GENETIC DIVERSITY AND BIOLOGY OF *Sirindhornia* H. Æ. Pedersen & Suksathan (ORCHIDACEAE)

KANOK-ORN SRIMUANG

DOCTOR OF PHILOSOPHY
PROGRAM IN BIOTECHNOLOGY

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ABSTRACT

The natural populations of *Sirindhornia* species were studied on their biology and genetic variation during 2006-2008 in Thailand. It was found that each species are self-compatible and depend on insects for the pollination. *Sirindhornia mirabilis* exhibited prolonged longevity of individual flowers and produced more flowers per inflorescence features that may have evolved in response to low visitation rates. In all three species, the female (and in *S. mirabilis* also the male) reproductive success decreased from the basal to the apical part of the inflorescence with differential fruit set in rewarding orchid species. Comparison of demographic and reproductive characteristics between the local endemics *S. mirabilis* and *S. pulchella* and the widespread *S. monophylla* found that the three species had similar demographic characteristics, but different reproductive attributes. The local endemics were more reproductively restricted than the widespread *S. monophylla.* Thus, the latter exhibited higher relative fruit set, higher seed production per inflorescence and more equal individual contributions of progeny. However, recruitment appeared to be more efficient in *S. pulchella* than the other two species. The observation of pollination biology in *S. mirabilis* was shown that the small carpenter bee in the genus *Ceratina* (Apidae, Xylocopinae) was very important pollinator, but visited infrequency. In

the roots of all species of *Sirindhornia* found most of Rhizoctonia-like fungi. Seed germination *in situ* of *S. mirabilis* could develop into seedling stage with very low number, whereas, seeds of *S. monophylla* could germinate into protocorms stage, but no further development. Only mature seeds of *S. monophylla* germinated after 16 month of incubation *in vitro*, while, only immature seeds (5 weeks after pollination) of *S. mirabilis* germinated. Thus, seed germination requires different factors for each species. The study of genetic variation of the genus *Sirindhornia* revealed that they have moderate genetic diversity ranged from 0.11-0.15, a little lower than other allogamous orchid species, due to sharing male gamete from the same plant. They also have rather similar genetic diversity between populations, which can be explained by high gene flow (pollen-mediated and seed mediated) or by the history of populations, such as recent colonization or continue connection between population in the past as a large population. Finally, it was found that *S. monophylla*, wider distribution has genetic diversity higher than *S. mirabilis* and *S. pulchella*, endemic species. For conservation point of view, natural populations, individuals and seeds need to be conserved in order to keep genetic resource of these vulnerable species. Cross hand pollination may help to increase genetic variation in population, but it needs to be confirmed by further study.

Keywords: *Sirindhornia* / Orchidaceae / Genetic diversity / Biology

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The genus *Sirindhornia* H. Æ. Pedersen & Suksathan is terrestrial orchid, endemic to Thailand and Southeast Asia. This genus comprises three species; *S. mirabilis* H. Æ. Pedersen & Suksathan, *S. monophylla* (Collett & Hemsl.) H. Æ. Pedersen & Suksathan and *S. pulchella* H. Æ. Pedersen & Indhamusika. In Thailand, these orchids are found only one locality of each. Furthermore, the natural habitats of *Sirindhornia* are becoming more well-known for tourists. The number of visitors that visit the National Park and Wildlife Sanctuary has been increasing every year. Moreover, all *Sirindhornia* are extremely disturbed by human activities e.g., deforestation, cattle feeding, or forest burning (for hunting, for mushroom collecting etc.), considered as risk for extinction. In addition, the environmental changing is likely to influence on their natural populations. The only way to protect these rare orchids is to understand their natures in the wild. Unfortunately, their biology and ecology still remain largely unknown. Until now, there is insufficient information and knowledge about *Sirindhornia* such as genetic diversity, breeding system, pollination biology, flowering phenology, flowering and fruit production including their conservation status. These data are the most important characteristics in the life history of any plants for determining fitness of their sexual reproduction. From this information, we might be able to explain how the nature shapes the phenology especially breeding system, which is considered one of the factors most strongly influencing the survival of plant species.

