± 1.5 ab	12.0 ± 3.3 b	22.5 ± 6.1 a	10.0 ± 0.0 ab	15.0 ± 2.3 ab	0.0	97.0 ± 0.5 a
VW + PE	Light	33.5 ± 9.4 bc	34.0 ± 1.5 bc	15.0 ± 3.1 b	10.5 ± 3.3 bc	5.0 ± 2.8 b	2.0 ± 1.4 d	0.0	66.5 ± 9.4 bc
	Dark	0.0 d	42.0 ± 1.1 a	12.5 ± 1.2 b	14.5 ± 1.5 ab	14.5 ± 1.2 a	16.5 ± 1.5 a	0.0	100.0 ± 0.0 a
VW + PE + CW	Light	49.5 ± 7.3 ab	26.5 ± 3.2 cd	8.0 ± 2.7 b	9.5 ± 1.5 bc	5.0 ± 2.8 b	1.5 ± 0.9 d	0.0	50.5 ± 7.3 cd
	Dark	17.0 ± 7.9 cd	44.0 ± 2.1 a	15.0 ± 3.0 b	7.0 ± 2.8 bc	9.0 ± 3.4 ab	8.0 ± 2.9 bcd	0.0	83.0 ± 7.9 ab

Values are means ± SE of 3 replications (100 seeds per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. Germination percentages were calculated from stage 2 to 6

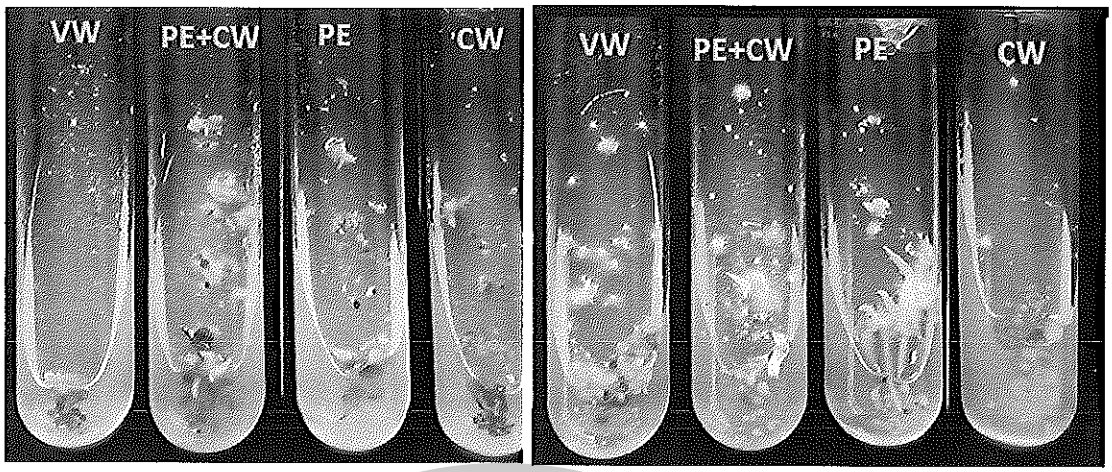


Figure 13 Asymbiotic germination of *P. mishmensis* × *P. tankervilliae* var. *alba* seeds under light (left) and dark (right) condition after 4 months of culture

CHAPTER IV

FACTORS EFFECTING SHOOT REGENERATION AND ACCLIMATIZATION OF CALANTHE GROUP AND CALANTHE HYBRIDS

Summary

The influence of organic supplements on growth and multiplication efficiency of seedlings devived from self-pollination of *P. mishmensis* and *P. tankervilliae,* and inter specific cross-pollination between this two species was investigated. Twelve week-old seedlings were cultured on half-strength semi-solid Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 8 g/L agar and various concentrations of CW (Coconut water) (0, 50, 100, 150 and 200 mL/L) combined with PE (Potato extract) (0, 25 and 50 g/L). The cultures were then kept under a constant photoperiod (16 h light: 8 h dark) at 25 ± 2 °C for 12 weeks. The highest number (3.03 shoots/explant) was found in *P. mishmensis* cultured in the medium with 50 ml/L CW and 50 g/L potato extract, whereas the highest number of leaves (5.9 leaves/explant) and roots (6.23 roots/explant) were observed on the medium, with 150 ml/L CW and 50 g/L PE. For *P. tankervilliae*, the highest number of shoots (3.93 shoots/explant) the was found when culturing on the medium added with 25 mL/L CW and 50 g/L PE. Meanwhile, the highest number of leaves (11.8 leaves/explant) was observed on the medium added with 100 mL/L CW and 25 g/L PE, whereas, the number of roots derived from various concentrations of organic substance was not significant difference. However, the highest number of roots (10.07 roots/explant) was observed on the medium supplemented with 50 mL/L CW and 50 g/L PE. For the putative interspecific hybrid seedling generated from *P. mishmensis* × *P. tankerviliae*, showed the highest number of shoots (2.43 shoots/explant) and leaves (8.23 leaves/explant) when culturing on the medium supplemented with 150mL/L CW and 50 g/L PE. Whereas the highest number of roots (8.50 roots/explant) was observed on the medium supplemented with 25 mL/L CW and 50 g/L PE. Additionally, plantlets derived from *P. mishmensis* were chosen and transferred to grow in the seven

different substances i.e., soil, sand, coconut husk chip, and four different combinations of these substances, soil-sand mix (1: 1), soil-coconut husk chip mix (1: 1), sand-coconut husk chip mix (1: 1) and soil-sand-coconut husk chip mix (1: 1: 1) for four weeks. The results showed 100% survival rate of acclimatized plants with leaf length approximately 3.2 leaves/explant in the combination of substances between sand and coconut husk chip mix. The number of shoots, shoot length, number of leaf and leaf width induced by pure soil and soil, sand-coconut husk chip combination was significantly higher than that planted in other substances (P<0.05). Meanwhile, no significant difference in new shoot formation among these substances was observed (P > 0.05). This precursory developing protocol was likely to be applied to the large scale of plant production as well as conservation of germplasm of this orchid *species*.

Introduction

Phaius is a genus of terrestrial orchids belonging to the subfamily Epidendroideae, tribe Collabieae. Less than 50 described species were recorded and widely distributed in Africa, Australia, Pacific and Indian Ocean islands and Asia including Thailand. Up to date, six known *Phaius* species (i.e. *P. tankervilleae*, *P. mishmensis*, *P. indigoferus*, *P. takeoi*, *P. flavus* and *P. epiphticus*) have been morphologically classified and exhibited throughout Thailand. *Phaius mishmensis* was listed as protected species; In addition, some countries use *P. tankervilleae* as folk medicines to treat rash, freckle, blemish and blister. This orchid species in natural habitat was speculated as rare and endangered spieces in Japan (Hirano et al., 2009), In Australia, it was now listed in CITES Appendix II, which somehow can be traded under a strict regulation (Acuña-tarazona et al., 2019). The extinction of *Phaius* and hybrid is now concerned because those orchids as rarely found in natural populations. Therefore, an efficient method for rapid propagation of this variety needed to be initially studied for conservation and sustainable utilization. Among those orchids, *P. tankervilleae* is one of the most ornamentally attractive species. Morphologically, their inflorescences are large, showy and distinctive. They are easy to grow and have a long duration of blooming, which is appropriate to use as potted plants and cut flowers.

There were reports that, *in vitro* culture technique can be used as an effective way to propagate many species of orchids using MS medium (Murasnige & Skoog, 1962) and VW medium the high potential of micropropagation from different explants (Chugh et al., 2009). Unfortunately, there were limited information related to large-scale multiplication of exquisite and rare hybrids using tissue culture techniques, the research studies of hybrid orchids may helped orchids occupying a position as one of the top ten cut flowers (Yong et al., 2009). In contrast, many previous researches regarding orchids *in vitro* propagations for instance, in terrestrial orchids have produced propagation in *P. tankervilliae*. In addition, many species of orchids (e.g., *P. tankervilliae, Paphiopedilum* sp. (Huang et al., 2001), *Eulophia nuda* (Panwar, et al., 2012), *Geodorum densiflorum* (Bhadra, & Hossain, 2003), *Haemaria discolor* (Shiau et al., 2005), Developed shoot of *Paphiopedilum* sp. (Chen, Chen, & Chang, 2004), *Pa. armeniacum* (Zhang et al., 2015), *P. tankuvillae*) were mass propagated using various techniques such as shoot development, somatic embryogenesis *in vitro* culture. These techniques were used for solving the problem of some threatened and Endangered orchid species, *Paphiopedilum* species, (Long et al., 2010) and of *Phalaenopsis* (Ishii et al. 1998), *Laelia speciosa* (Ávila-Díaz et al., 2009), *Cephalanthera falcate* (Yamato, & Iwase, 2008), *Calanthe tricarinata* (Godo et al., 2010). Moreover, induction of shoot regenerations were recorded in many orchid species such as *Arides maculosum* (Malabadi, et al., 2009),*Oncidium* sp. (Kalimuthu et al., 2007), *Cymbidium ensifolium* (Chang, & Chang,1998), *Vanda tessellate* (Rahman et al., 2009), *Phalaenopsis gigantean* (Yong et al., 2009).

In the culture medium used for micropropagation, the organic supplement was one of the key composition factors affecting the result of *in vitro* orchid culture, such as coconut water, potato, banana and tomato extracts. Organic substance was inducted the growth and multiplication rate of many orchid species that can be enhanced by adding some organic supplements like coconut water or potato extract to the culture medium (Pakum et al., 2016). Natural substances in tissue culture media of higher plants were adopted (Molnár, Virág, & Ördög, 2011). For composition of coconut water and potato extract, chemical composition and biological properties of coconut water for example in *Ph. hangianum* (Zeng et al., 2013), potato for *Ph. gigantean, Calanthe* hybrids (Baque, et al., 2011a), *Rhynchostysis retusa P. tankervilleae* and

Cymbidium pendulum (Kaur, & Bhutani, 2012) were detected. Moreover, sucrose can also affect on *Phalaenopsis*, *Doritaenopsis* and *Neofinetia* (Islam, & Ichihashi, 1999). In addition, they used activated charcoal *In vitro* germination and protocorm formation of *Calanthe* hybrids (Shin et al., 2011), especially in several orchids in Brazil. Moreover, they used plant growth regulator for hybrids such as using NPK and 6-Benzylaminopurine on growth and flowering of two orchid genera and applying auxin in orchid development (Novak et al., 2014). In addition, for intergeneric orchis hybrid in two rare and endangered orchids i.e. *Renanthera imschootiana* and *Vanda coerulea* (Kishor, & Sharma , 2009) was also used. However, the accomplishments are depending on explant types and orchid species (Molnár et al., 2011).

At present, few publications about the effect of organic supplements on shoot regeneration capability in the *Phaius* species were documented and no such reports in *Phaius species* and their hybrids. Therefore, this study firstly attempted to observe the influence of organic supplements on the shoot multiplications efficiency of *Calanthe* group as well as *Calanthe* hybrids. Although, micropropagation has been extensively used for the rapid multiplication of many plant species. However, its wider use is often restricted by the high percentage of plant loss or damaged when transferred to *ex vitro* condition. Therefore, the acclimatization is also very important technique for mcropropagation of plants. For instance, in orchids, *in vitro* acclimatization using uniconazole treatments and *ex vitro* adaptation of *Phalaenopsis* orchid (Cha-um et al., 2009) was reported. For research in the acclimatization of tissue cultured plants (Hazarika, 2003) as well as cryopreservation and low-temperature storage of seeds of *P. tankervilleae* (Hirano et al., 2009) efficient *in vitro* hardening technique of tissue culture raised plant (Deb, 2016).

This study the factors effecting shoot regeneration and acclimatization of *Calanthe* and *Calanthe* hybrids was carried out with various concentrations of potato extract and natural product on the *in vitro* growth and growth attributes of *Calanthe* hybrids that suitable for commercial exploitation. The acclimatization study is a novel technique for one step hardening of orchid which is successfully employed and established with a high degree of efficiency.

Materials and Methods

In vitro culture of *Calanthe* group and *Calanthe* hybrids

Plant materials

The pods of *Calanthe* group and *Calanthe* hybrids obtained from Romklao Botanical Garden under the Royal Initiative, Phitsanulok, Thailand were sterilized with 15% (v/v) Clorox® solution for 20 minutes. The seeds were selected from sterilized pod and cultured on modified VW (Blume et al., 2011) medium supplemented with 150 mL/L CW and 50 g/L PE. (Table 19)

Table 21 Orchid genus *Phaius* and hybrids

Orchid species	Explant
1. *P. mishmensis* × *P. mishmensis*	Pod
2. *P. tankervilliae* × *P. tankervilliae*	Pod
3. *Putative P. mishmensis* × *P. tankervilliae*	Pod

Culture medium and culture conditions for shoot formations

The media was solidified by 8 g/L agar and the pH was adjusted to 5.2. After 12 weeks of seed culture, the seedlings were cultured on half-strength MS medium (Murashige & Skoog 1962) supplemented with different concentrations of CW and PE. The media were added with 15 g/L sucrose, 8 g/L agar and adjusted the pH to 5.8. The seedlings were cultivated under the cool-white LED lamp (20 µmol/m^2/s) for 16 hrs/day at 25 ± 2 °C. Growth and development of seedlings were measured and recorded after 12 weeks of culture.

Table 22 Treatments for the study of factors affecting shoot regeneration and acclimatization of *Calanthe* and *Calanthe* hybrids

Treatments	Components
T1	½ MS
T2	½ MS + PE 25 g/L
T3	½ MS + PE 50 g/L
T4	½ MS + CW 50 mL/L
T5	½ MS + CW50 mL/L + PE 25 g/L
T6	½ MS + CW 50 mL/L + PE 50 g/L
T7	½ MS + CW 100 mL/L
T8	½ MS + CW 100 mL/L + PE 25 g/L
T9	½ MS + CW 100 mL/L + PE 50 g/L
T10	½ MS + CW 150 mL/L
T11	½ MS + CW 150 mL/L + PE 25 g/L
T12	½ MS + CW 150 mL/L + PE 50 g/L
T13	½ MS + CW 200 mL/L
T14	½ MS + CW 200 mL/L + PE 25 g/L
T15	½ MS + CW 200 mL/L + PE 50 g/L

Remark: (CW) coconut water and potato extract (PE)

Experimental design and statistical analysis

Each treatment was performed in 10 replicates and repeated 3 times. The statistical differences of data were analyzed using One-way ANOVA and Duncan's multiple range tests (DMRT).

Acclimatization of some Calanthe group and Calanthe hybrids plantlets

The *in vitro* rooted plantlets were washed with sterile water to remove the agar medium and immersed in a fungicide solution for 20 mins. The treated plantlets were then transplanted to plastic pots (5 cm wide and 5 cm height) containing the different planting substance and cultured under the greenhouse (28–34 °C relative 50–60% humidity and natural sunlight with 70% shade nets). After 4 weeks of transplantation, survival and growth rate of transplanted plants were recorded. The experiment was conducted with 3 replications of 10 platelets per replication.

Results

The influence of organic supplements on growth and multiplication efficiency of *Calanthe* group and *Calanthe* hybrids

Factors effecting shoot regeneration of *P. mishmemsis*

The seedlings of *P. mishmemsis* culturing on half-strength semi-solid MS medium supplemented with different concentrations of CW (0, 50, 100, 150 and 200 mL/L) combined with PE (0, 25 and 50) after 12 weeks of culture, all seedlings were and survived in all media. However, the different rate growth performances were found among the different PE and CW supplementations were observed (Figure 14).