The importance role of genetics is being increasingly accepted in the field of conservation biology. Conservation genetic is concerned with changes in genetic variation over a

long period of time. All conservation initiatives have to consider this information in all the action plans. Furthermore, the orchidologists and conservationists need to acquire a more comprehensive understanding of population processes and the genetics of the species that they are responsible for. Conservation genetics are a useful tool for solving problems in conservation biology especially genetics of the small populations such as *Sirindhornia.* Finally, the application of population genetics, together with the application of population ecology, will be significant to take comprehensive conservation action for this orchid genus.

1.2 Anticipated benefits of this research

Knowledge of genetic diversity is often critical for the identification of conservation achievement and management units, as well as for the conservation of evolutionary processes within and among population. Both results and information from this research will be analyzed and used as fundamental data to propose a sustainable conservation strategy for the genus *Sirindhornia.*

1.3 Objectives of the research

1.3.1 To study the ecology and biology of *Sirindhornia* in their natural habitats.

1.3.2 To study genetic diversity of each *Sirindhornia* species by AFLP technique.

1.3.3 To study the seed germination of *Sirindhornia.*

1.4 Period of the study

The studies were carried out between the early of 2006 - 2008.

CHAPTER 2

LITERATURE AND REVIEW

2.1 Orchid family

Orchidaceae is one of the largest families of flowering plants. Taxonomically, they represent the most highly evolved family among monocotyledons with 25,000-35,000 species. The Orchidaceae comprises approximately 850 genera, 70 subtribes and 22 tribes. The classification of orchids is well-studied in terms of phylogenetic relationships. Cameron *et al.* (1999) classified orchids into five subfamilies: Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae and Epidendroideae, base on molecular phylogeny.

Orchids exhibit an incredible range of diversity in sizes, shapes and colors of their flowers. Of its many species, approximately 25% are terrestrial, 70% are epiphytic, and the other 5% can grow on a variety of several substrates, including rocks. Orchids are widely distributed. They are almost ubiquitous, absent only from the Polar Regions, true deserts and the intertidal zones along the sea shores (Dressler, 1981). Their distribution is not uniform but skewed markedly towards the tropics. Even within the tropics the distribution varies widely between continents and within countries and regions (Cribb, 1999). They are known for high diversity of specialized pollination and ecological strategies and provide a rich system in which to study evolutionary patterns (Freudenstein and Rasmussen, 1999).

In Thailand, there are approximate a total of 177 genera and 1,125 species of which 150 species are considered endemic to the country. Among these 80% are epiphytic and most of the rest are terrestrial. Few species are saprophytic orchids. Some of the economically important

genera are *Dendrobium* spp., *Bulbophyllum* spp., *Cymbidium* spp., *Paphiopedilum* spp., and *Vanda* spp. (Nanakorn and Indharamusika, 1997).

2.2 Biology and ecology of orchids

2.2.1 Habitats of orchids

Orchids can be found in almost every condition especially in open grasslands, dense tropical jungles, cloud forests, on land slide and roadsides, on trees or rocks that hang over the ocean and are subjected to salt spray, underground, on floating vegetation "islands" in lakes, or even on the margins of deserts (Arditti, 1992; Cribb, 2003). They can be terrestrial, epiphytic or lithophytic. In the tropics, the majority of orchids are epiphytic on forest or woodland trees, or lithophytic on rocks and cliffs (Figure 2.1), and a significant minority is terrestrial, growing on the forest floor in seasonal woodland and in natural and secondary grasslands. The epiphytic orchids can be found on the trunks, branches or twigs of trees. Where the rain fall is sufficient, some epiphytic orchids can also be lithophytic. It is common to find the same species growing on trees and rocks in the same locality (Linder and Kurzweil, 1999; Cribb, 2003).

2.2.2 Growth types of orchids

There are two main growth types of orchids; sympodial (multiple stem) and the monopodial (single stem) (Figure 2.2). In order to grow orchids successfully, it is important to understand each type of growth. Sympodial growth is more common among orchids. They have pseudobulbs, which function as storage reservoirs for food and water. Each shoot grows and later gives rise to similar shoots from axillary buds. For monopodial growth, one main stem grows indefinitely from the center of the plants. The stem grows straight up and aerial roots sprout from where the stem and leaves meet. This is typical of the Vandeae, but has evolved independently in several orchid species (Arditti, 1992; Dressler, 1993; Linder and Kurzweil, 1999).

Figure 2.1 Terrestrial orchids: *Sirindhornia pulchella* growing on the limestone mountain.

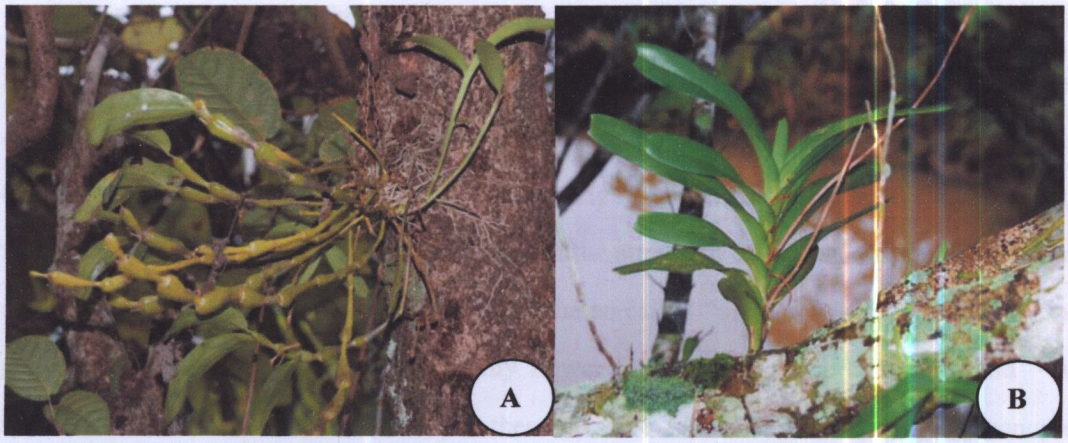

Figure 2.2 Growth habit: sympodial orchid (A), monopodial orchid (B).

2.2.3 Pollination of orchids

The importance of pollinators in the evolution of the Orchidaceae is reflected in the complex pollination mechanisms characteristic of this large family. The relationship between orchids and their pollinators has resulted in a large diversity of floral morphologies that are linked to pollination specialization (van der Pijl and Dodson, 1966; Tremblay, 1992). The pollination by deception is an unusual aspect of orchid biology. The ability to attract pollinators without offering them a reward has evolved independently in many angiosperm lineages, but usually in only a few species per family. In contrast, it has been estimated that around one-third of orchid species are pollinated by 'food-deception' mechanism (Ackerman, 1986; Nilsson, 1992; Renner, 2006; Waterman and Bidartondo, 2008). Many orchids offer flower parts or secretions (nectar, trichomes, wax-like or resin-like compounds, aromatic compounds, oils, etc.) that can be gathered/foraged by their pollinators, found in *Disperis* spp. (Steiner, 1989, 1991, Arditti, 1992; Dressler, 1993; Neiland and Wilcock, 1998; Reis *et al.*, 2004), *Stanhopea lietzeii* (Regel) Schltr., *S. insignis* Frost ex Hook. (Reis *et al.*, 2004), *Comparettia falcate* Poepp. & Endl. (Ackerman *et al.*, 1994) floral fragrances such as in *Catasetum* spp. (Kaiser, 1993), *Spiranthes odorata, Ophyrs speculum* (Liggio and Liggio, 1999) and occasionally pollens in *Cleistes* spp. (Gregg, 1991a, b) and *Psilochilus modestus* Barb. Rodr (Reis *et al.*, 2004). Yet, many orchids "deceive" their pollinators and offer no flower reward such as in *Tolumnia variegate* (Sw.) Braem (Ackerman *et al.*, 1997), *Calypso bulbosa* (L.) (Alexandersson and Agren, 1996), and *Disa nivea* H.P. Linder (Anderson *et al.*, 2005). Many orchids are merely "food-frauds", displaying a set of flower features (colors, fragrances) that apparently elicit the attraction of food-seeking animals. A much more sophisticated kind of deception involves the so-called "pseudocopulation". In this kind of pollination strategy, flower features (mainly flower fragrance, but also coloration and – when applicable - indument or pilosity) mimic these of some female insects (mostly Hymenoptera). Pollination is achieved when male insects attempt copulation with these "dummy females" (Singer, 2003).