The results showed that concentrations of CW (0 and 50 mL/L), with shoot number of *P. mishmensis* had a tendency to increase at this with the concentration of PE supplementation. On the other hand, shoot numbers tended to decrease with increasing of PE content at higher concentration CW (100 and 150 mL/L). Seedling plants cultureding on the medium supplemented with 50 ml/L CW and 50 g/L PE, produced the highest number of shoots (3.03 shoots/explant) (Figure 15A). Nevertheless, different concentration of PE did not affect the shoot number at the highest can of CW (200 mL/L). In summary, the most effective combination for shoot multiplication of *P. tankervilleae* var. *alba* seedling was 50 mL/L CW with 50 g/L PE (Figure 14 C).

Generally, PE and CW have been used in orchid culture media,that can promote the numbers of leaves of seedling PE (Figure 14 B). However, the high concentration (more than 150 mL/L) of CW without PE inhibited leave production. On the other hand, the leave production was obviously improved along with the increasing concentration of PE augmentation with high CW containing all culture media. Conversely, PE combination did not significantly increase the number of leave (5.9 leaves/explant) (Figure.14 D-F). When the concentration of CW lower than 150 mL/L. The highest average number of leaves per explant was achieved from the medium containing 150 ml/L CW and 50 g/L PE.

In this study, roots were successfully produced in every plantlet. As same as leave production. Without PE, and the increasing of CW concentration root was stopped germinating with (Figure 15 C). Similarly, stopping root germination was also observed with the increasing of PE concentration in media without CW. However, PE combinations were significantly improved root production in the media holding CW, especially the highest average roots (6.2 3 roots/explant) number per explant was achieved from the medium containing 150 mL/L CW and 50 g/L PE (Figure14 E).

Factors effecting shoot regeneration of *P. tankervilliae*

The seedlings of *P. tankervilliae* were cultured on half-strength semi-solid MS medium supplemented with various concentrations of CW (0, 50, 100, 150 and 200 mL/L) combined with PE (0, 25 and 50 g/L) after 12 weeks of culture, all seedlings were well grown in all media. However, diverse growth performances were found among the different concentration of PE and CW supplementations (Figure 16). At low concentration of CW (0 and 50 mL/L), the shoot number was likely to increase with the concentration of PE. On the other hand, shoot numbers tend to decrease with increasing PE content at higher CW (100 and 150 mL/L). Nonetheless, different concentration PE did not affect shoot number at the highest concentration CW addition (200 mL/L). In short, the most effective combination for shoot multiplication (3.93 shoots/explant) (Figure 17 B) of *P. tankervilleae* seedling was 25 mL/L CW with 50 g/L PE.

Although PE and CW have been employed in orchid culture media, numbers of leaves of seedlings are obviously affected by their organic supplement (Figure 16 B). The leave production was obviously improved along with the increasing concentration of PE augmentation in high CW containing media. When the cone of contrary, PE combination did not significantly increase the number of leave CW was lower than 150 mL/L. The highest average leaf number per explant was achieved from the medium containing 100 mL/L CW and 25 g/L PE. Meanwhile, the highest number of leaves (11.8 leaves/explant) (Figure 16 D) was obtained from the medium supplemented with 25 mL/L CW and 50 g/L PE.

In this study, without PE, root was retarded with the increasing of CW concentration (Figure 16 C). In the same way, root was also restrained with the increasing of PE concentration in media without CW. Interestingly, the highest

number of roots (10.07 roots/explant) was achieved from the medium containing 50 mL/L CW and 50 g/L PE but it was no significantly different (Figure 17 C) in every combination of media holding CW, especially the highest average root number per explant was achieved from the medium containing 50 mL/L CW and 50 g/L PE.

Factors effecting shoot regeneration of putative *P. mishmemsis* × *P. tankervilliae*

The hybrid seedlings of putative *P. mishmemsis* × *P. tankervilliae* cultured for 12 weeks on a half-strength semi-solid MS 1992 medium supplemented with various concentrations of CW (0, 50, 100, 150 and 200 mL/L) combined with PE (0, 25 and 50 mL/L) were well developed and survived in every combination of all organic supplemented media. However, diverse growth performances were observed among the different PE and CW supplementations (Figure 18). At the low concentration of CW supplementation (0 and 50 mL/L), shoot number had a tendency to increase with the concentration of PE supplementation. In contrast, shoot numbers tended to decrease with increasing PE content at high CW supplementation (100 and 150 mL/L). Notwithstanding, different PE supplementation did not effect on shoot number at the highest concentration of CW (200 mL/L). In conclusion, the most effective combination for shoot multiplication (2.43 shoots/explant) (Figure 19 A) of putative hybrid seedlings between *P. mishmemsis* and *P. tankervilliae* was 150 mL/L CW with 50 g/L PE.

Although PE and CW have been generally applied in orchid culture media, the numbers of leaves of seedlings still obviously affected by these organic supplementations. The leaf production was obviously developed along with the increasing concentration of PE augmentation in high CW containing media. On the other side, PE combination did significantly increase the number of leave at CW supplementation lower than 150 mL/L (Figure 19). The highest average leaf number, 8.23 leaves per explant (Figure 19 B), was achieved from the medium containing 150 mL/L CW and 50 g/L PE.

In this study, the medium without PE, root development was delayed with the increasing of CW concentration. On the opposite way, the root germination was likely to slow with the increasing of PE concentration in media without CW. However, PE combinations were not significantly improved the root production in the media

holding CW, especially the highest average roots number per explant was achieved from the medium containing 50 mL/L CW and 25 g/L PE (Figure 18 D), whereas the highest number of roots (8.50 roots/explant) (Figure 19 C) was observed in the medium supplemented with 25 mL/L CW and 50 g/L PE.

Survival and growth of acclimatized of *P. mishmemsis*

Additionally, plantlets of *P. mishmensis* were chosen and transferred to nurse for 4 weeks in the seven combinations of 3 substances i.e. soil, sand and coconut husk chip, soil-sand mix (1: 1), soil-coconut husk chip mix (1: 1), sand-coconut husk chip mix (1: 1) and soil-sand-coconut husk chip mix (1: 1: 1). The results showed 100% survival rate of acclimatized plants with leaf length approximately 3.2 leaves/ explant as shown in table 23 and figure 20 when soil, sand, and coconut husk chip mix was used as substances. The number of shoots, shoot length, number of leave and leave the width induced by pure soil and soil, sand-coconut husk chip mix was significantly higher than that planted in another substance ($P < 0.05$). Meanwhile, no significant difference in new shoot formation among these substances was observed ($P < 0.05$). (Figure 20) This preparatory developing protocol could be applied for more large scale of plant production as well as conservation of germplasm of this orchid species.

Discussion

This is the first report of the most suitable protocol for *in vitro* shoot multiplication using the half-strength MS supplemented with 150 mL/L CW and 50 g/L PE and acclimatization under greenhouse condition in the mixture soil-sand-coconut husk chip mix (1: 1: 1). This study provided the useful information applicable for commercial propagation and conservation. The combination effect of CW and PE on the number of leaves was also formerly reported in *Bulbophyllum nipondhii*; however, the number of leave did not decrease in high concentration of CW. (Pakum, et al., 2016). For *in vitro* orchid culture, CW and PE were extensively used because they effectively promote the growth and development of orchids (Molnár et al., 2011; Thorpe et al., 2008). In brief, the most effective combination for shoot multiplication of *P. mishmensis* seedling was 50 mL/L CW with 50 g/L PE. Although PE and CW have been adopted in orchid culture media, the effects of combinations of these organic supplements have been studied in only few orchid species (Pakum et al., 2016). Previously, growth enhancement of *Calanthe* hybrid plantlet by CW supplement has been reported (Baque et al., 2011b). The optimum concentration was 50 mL/L which was consistent with this study. However, the higher CW concentration promoted better growth and shoot multiplication in some orchid species (Pakum et al., 2016). Hence, the effect of CW might depend on species and explants. With regard, to the number of leaves and number of roots of seedling were obviously affected by the supplementation of CW and PE, without PE, adding high concentration (more than 150 mL/L) of CW can inhibit leaf production. However, the leaf production was obviously improved along with the increasing concentration of PE augmentation in high CW containing media. It was found that these three orchid explants were able to provide the highest number of leaves and roots in medium supplemented with PE 50 g/L combines with CW 150 mL/L especially, in *P. mishmensis*. Even though *P. tankervilliae* and *P. mishmensis* × *P. tankerviliae* were not significantly different, but the supplementation of both organic compounds tended to increase the number of leaves and roots. According to Punjansing et al., (2019), they studied the influence of organic supplements on growth and multiplication efficiency of *P. tankervilleae* var. *alba*. It was disclosed that the highest number of shoots was obtained when culturing on the medium added with 50 mL/L CW and 50 g/L PE. On one hand, PE combination

did not significantly increase the number of leave when the concentration of CW lower than 150 mL/L. The highest average leaves number per explant was achieved from the medium containing 150 mL/L CW and 50 g/L PE. This combination also effect on the number of *Bulbophyllum nipondhii* leaves (Pakum, et al., 2016); however, the number of leave did not decrease in high concentration of CW supplementation. *In vitro* plantlet with roots is preferred before transfer to ex vitro environment because the plantlets without root or poor roots quality hardly survive under ex vitro condition (Dohling et al., 2012). In this study, the roots were successfully produced in every plantlet as same as leaves production. The culture medium without PE, root germination was inhibited with the increasing of CW concentration. In the same manner, root development was also discontinued with the increasing of PE concentration in the media without CW. However, PE combinations were significantly improved root production in the media holding CW, particularly at a high CW concentration (more than 150 mL/L). Therefore, root production appeared to be induced by the combination of CW and PE. This effect was aforementioned in *Bulbophyllum nipondhii* (Pakum et al., 2016). The main compositions of CW were sugars, amino acids, minerals, vitamins and particularly plant hormones like auxin and cytokinin (Yong et al., 2009) which have been proposed as a keyingredients affecting explant organogenesis, growth and development. Whereas the main compositions of PE were carbohydrate, protein, and important vitamins (C, B_1, B_6) and mineral elements (potassium, iron, magnesium) (Bártová, & Bárta, 2009). Moreover, the extract of potato tubers contained carbohydrates, these components of orchids micropropagation effects on some species, such as *Phalaenopsis* sp and *Doritaenopsis* sp. However, the natural substances have beneficial effects on in vitro plant cell and tissue cultures. It was found that polyamine had properties to help slow down the growth of double cotyledons and monocotyledon plants. It prevents the formation of chlorophyll but promotes mitotic cell division, allowing plants to grow better. These findings could provide some information on the morphogenic response of different carbon sources and potato homogenate on the propagation of orchid *P. gigantea* (Murdad et al., 2010). Furthermore, organic nitrogen composition was easy to process by orchid protocorms in comparison to inorganic nitrogen because it might be bypassed some step in nitrogen metabolic pathways (Bártová, & Bárta, 2009). Thus,

the improvement of the shoot, leaf and root numbers of this orchid group probably due to the action of some phytohormones. However, the organic supplement was considered as being an undefined medium. Therefore, plant tissue culture medium in several researches normally used plant growth regulator for preparing medium. Moreover, the effect of basal medium and plant growth regulators on *in vitro* multiplication of *P. tankuvillae* has been examined. It was reported that VW solid medium supplemented with 0.004 mg/L triacontanol and 0.1 mg/L BA increased high number of shoots and roots (Blume et al., 2012). In this study, we observed a differential effect of CW concentration on the growth and growth attributes and we found that the optimum concentration that affected on growth and growth attributes for *Calanthe* group and *Calanthe* hybrids, was 50 mL/L related to the study of *Calanthe* hybrids when using higher concentrations of coconut water (100 mL/L), that decreased all the growth and morphological features, as well as induced abnormal platelet growth (Baque et al., 2011a). With the combination of CW and PE (50 to 150 mL/L) in the culture media, number of shoots, leave and root could be increased in both *Calanthe* and hybrids. Previous investigations regarding the beneficial effects of coconut water on orchid production in different plant species have been vastly reported. In addition, coconut water (10%) and peptone (2 g/L) were found to be effective in term of multiplication of PLBs and early plantlet development of *Cymbidium pendulum* (Kaur & Bhutani, 2012). In addition, *P. tankuvillae* plantlets culturing on MS medium supplemented with 3.0 mg/L TDZ gave the highest root length and leaf number with significant difference compared with the plantlets culturing on MS medium without PGRs and the medium supplemented with low concentration of BA. Additionally, as a source of natural product, 50 mL/L coconut water effectively enhanced plantlet growth in both hybrids compared to the relative control (without coconut water) (Baque et al., 2011). Several reports discovered that the medium supplemented with BAP (1.0 mg/L), after culture could increase shoots of *P. tankuvillae.*

Conclusion

This research was proposed as the first protocol for *in vitro* shoot multiplication of two major species of *Calanthe* group, namely *P. mishmensis* and *P. tankerviliae* and putative hybrids. The most suitable culture medium for *P. mishmensis* and *P. tankervilliae* and putative hybrids for shoot multiplication was half-strength MS supplemented with combinations of CW and PE, i.e. 50 mL/L CW and 50 g/L PE, 50 mL/L CW and 25 g/L PE and 150 mL/L CW and 50 g/L PE, respectively. These media provided the highest shoot number with fair leaves and roots. *In vitro* plantlets of *P. mishmensis* were successfully acclimatized and survived under the greenhouse. The mixture soil-sand-coconut husk chip mix (1: 1: 1) was the most appropriate substance for acclimatization. This study could provide the useful information applicable for commercial propagation and conservation of *Phaius* and their hybrids in the near future.