Pollination occurs when pollen is moved within flowers or carried from one flower to another of the same species. This must be followed by germination of the pollen (i.e. pollen-tube

growth) and fertilization, which leads to zygote formation. Few plant families rival the orchids in the complexity and ingenuity of their pollination mechanisms (Arditti, 1993). The precision of pollinaria transfer in most Orchidaceae demands a far closer morphological correspondence between flower and pollinator than in other plant families. Furthermore, orchids are subjects for the study of pollinator-induced morphological evolution (Johnson, 1997). The characteristics of the flowers usually are easily recognizable when listed, describe what is called a "syndrome". The syndromes are partly positive clearly showing the adaptations between the flowers and the animals which pollinated them (van der Pijl and Dodson, 1966).

Orchids are naturally pollinator-limited within a season but resource-limited over their lifetime as a result of trade-offs. It is clear that orchids require an ecosystem approach to their conservation and future research. Although, it is possible to utilize principles of the pollination biology to take positive steps to ensure the survival of orchid *in situ* (Roberts, 2003).

2.2.4 Breeding systems

The term 'breeding systems' includes all the aspects of sex expression in plants affected the relative genetic contributions to the next generation of individuals within a species. The breeding system is used in agriculture as a tool to regulate and canalize the components of fecundity for selection purposes in cultivated plants as well as in genetics. In pollination studies under natural conditions, knowledge of the sexual systems is an essential background for evaluation of the dependence of seed production on pollination rate and type towards the understanding of the mechanisms of gene flow within and between populations (Wyatt, 1983; Dafni, 1992). Traditionally, breeding systems have been treated in relation to the mechanisms which promote or reduce out crossing (Shivanna, 2003). Consequently, breeding systems have a significant effect on genetic diversity and the genetic structure of natural populations and therefore the formulation of conservation management plans. In the orchid family, it may surprise that the breeding system of orchids has received little detailed systematic attention (Roberts, 2003).

Breeding systems cover a spectrum from apomixes, with no sexual recombination, to obligate cross-pollination (Table 2.1), and influence variation and the pattern of evolution.

Table 2.1 Major breeding systems and their genetic consequences (Dressler, 1993).

Breeding system	Recombination and variation
Apomixis	none
Autogamy	little
Self-compatibility	moderate or high
Self-incompatibility	high
Dioecy (separate male and female plants)	very high

Dressler (1993) suggested that orchids are usually self-compatible but have mechanisms that favor cross-pollination to promote out-crossing. Breeding systems are often discussed as though each an absolute, but many plants are not 100 percent apomictic, autogamous, self-compatible or self-incompatible.

2.2.4.1 Apomixis

Apomixis is the reproductive process by which flowers produced seed without pollination. The embryos are not the result of sexual recombination but are maternal tissue. Apomictic plants maintain functional pollen, and via pollination. The genetic factors controlling apomixis can be potentially transferred to congeneric sexual populations. This unidirectional introgressive hybridization is expected finally to replace sexuality by apomixis and is thought to be a causal factor for the wide geographical distribution of apomictic complexes.

Apomixis offers a critical advantage to orchids that inhabit regions where pollinators are scarce or absent. The apomixis mechanism has been reported in some orchids such as *Nigritella* sp., *Orchis maculata* L., *Spiranthes hongkongensis* S.Y. Hu & Barretto and *Neotinea maculata* (Desf.) Steam (Sun, 1996; Teppner, 1996; Duffy, 2008).

A thorough knowledge of the genetic base for apomixis is important for the use of apomixis in breeding, as well as for fruit production discussions on evolutionary and ecological behavior.

2.2.4.2 Autogamy

Autogamy or automatic self-pollination is the formation of seeds derived by successful fertilization without a vector (Dressler, 1993), occurs in every tribe and subtribe of orchids. An estimated 5–20% of the Orchidaceae are thought to utilize autogamy as part of their mating system (Catling, 1990; Johnson and Edwards, 2000). Systematically, autogamy is most frequent in relatively primitive groups and least frequent in the advanced as "vandoid" groups (Catling, 1990; Dressler, 1993). Autogamy in orchids is facilitated by the close proximity of anther and stigma and many of the mechanisms of autogamy involve modification of the pollinarium.

In orchids with sectile pollinia, the massulae are often friable and fall onto the stigmatic surface. Caudicles in autogamous species are often either weak, allowing pollinia to flip across onto the stigma when the flower is jarred, or undergo a bending movement which brings the pollinium contact with the stigma of the same flower (Johnson and Edwards, 2000).

Autogamy is favored in certain situations, particularly in cases of environmental isolation such as on islands, mountain-tops and peninsulas, and occurs in habitats that are marginal for pollinator activity. Such species benefit by reproductive assurance, but depending on the possibilities of out-crossing, these plants may suffer from inbreeding depression or low levels of genotypic diversity. Autogamous species may be better suited to survive in the current changing climate due to reproductive assurance and the ability in some species to out-cross if conditions not permit (Roberts, 2003).

2.2.4.3 Self-compatibility

Self-compatibility is term usually refers to plant that are not automatically self-pollinated but may be either self-pollinated or cross-pollinated by flower visitors. Self-compatibility is common in the Orchidaceae. In some case, the fruit may abort or produce fewer or smaller seeds than those resulted by cross-pollination (Dressler, 1993).

2.2.4.4 Self-incompatibility

Self-incompatibility is a genetically controlled system which causes rejection of self-pollen. In sporophytic self-incompatibility, it is controlled by the genotype of the parent plant, not by the progeny genotypes, to distinguish from inbreeding depression as a result of self-pollination. Rejection of unsuitable pollen is by means of chemical recognition between the pollen grain and the stigma or style and is controlled by self-incompatibility loci (Dafni, 1992).

During the process of pollination, pollen grains are transported by wind, water or animal vectors to the stigma, the receptive surface of the pistil. The pistil possesses the capacity to discriminate between the different types of pollen it may receive. Thus, pollen belonging to a species other than that of the pistil is generally rejected, assuring maintenance of stability of the species. Beside these recognition mechanisms that prevent interspecific crosses, slightly more than 50 % of angiosperm species have the ability to identify and reject their own pollen following self-pollination. This mechanism that usually ensures obligated outbreeding, but it may carry the penalty of reproductive inefficiency (Richard, 1997; Gaude and Cabrillac, 2001).

Self-incompatibility in various orchid group has been reported from the Dendrobiinae, Oncidiinae, Vandeae and Coelogyninae (Johansen, 1990; Dressler, 1993; Agnew, 2006; Cheng *et al.*, 2009).

2.2.4.5 Dioecy

Dioecy, characterized by the presence of distinct male and female plants, is widespread in Angiosperm. No individual plant of the population produces both microspores and megaspores. Individual plants are either male (producing microspores) or female (producing megaspores). Dressler (1993) showed that true dioecy in orchids has not been reported yet.

2.2.4.6 Allogamy

Allogamy, also called cross-fertilization, is the usual and best-known way of sexual propagation. It guarantees genetic variability, and thus new combinations of alleles within a species. Allogamy is not restricted to flowering plants alone. Among spore plants, a type of allogamy using male gametes instead of pollen is common. The extent to which flowering plants

depend on external vectors for outcross pollen transportation is a central question in plant reproductive ecology. Allogamy is generally pollinated by animal vectors such as gnats, bees, beetles, wasps, moths, butterflies, skippers, flies and birds (Humana, 2008).

2.2.5 Orchid mycorrhizas

Orchid mycorrhizas are unique in that they occur only within the family Orchidaceae. Although they share some characteristics with other mycorrhizal types (e.g., root cells are colonized by fungi), they differ in that fungal associations are essential for both seed germination and seedling establishment in nature (Rasmussen, 1995; Peterson *et al.*, 1998). The seed produced by orchids are very small and resemble fine particle of dust. The seed is made up mainly of the embryo and a twisted testa, which protects the embryo. Most orchids produce hundreds of thousands of these small seeds each season (Scott, 2008). The main defining characteristic of orchid mycorrhiza is the formation of complex hyphal coils (pelotons) within host plant cells (Figure 2.3 and Figure 2.4) (Peterson *et al.*, 2004). The orchid/fungus association viewed as a compound organism presumably relies on organic debris for its subsistence. Since symbiotic culture of a range of orchid species has been achieved *in vitro* on organic substrates that contain cellulose, starch, proteins, etc., there is considerable evidence that orchid fungi can subsist as saprophytes on dead organic matters (Rasmussen, 1995).