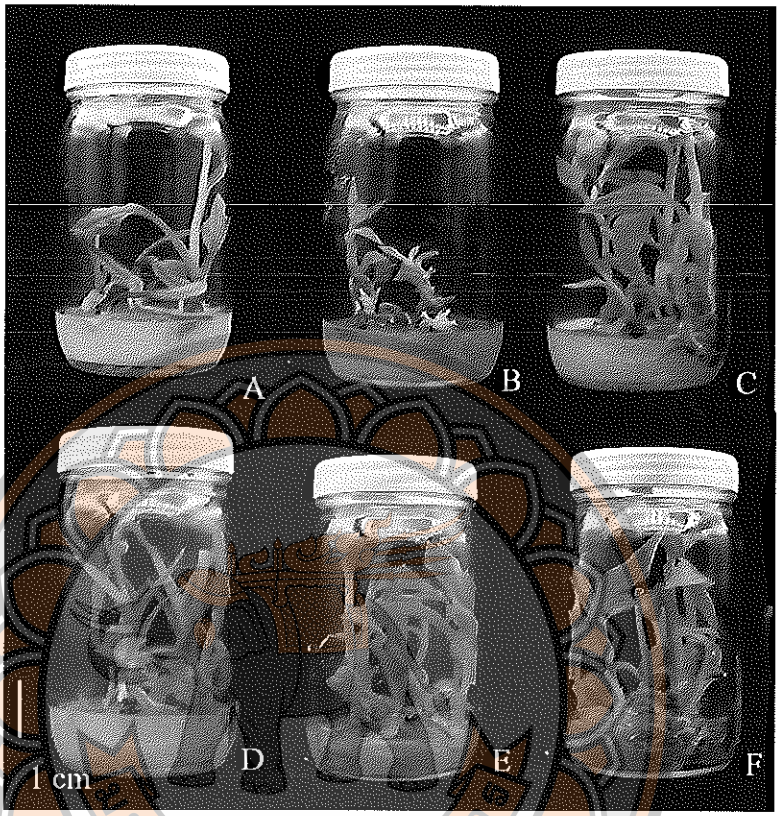


Figure 14 ***In vitro*** **plantlets of** ***P. mishmensis*** **12 weeks after cultured on (A) ½MS (Basal medium), (B) ½ MS supplemented with 150 mL/L CW, (C) ½ MS supplemented with 150 mL/L CW and 50 g/L PE (D- F) ½ MS supplemented with 150 mL/L CW and 50 g/L PE**

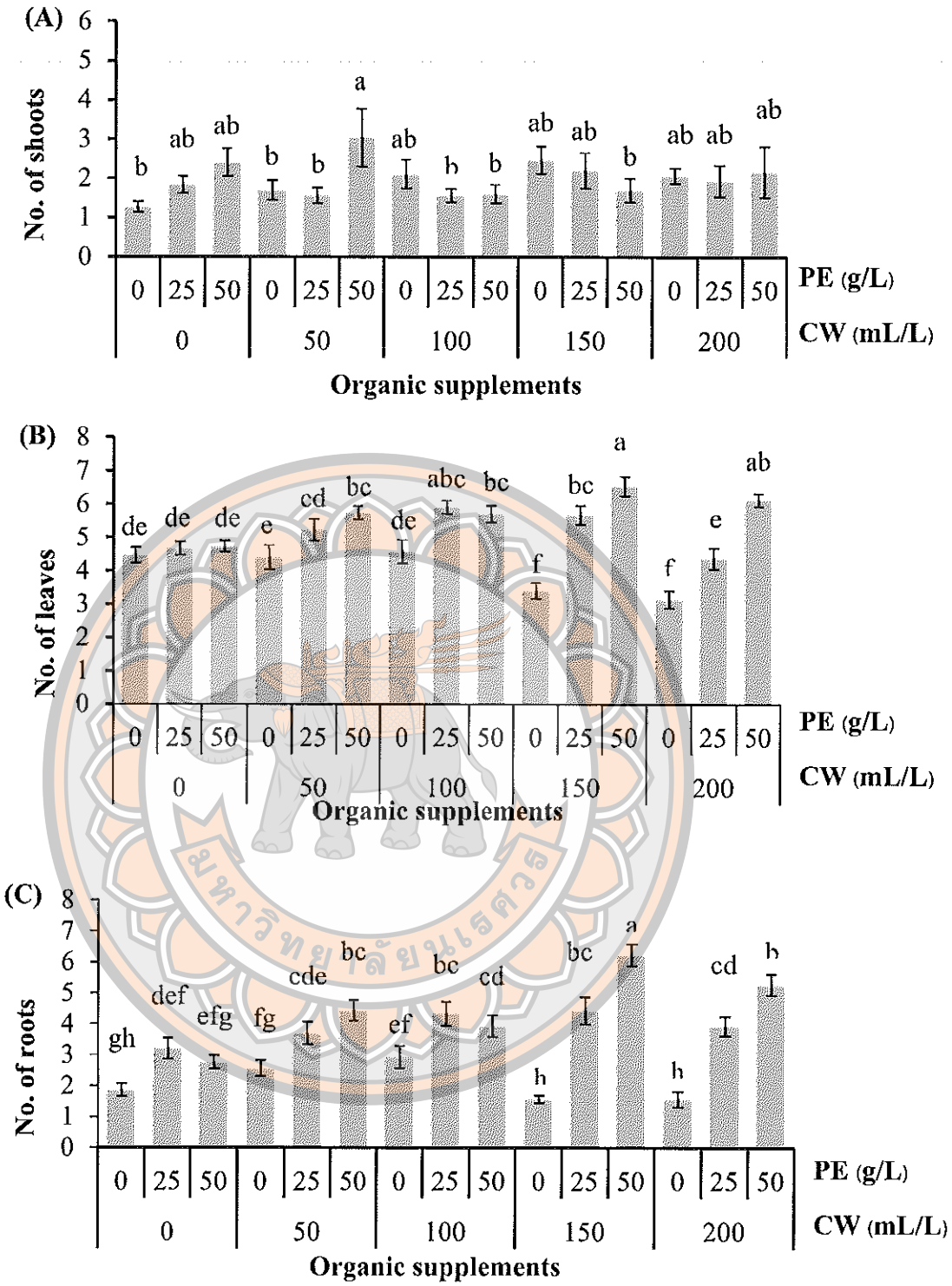


Figure 15 Effect of CW and PE supplementation on number of shoots (A), leaves (B) and root (C) per explant of *P. mishmensis* after culture for 12 weeks. The bars and error bars represent means and SE of 3 replicates (each with 10 plants), respectively. Different letters above the bar show significant differences analyzed by DMRT at $p \leq 0.05$

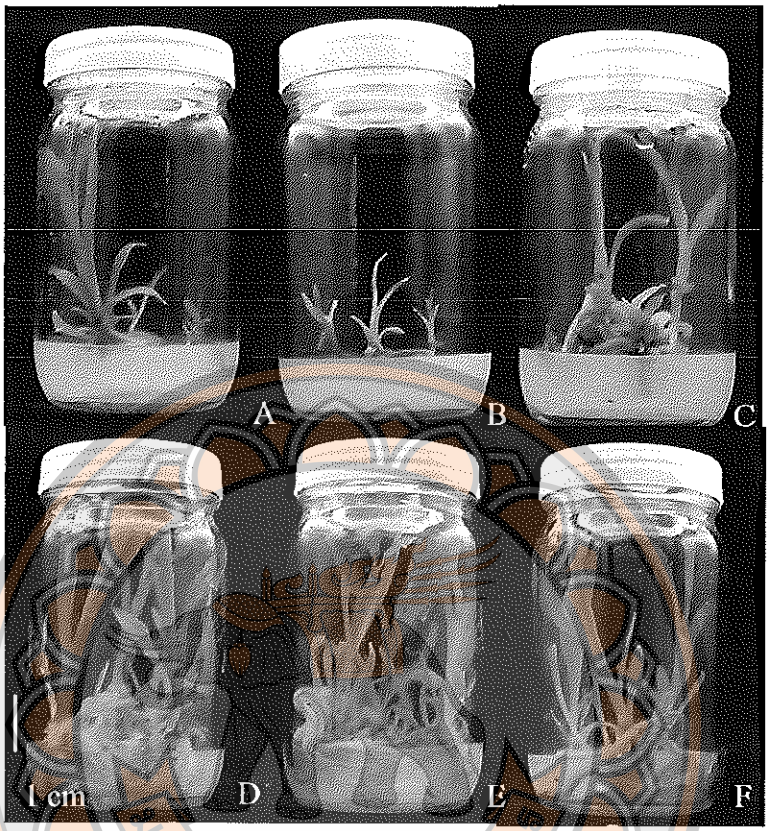


Figure 16 ***In vitro*** **plantlets of** ***P. tankervilleae*** **12 weeks after cultured on (A) ½MS (Basal medium), (B) ½ MS supplemented with 150 mL/L CW, (C) ½ MS supplemented with 150 mL/L CW combines with 50 g/L PE (D) ½ MS supplemented with 100 mL/L CW combine with 25 g/L PE (E- F) ½ MS supplemented with 50 mL/L CW combine with 50 g/L PE**

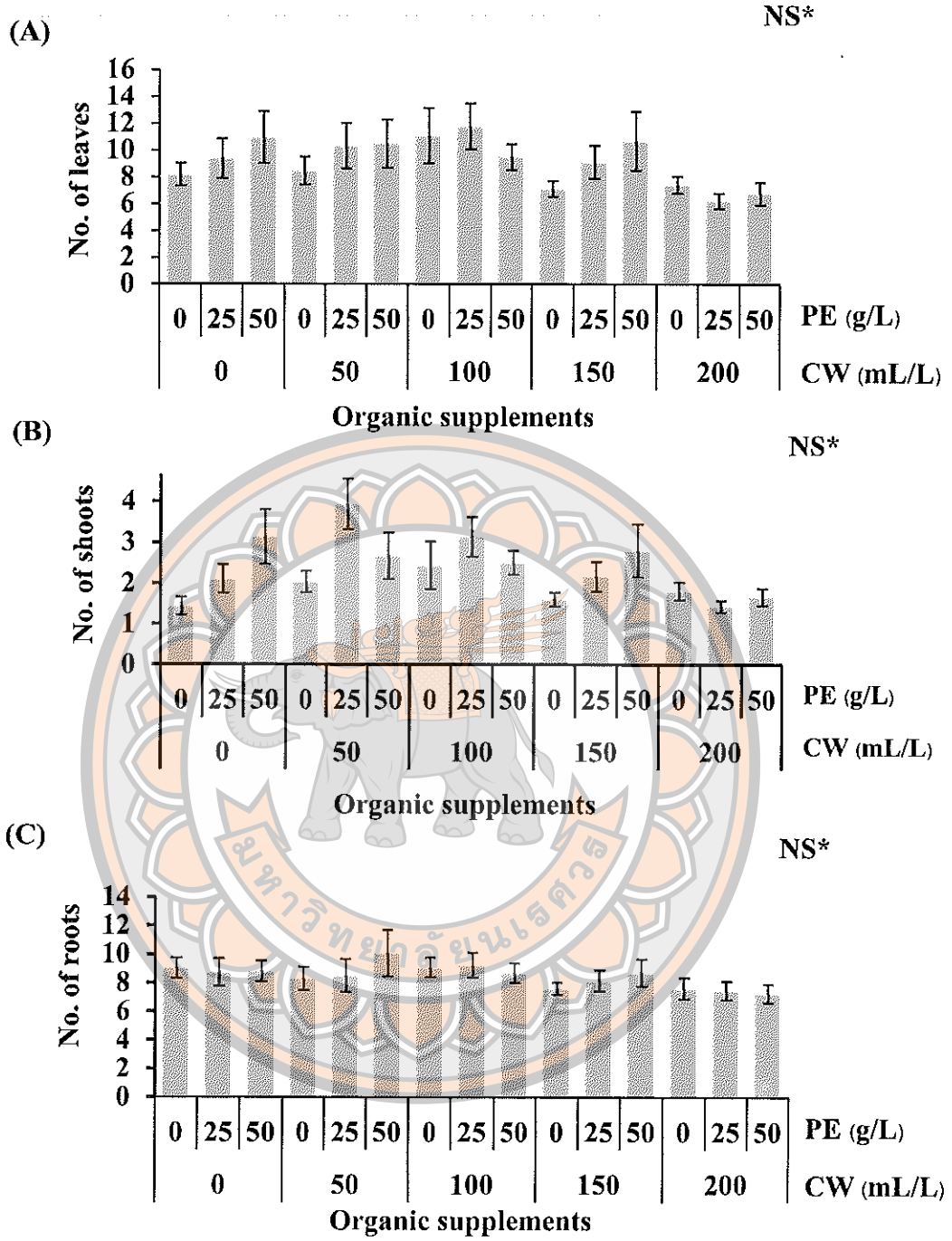


Figure 17 Effect of CW and PE supplementation on number of shoots (A), leaves (B) and root (C) per explant of *P. tankervilliae* after culture for 12 weeks. The bars and error bars represent means and SE of 3 replicates (each with 10 plants), respectively. Different letters above the bar show significant differences analyzed by DMRT at $p \leq 0.05$

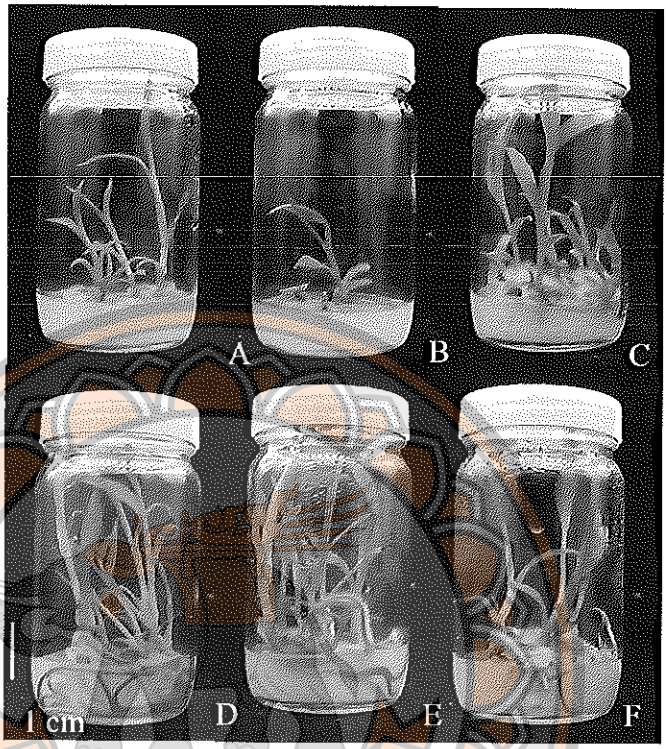


Figure 18 ***In vitro*** **plantlets of** ***P. tankervilleae*** **12 weeks after cultured on A) ½MS (basal medium), (B) ½ MS supplemented with 150 mL/L CW, (C) ½ MS supplemented with 100 mL/L CW and 50 g/L PE (D) ½ MS supplemented with 50 mL/L CW and 25 g/L PE (E) ½ MS supplemented with 50 mL/L CW and 150 g/L PE (F) ½ MS supplemented with 50 mL/L CW and 100 g/L PE**

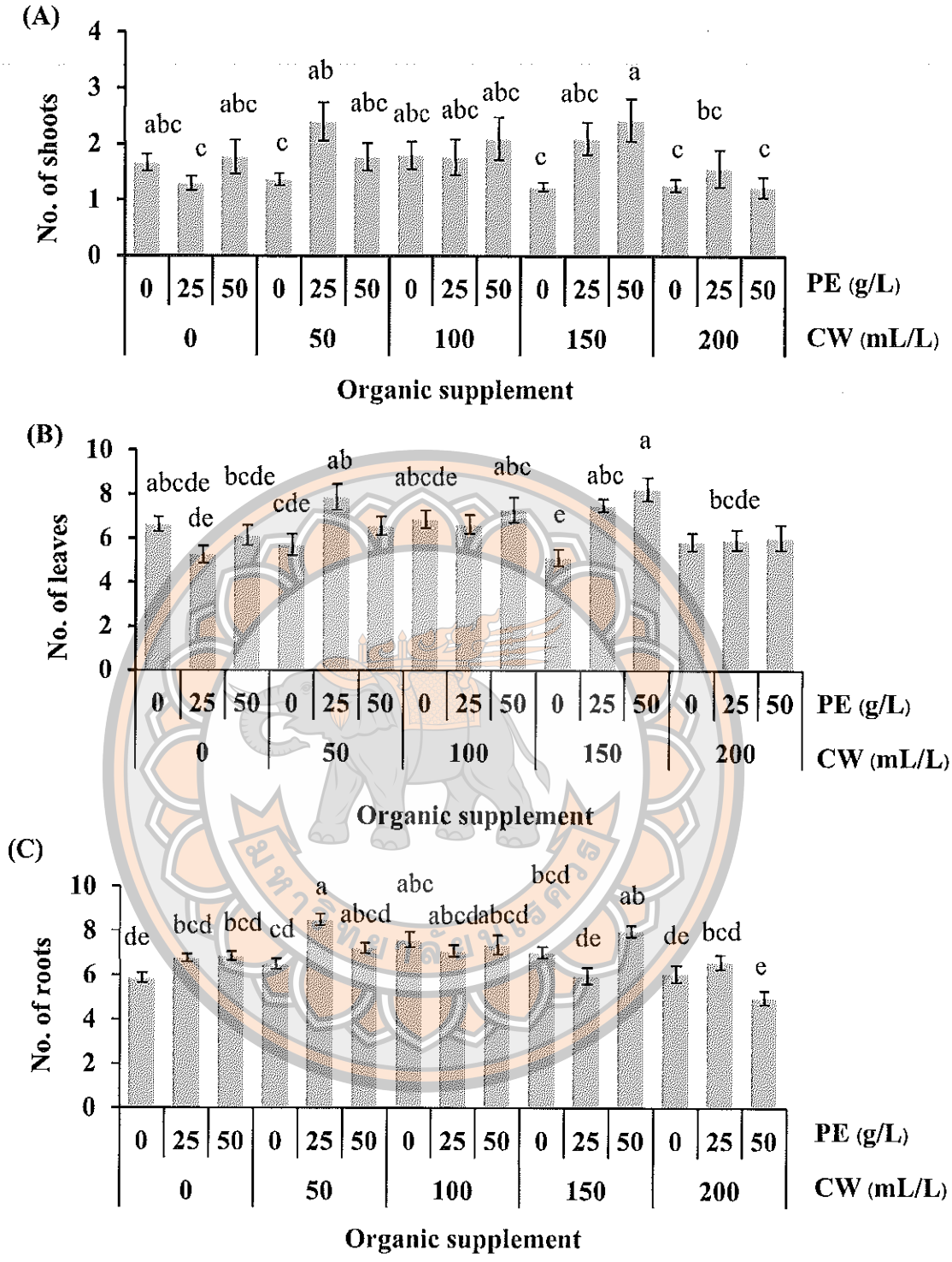


Figure 19 Effect of CW and PE supplementation on number of shootst (A), leaves (B) and root (C) per explant of *P. mishmensis* × *P. tankervilliae*. The bars and error bars represent means and SE of 3 replicates (each with 10 plants), respectively. Different letters above the bar show significant differences analyzed by DMRT at $p \leq 0.05$