Terrestrial orchid seeds are dispersed by air currents until they land on the soil, where weathering will take the seeds below the soil surface. If conditions are favorable and compatible fungus is in the soil, the seeds will germinates (Scott, 2008). The seeds contain a minute undifferentiated embryo lacking endosperm. These unique structural characteristics require that seeds must first become colonized by an appropriate fungal species that provides carbohydrates for further development of the embryo into a structure called a protocorm (Peterson *et al.*, 2004). The hyphal cytoplasm remains separated from the cytoplasm of the plants by an interface consisting of hyphal plasmalemma, hyphal cell wall, interfacial matrix, and plants plasmalemma serving as the location of nutrient exchange. The pelotons are eventually digested and the infected cells attain a characteristic appearance containing masses of hyphal remain (Rasmussen, 1995; Zettler *et al.*, 2003; Scott, 2008).

The majority of orchids are photosynthetic at maturity. However, more than 100 species of orchid are completely achlorophyllous (Leake, 2005) and are nutritionally dependent on their fungal partners throughout their lifetime. These latter orchids have previously been termed saprophytic but a more accurate designation is mycoheterotrophic (MH) (Leake, 1994; Bidartondo, 2005; Leake, 2005). Epiphytic species are easy to grow asymbiotically in complex nutrient media. In contrast, many terrestrial orchids, including both photosynthetic and MH species have not yet been cultivated success. Nowadays, many orchid species are in danger of extinction from the human activities which induced habitat loss. Although, conservation measures require a full understanding of the biology of each species in question for any plan to conserve the habitats of orchids (Dearnaley, 2007).

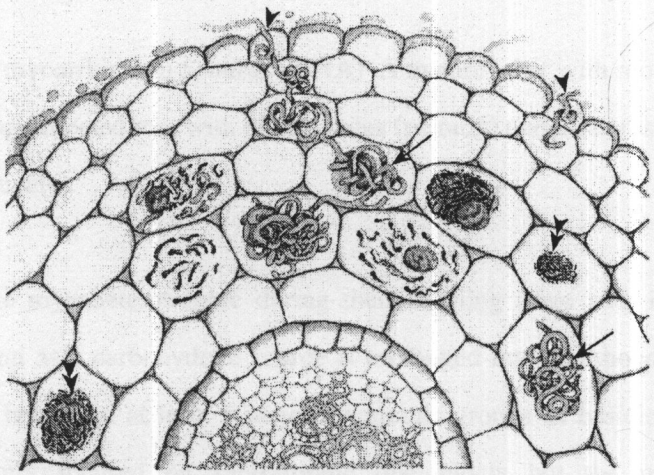

Figure 2.3 Diagrammatic representation of an orchid root showing the type of colonization typical of orchid mycorrhizas. Hyphae enter the root through the epidermis (arrowheads), and form hyphal coils (pelotons) in cortical cells (arrows). Hyphal coils undergo degradation over time (double arrowheads) (Peterson *et al.*, 2004).

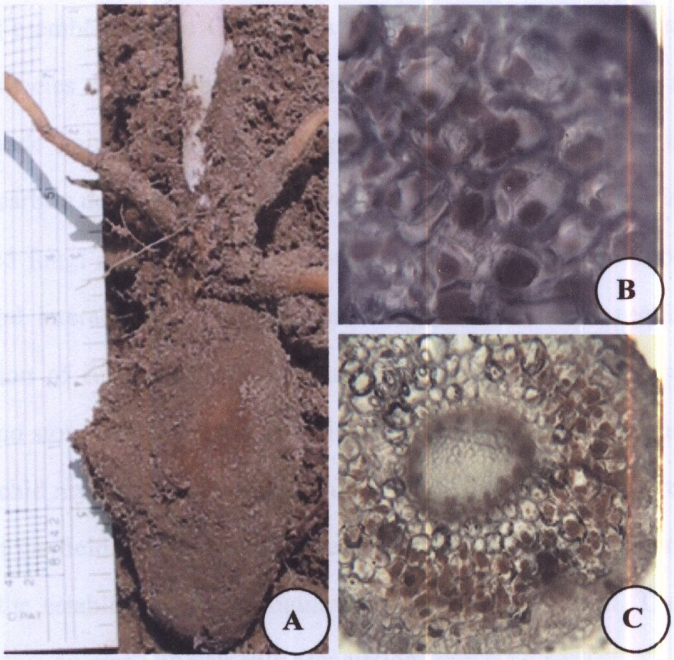

Figure 2.4 Orchid mycorrhizas in *S. mirabilis*. (A) A mature tuber with roots. (B) and (C) showing inflected root with hyphal coins (peloton) under microscope (10x and 4x, respectively).