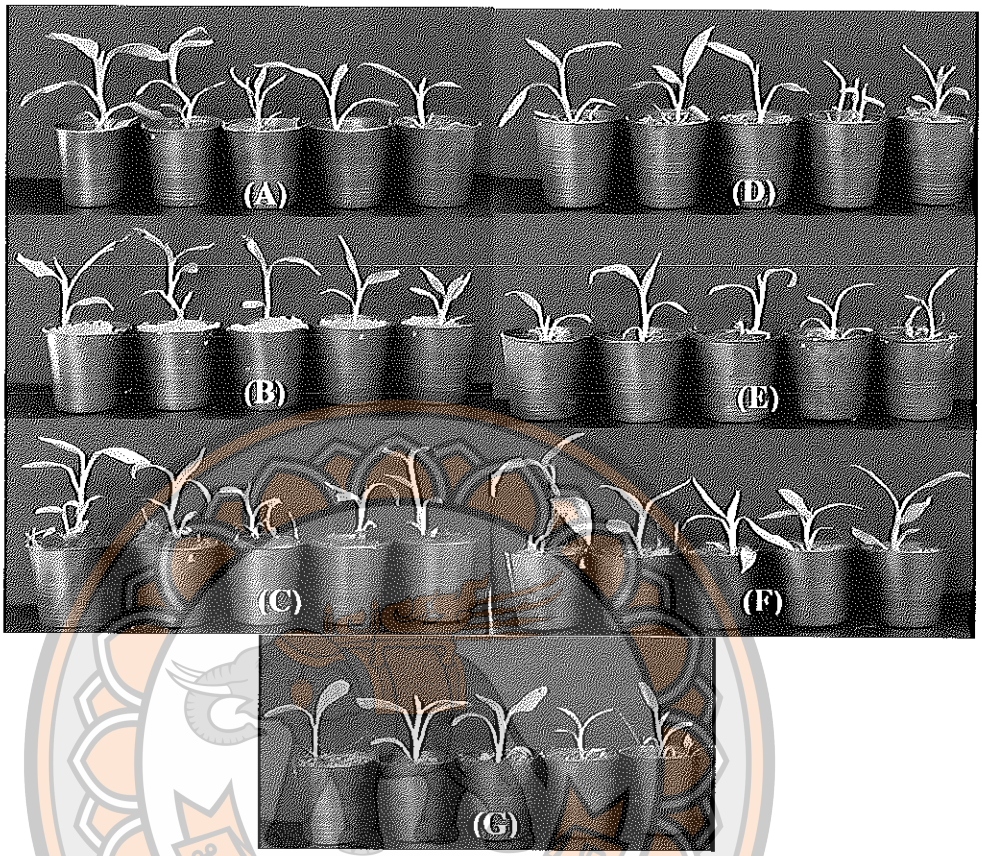


Figure 20 Growth of *P. mishmensis* with soil (A), sand (B), coconut husk chip (C), soil-sand mix, 1: 1 (D), soil-coconut husk chip mix, 1: 1 (E), sand-coconut husk chip mix, 1: 1 (F), coconut husk chip mix, 1: 1: 1 (G). The pictures were taken after 4 weeks of greenhouse acclimatization

Table 23 Effect of planting Substance on acclimatization of *P. mishmensis* in greenhouse for 4 weeks

Substance	Survival (%)	No. of shoots	Shoots Length	No. of leaves per plant	Leaf size (cm)[1]		New shoot Formation (%)
					Length	Width	
Soil	92.5	1.2±0.1 ns	3.2±0.3 ns	4.2±0.3 ns	3.1±0.2 a	0.8±0.1 ns	0.54
Sand	93.7	1.1±0.1	3.2±0.4	4.1±0.3	2.9±0.2 ab	0.9±0.1	0.32
Coconut husk chip	86.6	1.1±0.1	3.1±0.3	3.9±0.3	2.6±0.2 ab	0.7±0.1	0.32
Soil + Sand (1: 1)	91.1	1.2±0.1	2.9±0.3	3.2±0.3	2.4±0.2 ab	0.7±0.1	1.36
Soil + Coconut husk chip (1: 1)	93.9	1.2±0.1	2.3±0.2	3.9±0.2	2.5±0.2 ab	0.7±0.1	0.32
Sand + Coconut husk chip (1: 1)	91.8	1.2±0.3	2.4±0.2	3.6±0.3	2.1±0.2 b	0.7±0.1	0.00
Soil + Sand + Coconut husk chip (1: 1: 1)	100	1.2±0.1	3.6±0.3	4.1±0.3	3.2±0.2 a	0.8±0.1	1.05

Values are means ± SE of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] Data were obtained from the 3rd leaf of each plant

CHAPTER V

DEVELOPMENT SEQUENCE CHARACTERIZED AMPLIFIED REGION (SCAR) MARKER FOR IDENTIFICATIONS OF CALANTHE GROUP AND CALANTHE HYBRIDS

Summary

With regard to molecular markers, SCAR technique has been developed for species identification of orchids in particular the *Calanthe* group and their hybrids. A total of 17 species from *Calanthe* group and offspring obtained from interspecific crosses (putative hybrids) were used in order to develop species-specific molecular markers using a RAPD primer. Out of 20 random primers, four primers were initially amplified to show RAPD patterns. Afterwards, the RAPD fragments specific to orchid species were chosen for further cloning and sequencing. Then, all sequences were designed as SCAR primers. Thirteen SCAR primer pairs were developed. Herein, all primers were verified using PCR amplification. It was disclosed that five primer pairs could be specific to some group of these orchids; meanwhile, the rest were not enabled to employ. It suggested that the Pmis524 SCAR markers have been successful to determine these putative *Calanthe* hybrids while Pmis364 and Ptan285 should be clarify in further study.

Introduction

A molecular marker is a powerful tool for a basis of taxonomic identification for known and unknown species. Markers are recognized into two major types including protein and DNA markers. The former marker can detect the variation at the gene product level such as changes sequence of amino acid in protein. The latter marker is used to determine the variation at the DNA level such as nucleotide changes (deletion, duplication, inversion and insertion). Moreover, the DNA marker types can be divided into two different characteristics e.g. dominant (RAPD and AFLP) and co-dominant markers (RFLP and SSR). The molecular markers have been widely applied in several organisms including microorganisms, plants and animals. For instance, the

molecular marker was used for identifying the fungal resistance gene in eggplant (Mutlu, et al., 2008). DNA molecular markers were allowed for identification of hybrid plants in several studies e.g. chrysanthemum (Huang, et al., 2000), orange (mandarin) (Ahmad, et al., 2012), rice (Ye-Yun et al., 2005), mung bean (Khajudparn et al., 2012), rose (Nadeem, et al., 2016). Moreover, the hybrid confirmation among medicinal plants have also been addressed (Devaiah, & Venkatasubramanian, 2008).

At present, the new biotechnology concerning molecular markers for discriminating economic plants especially orchids (intergeneric cross pollination between *Aerides vandarum* and *Vanda stangeana*) was adopted (Kishor, & Devi, 2009). Former researches regarding molecular mark including RAPD have been extensively used for genetic variation, for example the use of RAPD maker for genetic relationship analysis between *Calanthe* species and some natural mutants *Calanthe* species (Paran, & Michelmore, 1993). However, RAPD marker is slightly limited due to low *reproducibility* (Kumar et al., 2009). The SCARs marker, therefore, was developed from the genomic DNA fragment at a single genetically defined locus so far. SCAR makers showed advantages over RAPD markers because its PCR amplification was less sensitive to reaction condition (Paran, & Michelmore, 1993). In addition, the PCR amplicons produced from SCAR were reproducible and can be readily scored (Weng et al., 1998). Hence, analysis using SCAR marker is straightforward, rapid and easy to conduct. This marker has been used for authentication of medicinal herb species like *Commiphora* species (Sairkar, et al.,2016)., *Artemisia* herbs (Lee, K., & Oh, 2006), *Pueraria tuberosa* (Devaiah, & Venkatasubramanian, 2008), *Nicotiana tabacum* (Julio, et al., 2006),. The SCAR markers were also used to identify in tree Sengon, (Yuskianti, & Shiraishi, 2010), *Brassica napus* (Barret et al., 1998) mildew resistance gene in grapevine (Akkurt et al., 2007) *Atractylodes* spp., *Echinacea* spp., *Phyllanthus* spp., *Jatropha curcas* and *Curcuma alismatifolia*, and other economic plants such as timber plants like bamboo, *Panax ginseng*, and *Piper longum* (Devaiah, & Venkatasubramanian, 2008). For developing hybrid orchids, the SCAR marker was also introduced to identify the species especially in the lady slippers, *Paphiopedilum* species and their hybrids (Sun et al., 2011). In this current study, the development of species-specific SCAR markers for discrimination of the *Calanthe* group was carried out. These obtained results could

be useful for classifying the *Calanthe* hybrid group. Suggesting that these markers could provide for further other inauthentic plants.

Materials and Methods

Plant materials

The samples of *Calanthe* group were collected from different localities throughout Thailand. Herewith, Ban Romklao Botanical Garden (Phitsanulok province) and Queen Sirikit Botanical Garden (Chiang Mai province) were the main stations for these orchid collection. All samples were morphologically identified into species level using the main published literature of Kurzweil et al. (2010). The specimens were also compared with those type specimens deposited in the Herbarium Queen Sirikit Botanical Garden (QSBG), Chiang Mai province and confirmed by experts.

The *Calanthe* samples came from three genera, namely *Calanthe* (9 species), *Phaius* (6 species) and *Cephalantheropsis* (2 species). (Table 24)

The samples for primer screening via RAPD PCR composed of 10 species comprising 4 members in genus *Calanthe* ; *C. cardioglossa*, *C. lyroglossa*, *C. masuca,* and *C. triplicata*, 3 species 1 variety in genus *Phaius*; *P. tankervilliae*, *P. tankervilliae* var. *alba*, *Phaius flava* and *P. mishmensis* and 2 species of *Cephalantheropsis*; *Ce. obcordata* and *Ce. longipes* as well.

DNA extraction

The genomic DNA was isolated from young fresh leaves of all samples *in Calanthe* group and putative hybrids (Table 24), following as extraction CTAB method of Doyle and Doyle (1990) with some modification. After that, the DNA quality was determined using Multimode Microplate Readers machine (Synergy H1 Biotex, Vermont, and U.S.A), in additions, the size of extracted DNA was verified using 0.8% agarose gel electrophoresis. Finally, each DNA sample was adjusted to a concentration of 100 ng/μL prior to RAPD amplification.

RAPD Amplification with random primers

The PCR was carried out according to the method of Williams et al. (1993) and twenty ten mer random primers were used in the RAPD amplification. Twenty

RAPD primers were chosen in accordance with Punjansing et al. (2017). Consequently, the amplification was performed using a total volume of 25 µL containing of 100 ng DNA, 1X PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTP, 0.4 mM RAPD primer and 1 unit *Taq* DNA polymerase (RBC Bioscience, Taiwan), then the reaction was adjusted to 25 µL final volume with ddH_2O. The PCR protocol for DNA amplification started with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation 94°C for 1 min, annealing at 40 °C for 2 min and extension at 72 °C for 2 min, and last step with further extension at 72 °C for 5 min. Then the amplified products were examined using 1.5% agarose gel electrophoresis in the 1X TAE buffer for 30 min at 100 volts and compared fragment size with the 100 bp DNA ladder (Gene Direx, Taiwan) and stained with ethidium bromide. The polymorphic bands were selected to excise from the agarose gel and purified using the PureDirexPCR Clean-up & Gel Extraction Kit (GeneDirex, Taiwan). Before further method, the purified fragments were check quality again in 1% agarose gel electrophoresis.

DNA Cloning

Purified DNA fragments were ligated at temperature of 16°C for 12 hours so that the vector plasmid pTZ57R/T cloning vector (Figure 21) (Thermo Scientific, USA.) The the ligation was transformed into the $CaCl_2$ competent cell, *Escherichia coli* DH5α using the heat shock method. The recombinant clones were selected on LB agar plates containing 100 µg/mL of ampicillin, 20 mg/ml of X-gal solution and 100 µg/mL of IPTG using the blue-white screening method. The white colonies were screened about three white colonies per plate were chosen and verified using T7/SP6 primer pairs near to the ligation ends. The positive plasmids were extracted using PureDirex Plasmid miniPREP Kit (Bio-Helix,) and sequenced by Macrogen Company (*Seoul,* South Korea) with Universal Primers M13F and M13R.

SCAR primer design

BLAST program (ncbi.nlm.nih.gov/BLAST) was employed for checking the obtained sequences with reference sequences in database. The representative sequence of each species was designed as specific SCAR primers using website IDTDNA (https://sg.idtdna.com/site) and rechecked those designed primers using Primer 3 program (http://bioinfo.ut.ee/primer3-0.4/primer3/). Those required SCAR primer pairs were synthesized Macrogen Company (*Seoul,* South Korea).

Validation of SCAR primer

Afterwards, the specificity of SCAR primers was conducted. Ten species of *Calanthe* were tested for three pair of Cma markers and 8 species of *Phaius* (6 species) and *Cephalantheropsis* (2 species) were carried out for five pair of each Pmis and Ptan markers. In terms of PCR condition, the annealing temperature was ranged from 50-60 °C depending on each primer pair.

Results

DNA Extraction

The DNA was extracted from the orchid leaves in the *Calanthe* group namely *Calanthe* (10 species), *Phaius* (6 species) and *Cephalantheropsis* (2species), as well as putative *P. mishmensis* x *P. tankervilliae* and reciprocal cross- pollination, using CTAB method (Doyle, & Doyle, 1987) with slightly modifications. It was revealed that most of the DNA solution was clear and colorless whereas some samples showed brown color due to phenol containing. When the DNA solution was examined using a method of electrophoresis on a 0.8 % agarose gel, the obtained DNA band was more than 10 Kb, when compared to the standard DNA band (Gene Ruler 1 kb DNA. Ladder, Thermo Scientific) and it was noticed the smears in some samples were obviously occurred, suggesting that there was a fracture of the genomic DNA. Nonetheless, the acquired concentration was diluted to 100 ng/µL, the PCR products could be successfully generated. Therefore, all orchid members in this *Calanthe* group were considered as being suitable samples for being applied concerning DNA extraction by using this technique.

DNA pattern from RAPD technique

For RAPD primers screening, a total of 10 orchid species in the *Calanthe* group viz. *Calanthe* (4 species) *Phaius* (4 species) and *Cephalantheropsis* (2 species) were PCR amplified using 20 selected RAPD primers. It was found that DNA fragments were successfully amplified from four primers, particularly OPX12, OPX13, OPY02 and OPY20. Sixteen out of 22 bands were produced as the highest polymorphic bands using OPY20 (Figure 22D). Next, OPX13 held 14 polymorphic bands (Figure 22B) from 17 bands and OPY02 displayed 11 polymorphic bands (Figure 22C) from 15 bands. Meanwhile, OPX-12 gave the least of polymorphic band at 10 out of 16 bands. On contrary, 16 primers could not produce in some species so they were discarded. Herein, the DNA fragments size was ranged from 200-1800 bp in all four primers. Fifty-one out of 70 were polymorphic bands (72.85%) and the average amplicons were obtained at 17.5 band/primer. (Table 26)

Cloning and sequencing of the putative species-specific markers

The 4 primer of RAPD markers was showed the specific DNA bands which may be efficient to use for the development of SCAR markers, including 2 bands (A1 and A2) from the primer OPX 12 were selected, (Figure 22A), and 3 bands (B1, B2 and B3) from primer OPX-13 (Figure 22B) and two bands (C1 and C2) from primer OPY02 (Figure 22C). Totally 7 specific bands were cut from agarose gel electrophoresis. All of them were successful cloned. The recombinant DNA from 22 fragments was sequenced but only 12 fragments were attempt (Table 26).

Nucleotide BLAST

The 5 sequences of DNA fragment from *C. masuca* via OPX 12 gave the size about 382 bp and showed identity to complete genome of *Heuchera*. Meanwhile, the DNA fragment size from *P. tankervilliae* and *P. mishmensis* using OPY 02 held about 600 and 750 bp, respectively. Based on database comparison, both sequences presented no match with others (Table 27). From failure cloning and sequencing, it might mistake due to bacterial contamination or quality of extracted plasmids.

Develop SCAR primer

After nucleotide BLAST, a total of 13 primer pairs were designed from three cloned fragment to develop the SCAR markers (Table 28). The three primer pairs consisting of Cma234, Cma262 and Cma307 of *C. masuca* were designed from 382 bp size of RAPD with OPX12 and the expected sizes of each were 234, 262 and 307 bp, respectively (Figure 23). The five primer pairs consisting of Pmis 364, Pmis 565, Pmis 466, Pmis 524 and Pmis 525 of *P. mishmensis* were designed from 750 bp size of RAPD with OPY02 and the expected sizes of each were 364, 565, 466, 524 and 525 bp, respectively (Figure 24). The five primer pairs consisting of Ptan362, Ptan285, Ptan285/1, Ptan346 and Ptan393 of *P.tankervilliae* were designed from 750 bp size of RAPD with OPY02 and the expected sizes of each were 362, 285, 285, 346 and 393 bp. (Figure 25).

Validation of SCAR primer design

The Cmar SCAR primers were verified only in genus *Calanthe* at optimal annealing ranging from 56-65 °C. The Cmar234 SCAR primer pair could amplify in all samples but show monomorphic band at 234 bp (Figure 26A). It meant that this SCAR marker showed failure. The other two SCAR primers showed the similar results which there were two amplicons from each primer. The Cmar261 SCAR marker was specific to *C. masuca*, *C. triplicata* and *C. herbacea* in size of 261 bp while other species presented in about 500 bp in size (Figure 26B). Furthermore, the Cmar307 SCAR marker could detect the correct amplicons size in *C. masuca*, *C. triplicata* and *C. herbacea* whereas others showed approximately 600 bp in size (Figure 26C). The overall result presented that SCAR marker for only *C. masuca* was not successful; however, Cmar261 and Cmar307 markers could separate 3 species in the same evolutionary clade from other species in the same genus.

Five SCAR primers developed from *P. mishmensis*, including Pmis525 (525 bp), Pmis364 (364 bp), Pmis565 (565 bp), Pmis466 (466 bp) and Pmis524 (524 bp) were validated with the six *Phaius* species including *P. mishmensis*, *P. tankervilliae*, *P. tankervilliae* var. *alba*, *P. flava*, *P. indochineis* and *P. takeoi* and two species of genus *Cephalantheropsis* i.e. *Ce. obcordata* and *Ce. longipes*. The results showed that Pmis525 and Pmis364 SCAR primers were unsuccessful to amplify and showed unclear band in many species so both were discarded. The Pmis466 generated the 466 bp amplicons in 2 species, namely *P. mishmensis* and *P. indochinensis*; however, all species could be amplified the fragment size about 400 bp (Figure 27A). The Pmis524 for 524 bp size showed in *P. mishmensis* and *P. indochinensis* while the fragment of others was about 420 bp (Figure 27B). The last Pmis364 SCAR primer could detect monomorphic bands in most of *Phaius* and *Cephalantheropsis* species while *P. tankervilliae* and *P. tankervilliae* var. *alba* were not amplified (Figure 27C). From all results, *P. flava* could not show any bands because it was too low DNA quality to amplify. To sum up, three SCAR markers may be good markers for *P. mishmensis* and *P. indochinensis* identification.

Five SCAR primers were developed from *P. tankervilliae* size 600 bp, namely Ptan362 (362 bp), Ptan285 (285 bp), Ptan346 (346 bp), Ptan285/1 (285 bp) and Ptan393 (393 bp) were validated with six *Phaius* species (*P. mishmensis*, *P.*

tankervilliae, *P. tankervilliae* var. *alba*, *P. flava*, *P. indochineis* and *P. takeoi*), and two *Cephalantheropsis* species (*Ce. obcordata and Ce. longipes*). The four SCAR primer, namely Ptan362, Ptan285, Ptan346 and Ptan393 were unsuccessful to amplify and present unclear bands. The Pmis285/1 could display specific 285 bp band only *P. tankervilliae*, although *P. tankervilliae* var. *alba* could produce also but it is the same species (Figure 28).

From our experiment, we were successfully to develop 6 SCAR primers pairs for orchids in *Calanthe* group. The only 3 SCAR primers were chosen to identify hybrids in interspecific cross pollination between *P. mishmensis* (♀) × *P. tankervilliae* (♂) and their reciprocal for confirmation of SCAR primer with parental plants and putative hybrids.

Confirmation of SCAR primers with hybrids

Determination and confirmation of putative hybrids were conducted using three SCAR primers. The 21 putative hybrids from *P. mishmensis* (♀) × *P. tankervilliae* (♂) and another 21 putative hybrids from reciprocal cross- pollination of both species were randomly selected from seedling under tissue culture condition.

For Pmis524 SCAR primer targeting to 524 bp of *P. mishmensis*, the PCR amplification showed a fragment of *P. mishmensis* at size 524 bp and *P. tankervilliae* at 400 bp. It pointed that maternal and paternal species showed different fragment size; hence, 21 putative hybrids should present both fragment size. From the result, all hybrids gave both size of fragments not only *P. mishmensis* (♀) × *P. tankervilliae* (♂) but also *P. tankervilliae* (♀) × *P. mishmensis* (♂) (Figure 29).

For Pmis364 SCAR primer focusing on 364 bp of *P. mishmensis*, the PCR products showed a single band specifically in *P. mishmensis* at 364 bp size while there was no band in *P. tankervilliae*. The 42 putative hybrids between *P. tankervilliae* × *P. mishmensis* could produce as a single band according to expected size in both crosses not only *P. mishmensis* as mother or as father (Figure 30).

For Ptan285 SCAR primer for 285 bp of *P. tankervilliae*, the PCR products showed a single band specifically in *P. tankervilliae* at 285 bp size while there was no band in *P. mishmensis*. The 42 putative hybrids between *P. tankervilliae* × *P. mishmensis* could produce as a single band according to expected size in both crosses not only *P. tankervilliae* as mother or as father (Figure 31).

In overall, the Pmis524 was the good SCAR primer because the single primer use could detect hybrids completely. Meanwhile, Pmis364 and Ptan285 should be used together to amplify for hybrid confirmation; therefore, the duplex or multiplex PCR should be recommended in further study.

Discussion

This present study focused on development of molecular markers in order to identify *Calanthe* hybrids. Regarding DNA extraction of *Calanthe* group, DNA extraction from this study was applied according to CTAB method of Doyle & Doyle (1990) with slightly modification. It was showed that leaves of these species are suitable to extract using this method because of thinness and low phenolic containing even though some DNA samples was brown due to cell disruption and cytoplasmic compounds with nuclei and other organelles contraction (Loomis, 1974). In addition, if there are more phenol contamination, polyphenols covalently will bind to DNA giving a brown colour and making useless for research applications. (Katterman & Shattuch, 1983).

DNA pattern using RAPD primers was amplified and modified from the protocol of Punjansing et al. (2017) and twenty primer pairs were selected for this study though these primers were only relied on previous detection of 3 orchid species via RAPD technique. It was found that 20 percent of selected RAPD primer could amplify with 10 selected species and gave total 70 bands. The 72.9 percent of polymorphic bands were presented from 4 RAPD primers whereas 99.45 percent of polymorphic bands were showed from 18 SRAP primers (Nutthapornnitchakul et al., 2019). This is mentioned that the more primers use, the more amplicons are increased.

For cloning and sequencing of the putative species-specific markers, seven bands were cut and cloned to plasmid which 100% were successful. In initial experiment, the cloning gave small amount of white colony because the vector-to-PCR ratio calculation should be needed (Peyachoknagul, 2009). In addition, the transformation process to insert DNA strands into the cells of bacteria using heat shock method made the low efficiency because of DNA size limitation so the electroporation should be suggested (Peyachoknagul, 2009).

The DNA sequences from polymorphic bands were compared with databases in Genbank. Most of them are not compatible with previous sequences in databases. This affects from RAPD marker generated random fragments of whole genome. It is not specific to gene or anything else.

The most of SCAR primers showed the relationship within *Calanthe* group which consistency with the morphological and evolutionary studies of Zhai et al. (2014) and Nutthapornnitchakul et al. (2019). According to the study of flora of Thailand based on the morphology suggested that these orchids should be divided into three main groups (Santisuk, et al., 2011), namely *Calanthe*, *Phaius* and *Cephalantheropsis*. For Zhai et al. (2014) study, the phylogenetic tree based on morphological and molecular data purposed that this Calanthe group composes of 9 clades. Next, Nutthapornnitchakul et al. (2019) generated dendrogram from SRAP marker and showed 5 clades. The two SCAR markers for *C. masuca* could not separate *C. triplicata*, *C. masuca* and *C. herbacea* because three species are closely related in evolutionary trait so it is hard to identify each (Zhai et al., 2014; Nutthapornnitchakul et al., 2019). Alike, the two SCAR markers for *P. mishmensis* could not separate it and *P. indochinensis* due to placing in the same clade (Nutthapornnitchakul et al., 2019). In previous study, *P. indochinensis* was classified as *P. mishmensis* with the highest similarity of inflorescence on the lateral side of the cane-like stem (Pedersen et al., 2014).

The SCAR marker is the molecular marker to show advantage for species and hybrid identification such as in orchids (Sun et al., 2011), bamboo (Devaiah, & Venkatasubramanian, 2008) The application of SCAR marker to confirm hybrid was developed in orchids of Genus *Paphiopedilum* in success from 3 species based on ITS region (Sun et al., 2011) including multiplex PCR to test hybrids as well. Therefore, our studies are more beneficial if the multiplex PCR will be approved.

Conclusion

In brief, the development of the molecular markers for the identification of 17 species orchids in *Calanthe* group and 2 pairs of hybrids was carried out using random RAPD primer. The pattern from RAPD showed polymorphism. Afterwards, thirteen

pairs of SCAR primers could be designed and gave specific bands of PCR products. The five SCAR primers (Cmas261, Cmas307, Pmis466, Pmis524 and Pmis364) were specific to closely evolutionary clade whereas only one SCAR primer (Ptan285) was specific to species identification to *P. tankervilleae* in both varieties. Moreover, our SCAR primer (Pmis524) can be used as tester for hybrid confirmation.

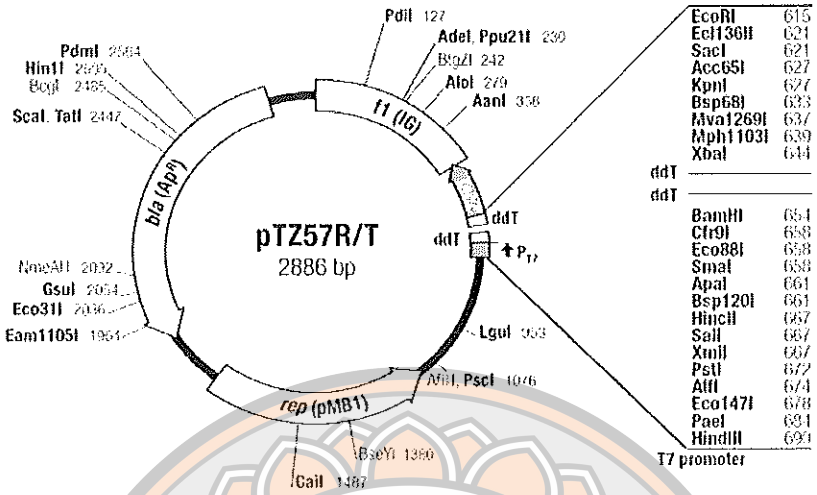


Figure 21 Map of the pTZ57R/T cloning vector (Thermo Scientific, USA.)

Table 24 List of plants in *Calanthe* group used in this study

Genus	Species	Abbreviation
Calanthe	1. *Calanthe masuca* (D. Don) Lindl.	CMA
	2. *C. triplicata* (Willemet) Ames	CTR
	3. *C. clavata* Lindl.	CCL
	4. *C. rubens* Ridl.	CRU
	5. *C. cardioglossa* Schltr.	CCA
	6. *C. vestita* Wall. Ex Lindl.	CVE
	7. *C. herbacea* Lindl.	CHE
	8. *C. densiflora* Lindl.	CDE
	9. *C. rosea* (Lindl.) Benth.	CRO
Phaius	1. *Phaius tankervalliae* (Banks) Blume	PTA
	2. *P. tankervalliae* (Banks) Blume var. *alba*	PTAA
	3. *P. mishmensis* (Lindl. & Paxton) Rchb.f.	PMI
	4. *P. flava* (Blume) Lindl.	PFL
	5. *P. indochainesis* Seidenf. & Ormerod	PIN
	6. *P. takeoi* (Hayata) H. J. Su	PTAK
Cephalantheropsis	1. *Cephalantheropsis obcordata* (Lindl.) Ormerod	CEOB
	2. *Ce. longipes* Hook.f. Ormerod	CELO
Putative hybrids	*P. mishmensis* x *Phaius tankervalliae*	PMI x PTA
	Phaius tankervalliae x *P. mishmensis*	PTA x PMI

Note * only for checking SCAR marker

Table 25 List of 10-mer oligonucleotide primers for RAPD analysis

Primer name	Nucleotide sequences (5' to 3')	Primer name	Nucleotide sequences (5' to 3')
OPP-01	GTAGCACTCC	OPY-02	CATCGCCGCA
OPP-02	TCGGCACGCA	OPY-04	GGCTGCAATG
OPP-07	GTCCATGCCA	OPY-14	GGTCGATCTG
OPX-06	ACGCCAGAGG	OPY-15	AGTCGCCCTT
OPX-11	GGAGCCTCAG	OPY-16	GGGCCAATGT
OPX-12	TCGCCAGCCA	OPY-18	GTGGAGTCAG
OPX-13	ACGGGAGCAA	OPY-20	AGCCGTGGAA
OPX-17	GACACGGACC	OPF-04	GGTGATCAGG
OPX-19	GTGGCATCTC	OPF-07	CCGATATCCC
OPY-01	GTGGCAGCTC	OPF-08	GGGATATCGG

Remark: RAPD primers were synthesized from Macrogen Company in South Korea.