Orchids are mycoheterotrophic during their seedling stage and, in many species, the dependency on fungi as a carbohydrate source is prolonged into adulthood. The mycobionts in orchid mycorrhiza belong in at least 5 major taxonomic groups of basidiomycetes. Traditional records have mainly focused on saprotrophic mycobionts but the participation of both ectomycorrhizal and parasitic fungi in orchid mycorrhiza have been corroborated (Rasmussen, 2002). There is an increasing evidence of specific relationships between orchids and fungi. Physiological compatibility demonstrated under artificial conditions, as *in vitro*, may be not only a species-to-species level but much broader. Recent development of field sowing techniques has improved the possibilities of evaluating orchid-fungal relations in an ecological context (Rasmussen and Whigham, 1993).

Dearnaley (2007) summarized that orchid mycorrhizas are mutualistic interactions between fungi and members of the Orchidaceae. The majority of orchids are photosynthetic, a small number of species are mycoheterotrophic throughout their lifetime, and recent research indicates a third mode (mixotrophy) whereby green orchids supplement their photosynthetically fixed carbon with carbon derived from their mycorrhizal fungus. Molecular identification studies of orchid-associated fungi indicate that common fungal taxa across the globe and some orchids have specific fungal interactions. Confirmation of mycorrhizal status requires isolation of the fungi and restoration of functional mycorrhizas. New methods may be used to store orchid-associated fungi, and store and germinate seed, leading to more efficient culture of orchid species. However, many orchid mycorrhizas must be synthesised before conservation of these associations can be attempted in the field. Further gene expression studies of orchid mycorrhizas are needed to better understand the establishment and maintenance of the interaction. These data will add to efforts to conserve this diverse and valuable association.

Mycorrhizal fungi may be a key source of water for orchids such as *Platanthera integrilabia* (Correll) Luer and *Epidendrum conopseum* R. Br. that water content was higher for mycorrhizal seedlings than uncolonised controls (Yoder *et al.*, 2000). Overall, nutrient exchange in at least photosynthetic orchids appears more complete. All orchids need fungi to provide inorganic and organic nutrients for seed germination and/or early protocorm development. In adult photosynthetic orchids, nitrogen (N), phosphorus (P) and water continue to flow from the fungal partner but carbon exchange is essentially reversed with photosynthetic providing incentive for continued fungal colonization. The reward for fungi at the seed/protocorm stage is still a matter for conjecture (Alexander *et al.*, 1984; Yoder *et al.*, 2000; Dearnaley, 2007).

The understanding of the orchid/fungus association is important to management and conservation, not only because of the potential application of symbiotic methods to the propagation of endangered orchid species, but also in relation to the ecological characteristics associated with the natural occurrence of symbiotic fungi and their colonization in roots of mature orchids (Hadley and Pegg, 1989).

2.3 *Sirindhornia*, an endangered orchid genus from Thailand

The orchid genus *Sirindhornia*, named after Her Royal Highness Princess Maha Chakri Sirindhorn of Thailand to mark Her Royal Highness Princess 48th birthday in 2003. *Sirindhornia* is strictly distributed in Thailand (Chiang Mai and Tak provinces), Myanmar (Shan State), and Southern China (Yunnan province). It comprises three species: *S. mirabilis* H. Æ. Pedersen & Suksathan, *S. monophylla* (Collett & Hemsl.) H. Æ. Pedersen & Suksathan and *S. pulchella* H. Æ. Pedersen & Indhamusika. *S. monophylla* is found almost throughout the range, while the others seem to be endemics to Thailand (Pedersen *et al.*, 2002).