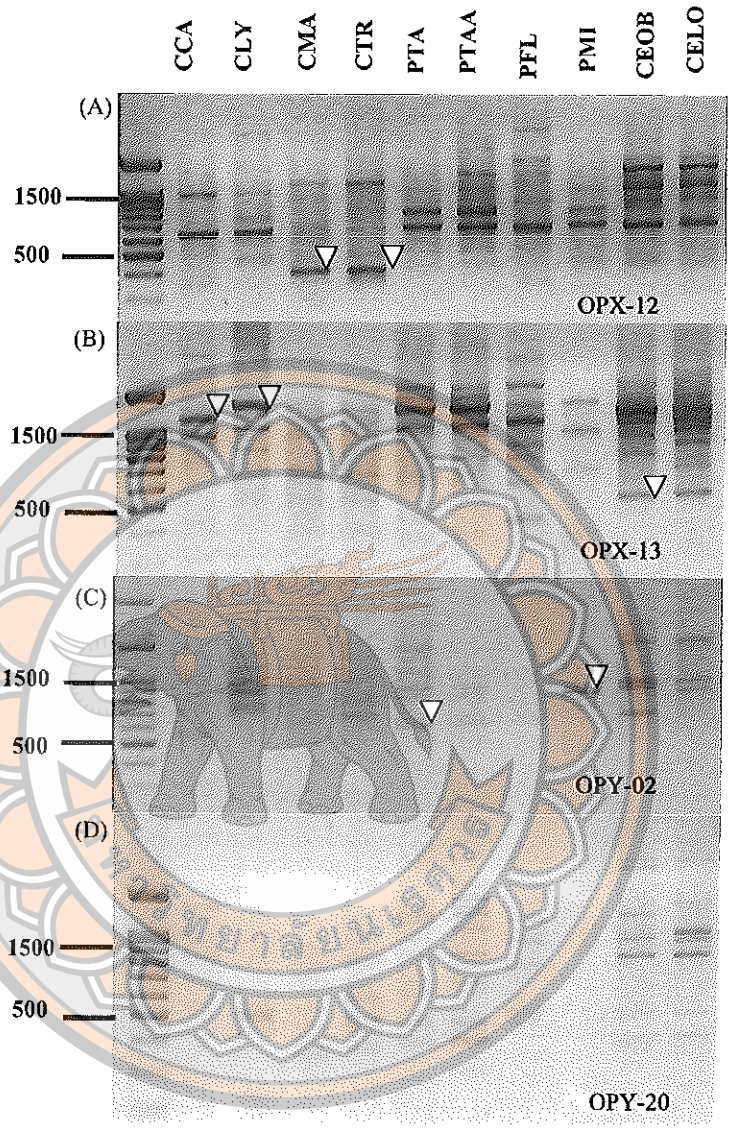


Figure 22 PCR amplification with OPX-12 (A), OPX- 13 (B), OPY-02 (C), primers OPY- 20 (D). Arrows represent cut specific bands. M: 100 bp DNA ladder (Gene Direx, Taiwan)

Table 26 RAPD amplification and their successful in cloning

Primer RAPD	No. of total band	No. of polymorphic band	Code DNA fragment	No. of band cutting	No. of band Cloning attempt	Size of clone ~ (bp)	Species	No. of colony to Sequencing attempt	No. of Sequencing Successful attempt
OPX-12(A)	16	10	A1	1	1	300	*C. masuca*	6	5
			A2	1	1	300	*C. triplicata*	3	0
OPX-13(B)	17	14	B1	1	1	1,200	*C. cardioglossa*	1	0
			B2	1	1	1,400	*C. lyoglosa*	1	0
			B3	1	1	400	*Ce. obcordata*	1	0
OPY-02(C)	15	11	C1	1	1	600	*P. tankevalliae*	6	4
			C2	1	1	750	*P. mishmensis*	4	3
OPY-20(D)	22	16	0	0	-	-	-	-	-
Total	70	51		7	7	-	-	22	12

Table 27 Blast results of bioinformatics analyses of sequences acquired from RAPD

RAPD marker	*Species*	Size (bp)	Code	BLASTN	Percent Identity (%)
OPX-12(A)	*C.masuca*	382	A1/1	*Heuchera parviflora* var. *saurensis* voucher Folk 97 (OS), complete genome	92.00
	C.masuca	382	A1/2	*Heuchera parviflora* var. *saurensis* voucher Folk 97 (OS), complete genome	90.25
	C.masuca	382	A1/3	*Heuchera parviflora* var. *saurensis* voucher Folk 97 (OS), complete genome	93.00
	C.masuca	382	A1/4	*Heuchera parviflora* var. *saurensis* voucher Folk 97 (OS), complete genome	91.91
	C.masuca	382	A1/5	*Heuchera parviflora* var. *saurensis* voucher Folk 97 (OS), complete genome	91.91
OPY-02(C)	*P. tankervilliae*	600	C1/1	No significant similarity found.	-
	P. tankervilliae	600	C1/2	No significant similarity found.	-
	P. tankervilliae	600	C1/3	No significant similarity found.	-
	P. tankervilliae	600	C1/4	No significant similarity found.	-
OPY-02(C)	*P. mishmensis*	750	C2/1	No significant similarity found.	-
	P. mishmensis	750	C2/2	No significant similarity found.	-
	P. mishmensis	604	C2/3	No significant similarity found.	-

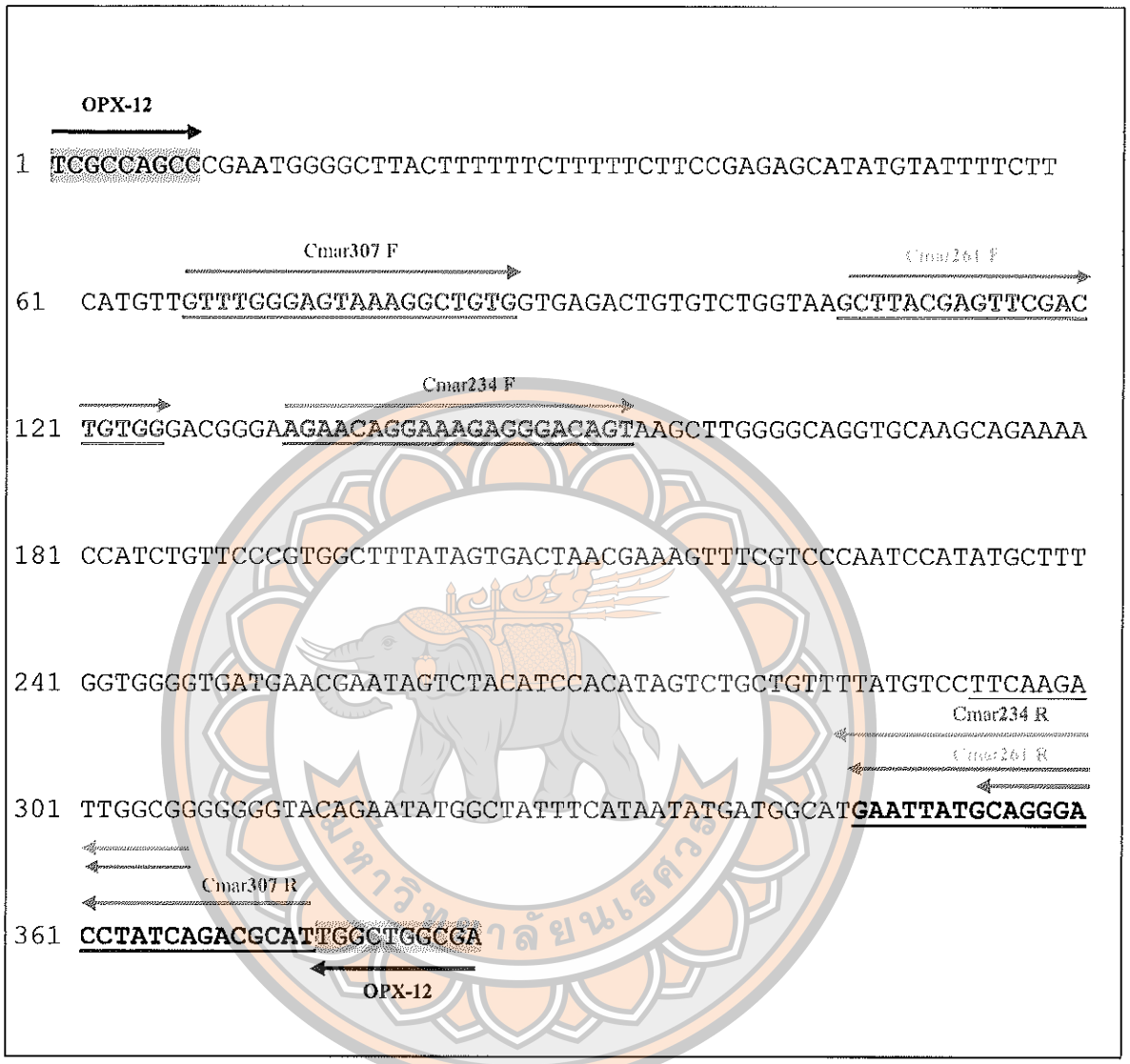


Figure 23 DNA sequence size 382 bp of *C. masuca* generated from OPX-12 RAPD primer. The gray highlight represents binding site of OPX-12 primer. The arrows mean binding site of designed SCAR primer.

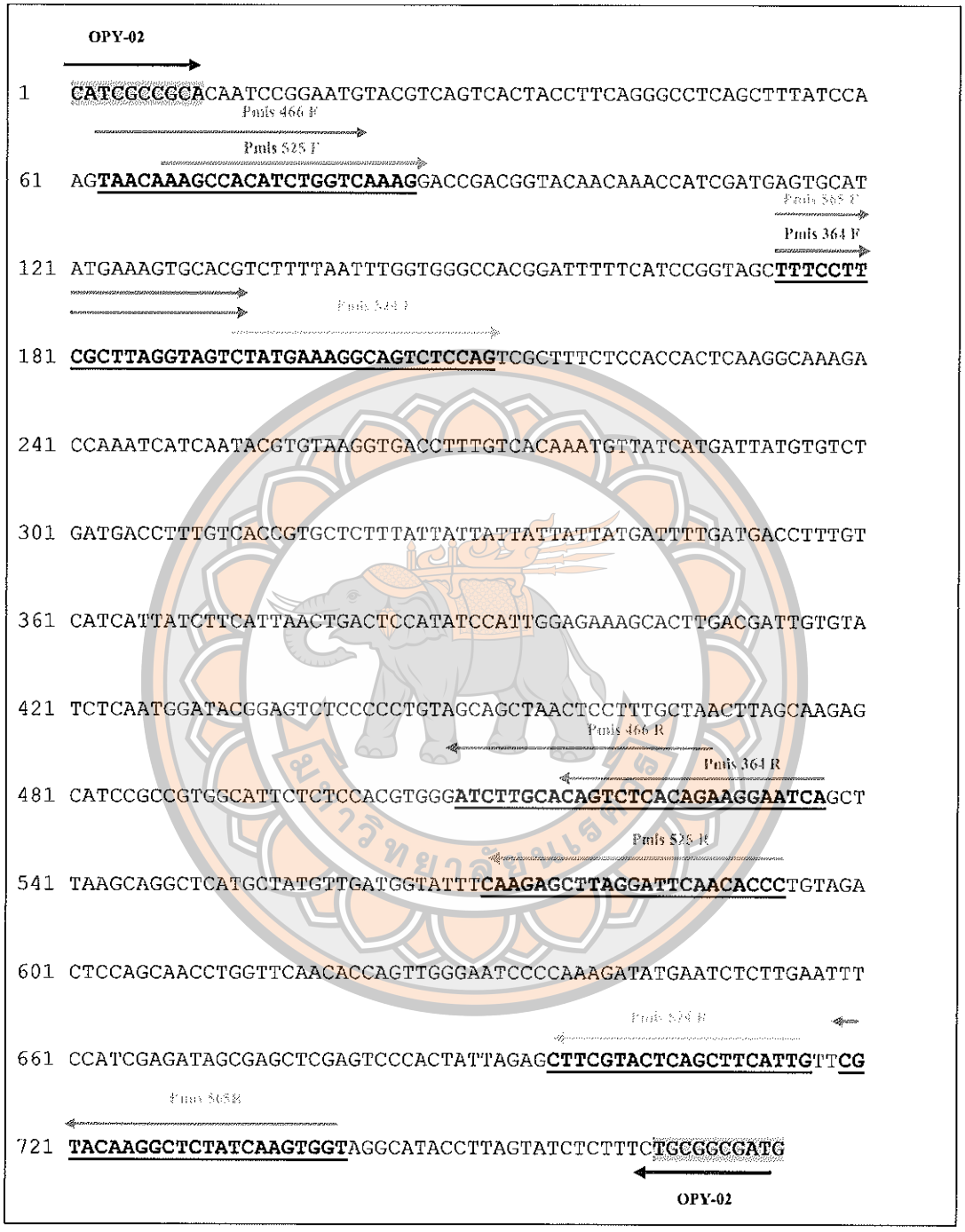


Figure 24 DNA sequence size 716 bp of *P. mishmensis* generated from OPY-02RAPD primer. The gray highlight represents binding site of OPY-02primer. The arrows mean binding site of designed SCAR primer.

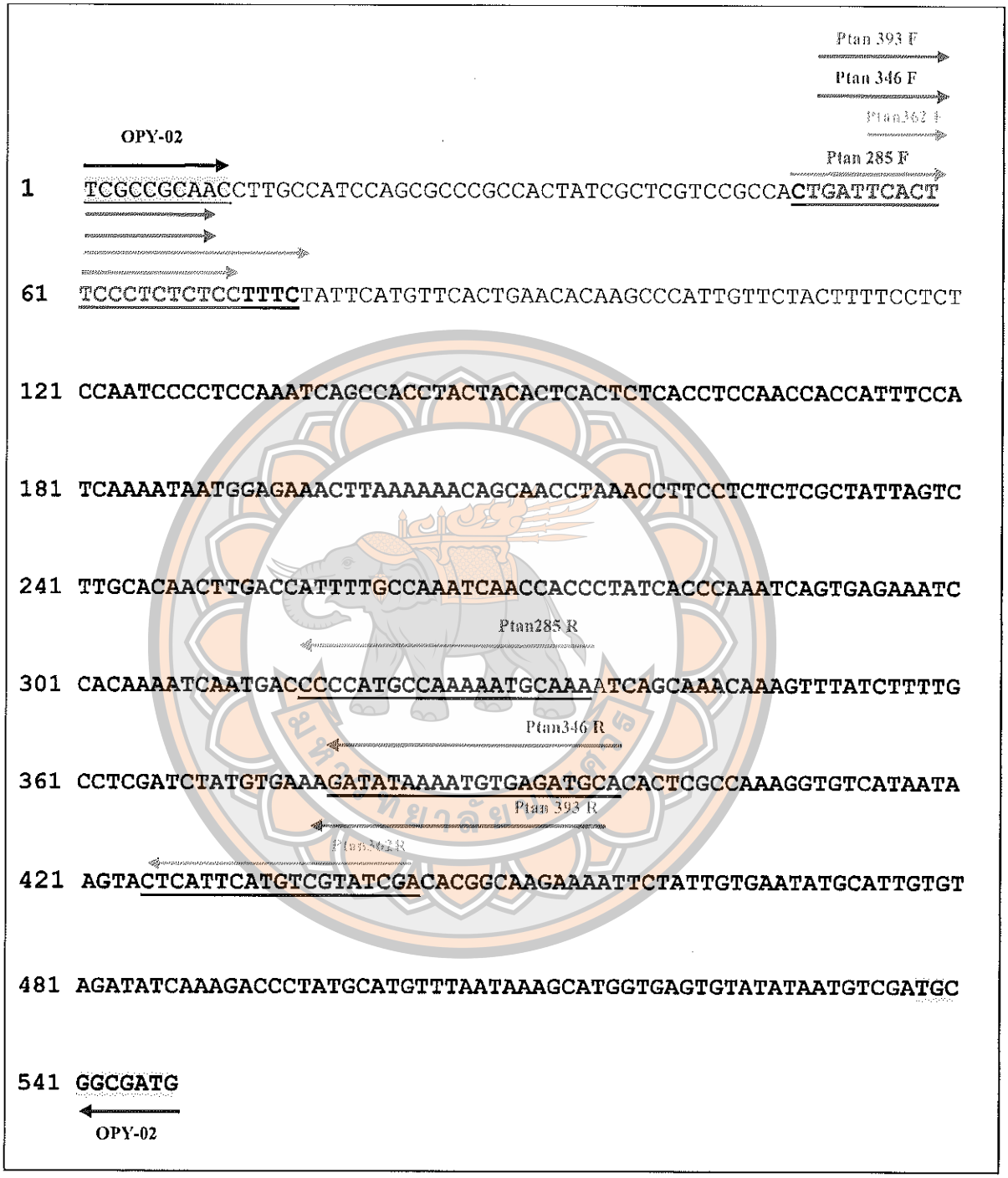


Figure 25 DNA sequence size 514 bp of *P. tankervilliae* generated from OPY-02RAPD. The gray highlight represents binding site of OPY-02primer. The arrows mean binding site of designed SCAR primer.

Table 28 Characteristics of SCAR primers in this study

Species	SCAR primer	Sequence(5'-3')	Expected size (bp)	Tm. (° C)	Specificity
C. masuca	Cmar 234F	AGAACAGGAAAGAGGGACAG	234	58.4	No
	Cmar 234R	GATAGGTCCCTGCATAATTC		58.4	
	Cmar 307F	GTTTGGGAGTAAAGGCTGTG	307	58.4	**Yes**
	Cmar 307R	TGCGTCTGATAGGTCCCTGC		62.5	
	Cmar 261F	GCTTACGAGTTCGACTGTGG	261	60.5	**Yes**
	Cmar 261R	GATAGGTCCCTGCATAATTC		56.4	
P. mishmensis	Pmis525F	AAAGCCACATCTGGTCAAAG	525	56.4	No
	Pmis 525R	TGTTGAATCCTAAGCTCTTG		54.3	
	Pmis 364F	TTTCCTTCGCTTAGGTAGTC	364	56.4	No
	Pmis 364R	TGATTCCTTCTGTGAGACTG		56.4	
	Pmis 565F	TTTCCTTCGCTTAGGTAGTC	565	56.4	**Yes**
	Pmis 565R	ACTTGATAGAGCCTTGTACG		56.4	
	Pmis 466F	TAACAAAGCCACATCTGGTC	466	56.4	**Yes**
	Pmis 466R	CTGTGAGACTGTGCAAGATC		58.4	
	Pmis 524F	CTATGAAAGGCAGTCTCCAG	524	58.4	**Yes**
	Pmis 524R	GAACAATGAAGCTGAGTACG		56.4	
P. tankervaliae	Ptan362F	TCACTTCCCTCTCTCCTTTC	362	58.4	No
	Ptan 362R	TATGACACCTTTGGCGAGTG		58.4	
	Ptan 285F	CTGATTCACTTCCCTCTCTC	285	58.4	**Yes**
	Ptan 285R	TTTGCATTTTTGGCATGGGG		56.4	
	Ptan 346F	TGATTCACTTCCCTCTCTCC	346	58.4	**No**
	Ptan 346R	TGCATCTCACATTTTATATC		50.2	
	Ptan 285/1F	CTGATTCACTTCCCTCTCTC	285	58.4	**No**
	Ptan 285/1R	TTTTGCATTTTTGGCATGGG		54.3	
	Ptan 393F	TGATTCACTTCCCTCTCTCC	393	58.4	No
	Ptan 393R	GTCGATACGACATGAATGAG		56.4	

Remark: Specificity means the successful primer to show differences.

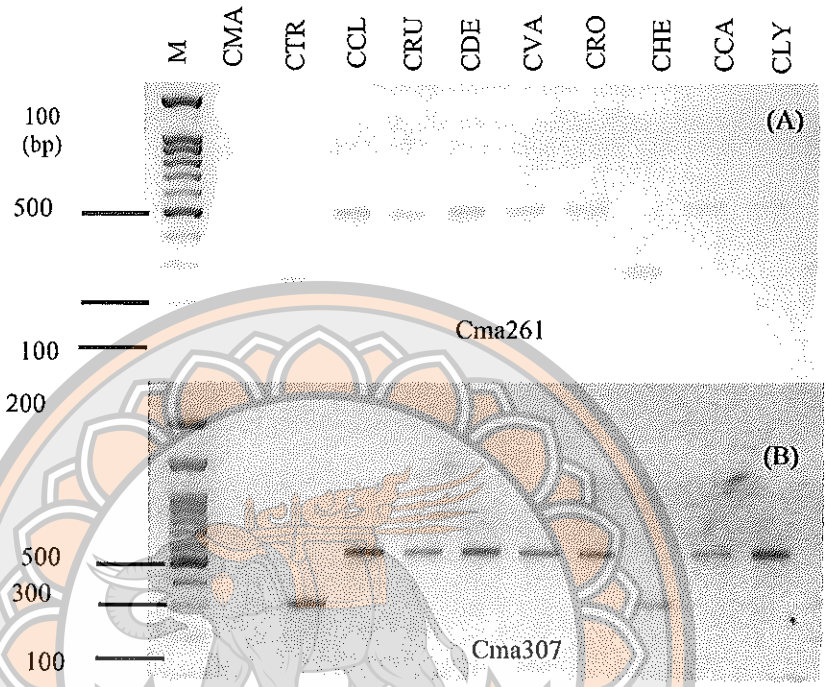


Figure 26 Agarose gel of PCR product from Cma234 (A), Cma 261 and Cma 307 (B), SCAR primer. 100 bp DNA ladder (Gene Direx, Taiwan)

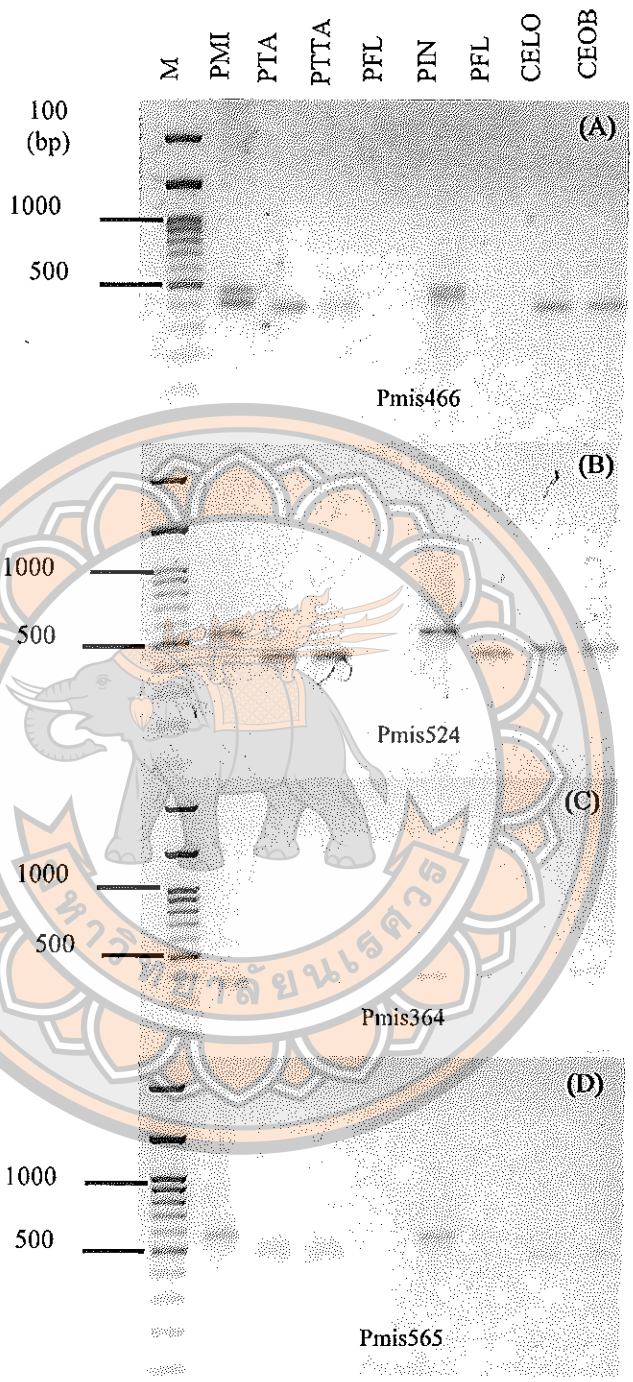


Figure 27 Agarose gel of PCR product from Pmis466 (A), Pmis524 (B), Pmis364 (C) Pmis565 (D) SCAR primer. 100 bp DNA ladder (Gene Direx, Taiwan)

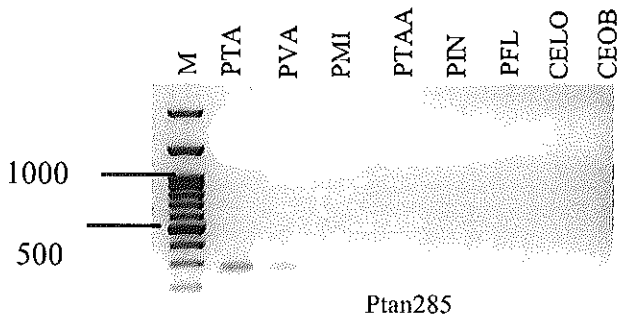


Figure 28 Agarose gel of PCR product from Ptan 285 (A), SCAR primer. 100 bp DNA ladder (Gene Direx, Taiwan)

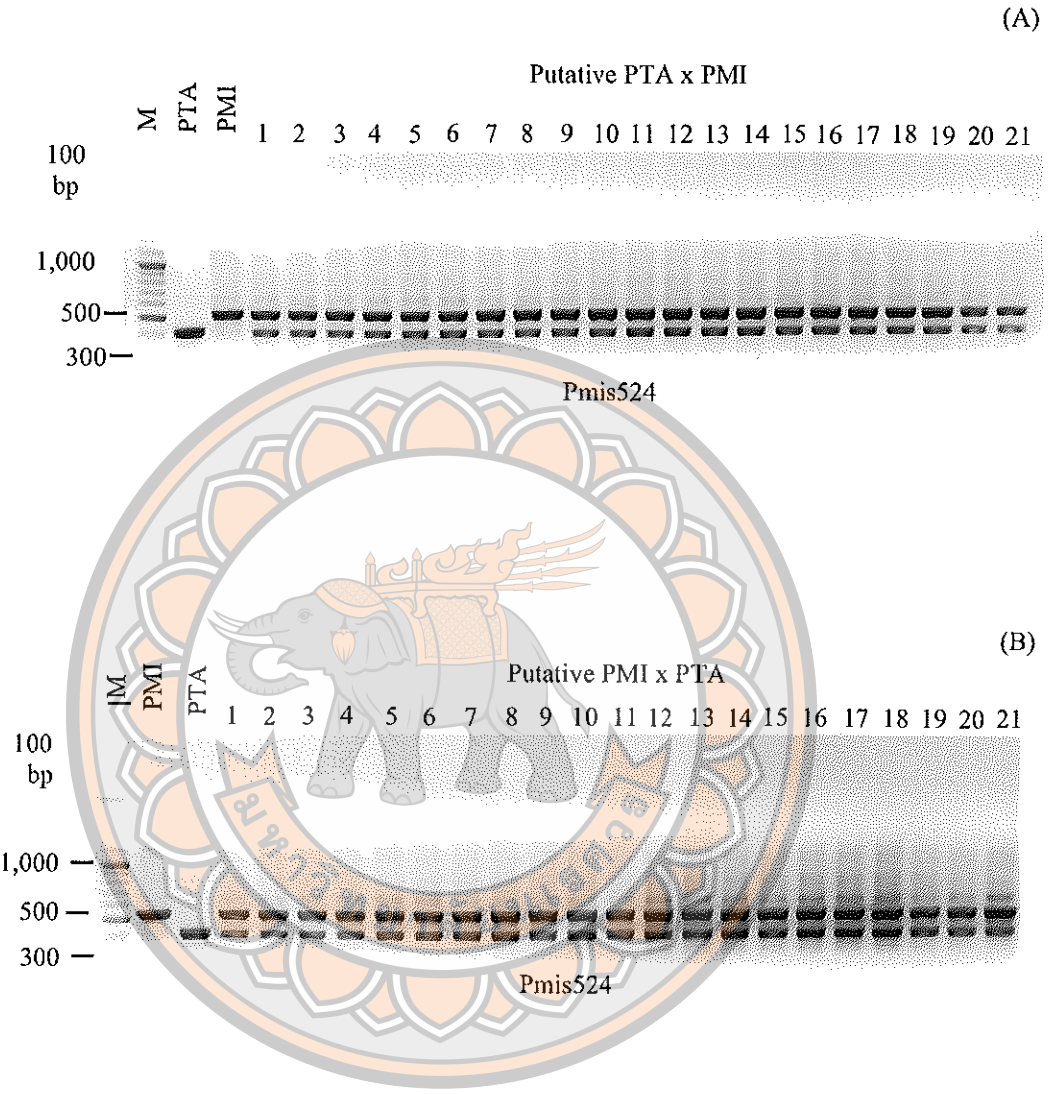


Figure 29 Confirmation of interspecific hybrids by PCR using Pmis524 SCAR primers designed for differentiation of *P. mishmensis P. tankervilliae* and their Putative hybrids of *P.tankervilliae* (♀) x *P. mishmenesis* (♂) (A) and *P. mishmenesis* (♀)x *P.tankervilliae* (♂) (B). 100 bp DNA ladder (Gene Direx, Taiwan)

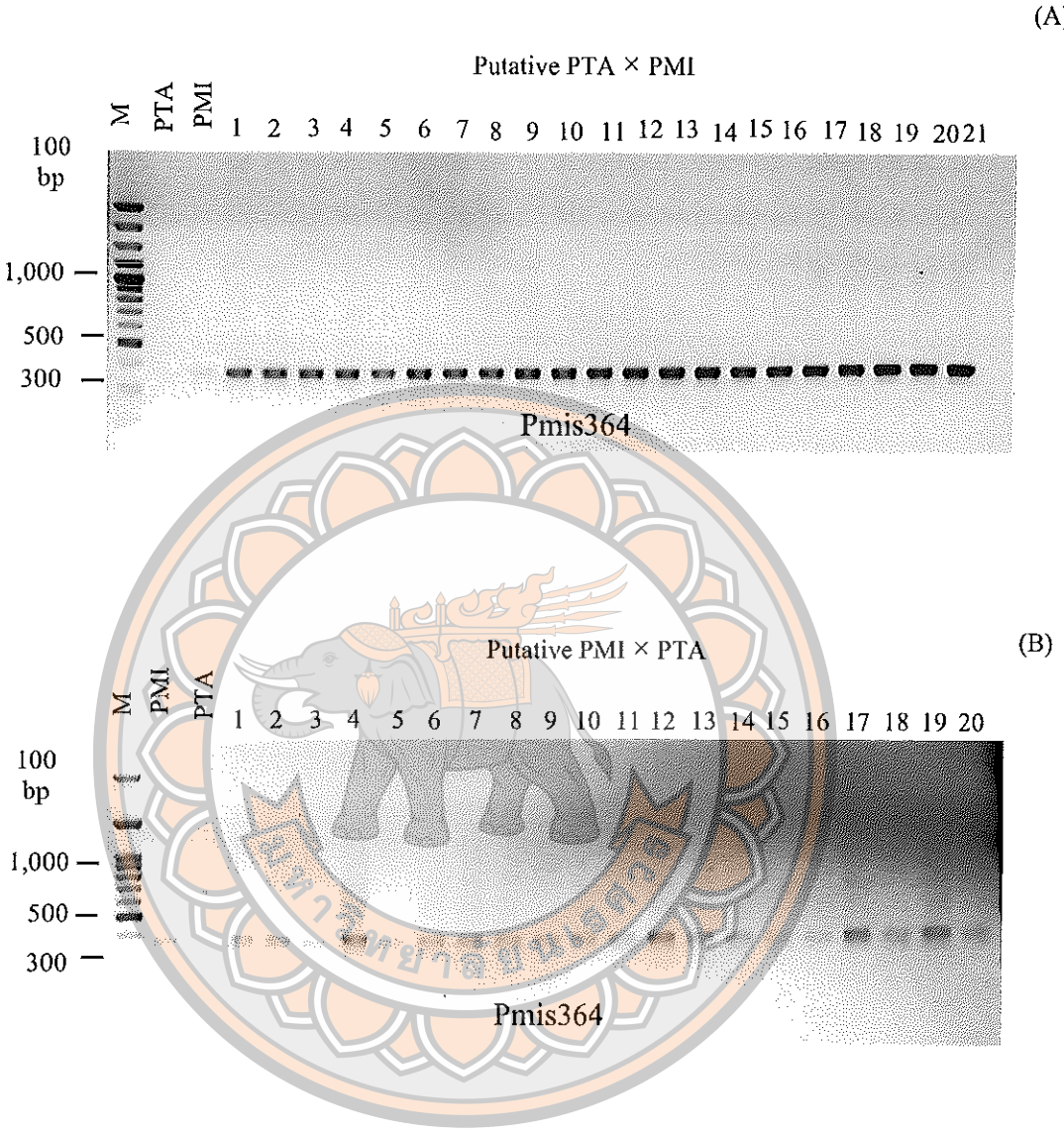


Figure 30 Confirmation of interspecific hybrids by PCR using Pmis364 SCAR primers designed for differentiation of *P. mishmensis P. tankervilliae.* and their Putative hybrids.(A) Cross of *P. tankervilliae* (♀) and *P. mishmenesis* (♂) .(B) Cross of *P. mishmenesis* (♀) and *P. tankervilliae* (♂) 100 bp DNA ladder (Gene Direx, Taiwan)

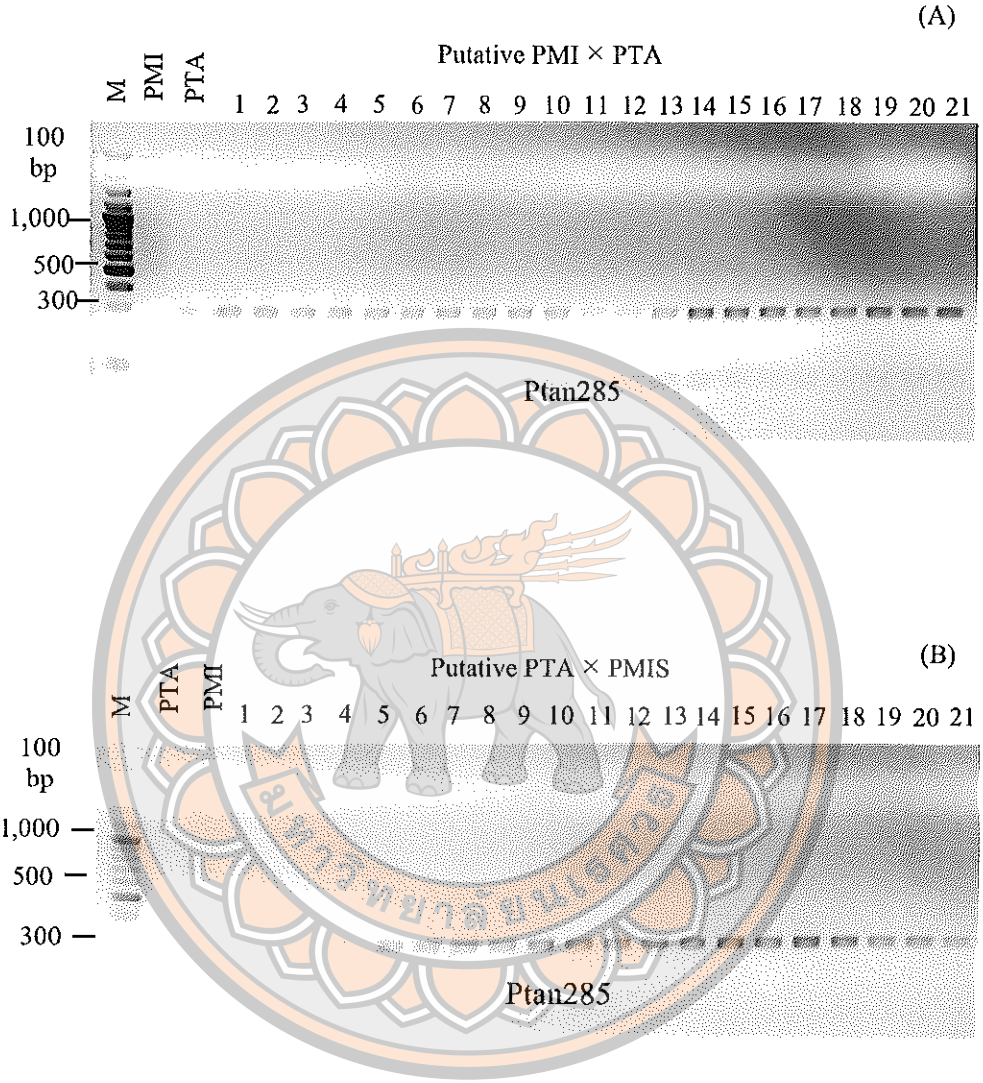


Figure 31 Confirmation of interspecific hybrids by PCR using Ptan285 SCAR primers designed for differentiation of *P. mishmensis P. tankervilliae.* and their putative hybrids. (A) Cross of *P.tankervilliae* (♀) and *P. mishmenesis* (♂). (B) Cross of *P.tankervilliae* (♀) and *P. mishmenesis* (♂). 100 bp DNA ladder (Gene Direx, Taiwan)

CHAPTER VI

DISCUSSION

Conclusion

Orchids in the *Calanthe* group are now cultivated as ornamental potted plants and successfully commercialized worldwide. Many Thai *Calanthe* orchids display remarkable features and can be an alternative choice for future prospective characteristics. Therefore, the value added of the *Calanthe* improvement through breeding program can be an alternative way for further new hybrid construction interesting. Although some Thai *Calanthe* orchids are now available for commercialization in the floricultural and ornamental markets, but almost of the commercialized potted plants are wild or true orchid species. This makes Thai *Calanthe* orchids risks of over collection from natural site and may dealt with the injury consequences. Hence, an efficient *in vitro* culture technique for conservation and rapid propagation of both *Calanthe* species and some their hybrids also need to be urgently explored and conserved for further sustainable use. Because of time consuming for conventional determination of hybrid improvement, molecular marker technique is one of efficient method for rapid hybridization determination and have been continuously developed for many plant species included orchids (Agrawal et al., 2008). In our hybrid investigation, successfully determination was performed using SCAR markers. However, this molecular markers method can be detected and determined only once for each primer pairs. Further development method using marker technique for molecular identification need to be established.

Specific morphological characteristics of orchids are very important for the initial selection step before deciding to the process of breeding program in order to receive desirable expected form of new orchid hybrids. In Thailand, some orchid species in the genus *Aerides*, *Bulbophyllum*, *Dendrobium*, *Rhynchostylis* and *Vanda* etc. (Thammasiri, 2016) were successfully improved into new hybrids. The first step for breeding program of these genera started from the morphological characteristic studying follow by selection of dominant and/or desirable characteristics before heading to the hybridization process. From the study, 7 different orchid species in the

Calanthe group were considered to be selected for hybridization experiment because of their specific and attractive morphological characteristics. The decision for morphological selection was also concerned about the future imagination of the expected appearance both in the same species and between species and genera. Thus, different pollination methods included intraspecific self-pollination as experimented for autogamous type and interspecific and intergeneric cross pollination methods as performed by geitonogamous and xenogamous in the Thai *Calanthe* group were conducted and investigated. The relative orchid hybridization studying had been previously reported and succeeded in *Bletilla striata* (Chung & Chung, 2005), *Chloraea* sp. (Human˜a et al., 2008) and *Bulbophyllum nipondhii* (Pakum et al., 2016). From the results, all of the studying orchids including *Calanthe cardioglossa*, *C. masuca*, *C. triplicata*, *C. lyoglossa*, *P. mishmensis*, *P. tankervilliae* and *P. tankervilliae* var. *alba* were successfully performed by intraspecific hybridization pollination whereas an interspecific hybridization between *C. masuca* (♀) × *C. lyroglossa* (♂), *P. mishmensis* (♀) × *P. tankervilliae* (♂), reciprocal cross and *P. mishmensis* (♀) × *P. tankervilliae* var. *alba* (♂) were successfully created. In case of the genus *Phaius*, successful hybrid production in the selected species of *Phaius* might be due to the close genetic relationship as previously studied in sequence analysis of ITS gene and reported in phylogenetic analysis of sheds new light on the relationship in *Calanthe* and alliance in China (Zhai et al. 2014). In our results, interspecific hybridization between *P. mishmensis* (♀) × *P. tankervilliae* (♂) as parental mating could produce new hybrids. The new hybrids could also develop into seedlings. This might be due to the adjacent chromosome number in *P. mishmensis* (2n = 44) (Teoh, 1980) and *P. tankervilliae* (2n = 48) (Jones, 2017). Two other intergeneric hybrids of *P. mishmensis* (♀) × *P. masuca* (♂) and *P. mishmensis* (♀) × *C. cardioglossa* (♂) were also achieved. However, no embryo formation was found on *P. mishmensis* x *C. cardioglossa* hybrids. This might be due to the close genetic relationship between *P. mishmensis* and *C. masuca* whereas other intergeneric crosses produced no embryo inside fruits. This could be assumed that no fertilization between pollinia and egg was occurred between pollination and fertilization process (Proctor, & Harder, 1994).

In vitro culture for asymbiotic germination in orchids is an efficient technique for not only improve better rapid propagation but also impact on conservation (Arditti, & Ernst, 1993 & Arditti, 2008). Successful reports on *in vitro* seed germination in orchids has been studied in many orchid species included the *Calanthe* species (Godo et al., 2010). From our experiment, seeds derived from interspecific cross pollination between *P. mishmensis* (♀) × *P. tankervilliae* (♂), and their reciprocal cross and *P. mishmensis* (♀) × *P. tankervilliae* var. *alba* (♂) were *in vitro* asymbiotic germinated. The results revealed that *in vitro* culture method could improve rapid hybrid seed germination in the *Calanthe* group. An advantage of *in vitro* culture method improved propagation ability of several plants especially in orchid species (Arditti, 2008).

Several molecular marker methods for plant identification, for instance, RAPD, AFLP, RFLP, and SRAP have been developed for specific identification purposes (Agrawal et al., 2008). But these techniques encounter some limitation about reproducibility in RAPD, high cost and complicated process in AFLP and RFLP. Hence, the development of SCAR marker for increasing reproducibility and repeatability improvement was created and successfully used for identification of several medicinal plants for example in *Artemisia* sp., (Lee et al., 2006) *Nicotiana* sp., (Julio et al., 2006) and *Curcuma* sp. (Devaiah, & Venkatasubramanian, 2008). However, molecular identification using SCAR markers in orchids has only been studied in *Paphiopedilum* sp. and their hybrids (Sun et al., 2011) etc. In our experiment, we succeeded to develop 3 SCAR primers pair for *Calanthe* hybrid identification in interspecific cross pollination between *P. mishmensis* (♀) × *P. tankervilliae* (♂) their reciprocal. This information indicated that a SCAR marker is an optimal and reliable method for molecular identification of the orchid species and hybrid in the *Calanthe* group.

CHAPTER VII

SUMMARY

Conclusion

The desirable morphological characteristics for hybrid establishment of *Calanthe* group could be selected. The establishment of interspecific-cross pollination hybrids originated from *P. mishmensis* x *P. tankervilliae*, reciprocal cross-pollination hybrids and *P. mishmensis* x *P. tankervilliae* var. *alba* was successfully created. Two other intergeneric hybrids of *P.mishmensis* x *C. masuca* and *P. mishmensis* x *C. cardioglossa* were also achieved. Dark incubation condition and adding organic supplements to the medium improved better *in vitro* asymbiotic seed germination rate and light condition triggered better seedling development of the *Calanthe* hybrids. Application of SCAR markers method for determination of the *Calanthe* orchid hybridity was successfully developed and identified parental and their putative hybrids. Three designed SCAR primers including Pmis524, Pmis364 and Ptan285 were successfully developed for identification of parental and their hybrids. This research study implies the potential of the *Calanthe* hybrid improvement and development of SCAR marker are reliable and suitable for *Calanthe* hybrid identification and can be applied as a model for other orchid hybrid establishment.

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APPENDIX

1. **Media for *in vitro* propagation**

Table 29 Components of modified Vacin and Went (Vacin, & Went, 1949) medium

Components	mg/L
Macronutrients	
$Ca_3(PO_4)2$	200
KNO_3	525
KH_2PO_4	250
$MgSO_4.7H_2O$	250
$(NH_4)2SO_4$	500
Micronutrients	
$MnSO_4.H_2O$	5.7
Iron	
$Fe_2(C_4H_4O_6)32H_2O$	28
Sucrose	20,000 (20 g.)
Coconut water	150 mL (15%)
pH	5.2