2.3.1 Taxonomy of *Sirindhornia* (Pedersen *et al.*, 2002)

Family : Orchidaceae

Subfamily : Orchidoideae

Tribe : Orchideae

Subtribe : Orchidinae

Genus : *Sirindhornia* H. Æ. Pedersen & Suksathan

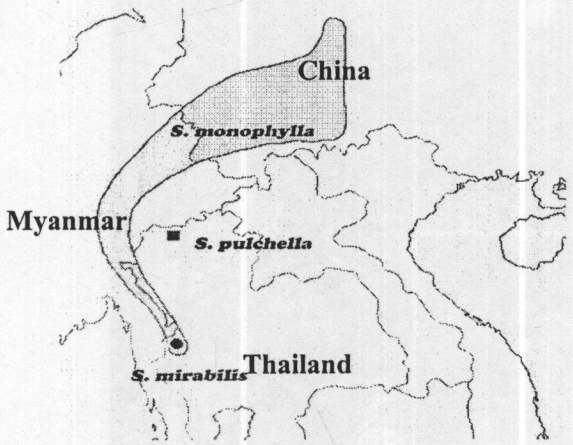

Figure 2.5 Distribution of the genus *Sirindhornia* showing localities of *S. pulchella* (square), *S. mirabilis* (dot) and distribution range of *S. monophylla* (Pedersen *et al.* 2002).

***Sirindhornia mirabilis* H. Æ. Pedersen & Suksathan**

S. mirabilis is known in Thai name as Ueang Sri Prajim. This species was firstly collected by Dr. Piyakaset Suksathan in 2001. It occurs in small numbers among limestone crevices and on grassy slopes near limestone outcrops at approximately 8000 m altitude.

The heights of *S. mirabilis* plants are between 13.0 - 70.0 cm. The inflorescences have the number of flowers between 2 to 48 flowers (Figure 2.6 A), rachis 12.7 cm long, the floral bracts ovate-triangular shape. The tubers are ellipsoidal to narrowly ovoidal-ellipsoidal (Figure 2.6 B). The stems are relatively robust with mottled purple/green. The floral size is approximate 1.5 cm in diameter (Figure 2.6 C). The sepals are more or less papillose-ciliate. The lateral sepals are reflexed. The labellum is deeply 3-lobed in proximal part; including green sidelobes, midlobe spathulate and spur straight with abruptly downcurved apex. The pollinaria are situating with one common viscidium; bursicle very broadly hinged and ovary recurves. The concentration of nectar is approximately 5 mg/ml. Flowering period is between April - June. The distribution of this species is restricted only at Doi Hua Mot, Tak province, Thailand (Figure 2.7) (Pedersen *et al.*, 2002).

Figure 2.6 *S. mirabilis* (A) inflorescences (B) an ellipsoidal-shape tuber (C) a flower.

***Sirindhornia monophylla* (Collett & Hemsl.) H. Æ. Pedersen & Suksathan**

S. monophylla is known in Thai name as Ueang Sri Arkane. This orchid species grows on limestone mountains at 800 to 2,200 m altitude, ranging from the tropical to the mountain zones. It seems to occur mainly on open and grassy slopes, but has been found under shrub as well. *S. monophylla* can be found frequently near *S. mirabilis* (Pedersen *et al.*, 2002).

Figure 2.7 The habitats of *S. monophylla* and *S. mirabilis* (A) the over view of Doi Hua Mot (B) the area of study site.

The heights of *S. monophylla* are between 12.0 to 40.0 cm. The stems are slender with mottle purple/green, bract-like leaves which are glabrous on their adaxial face. The inflorescences have the numbers of flowers between 6 to 29 flowers (Figure 2.8 A). The tubers are ellipsoidal or

ovoidal to subterete (Figure 2.8 B). The floral size is approximately 0.7 cm in diameter. The labellum is less 3-lobed in distal part; including rose-colored to white with numerous tufts of purple papillae on the adaxial side (Figure 2.8 C). The pollinia are situating with separate viscidia. The concentration of nectar is approximately 5 mg/ml. Flowering period is between May – June. The distribution in Thailand of this species is restricted only at Doi Hua Mot, Tak province, Thailand (Figure 2.7) (Pedersen *et al.*, 2002).

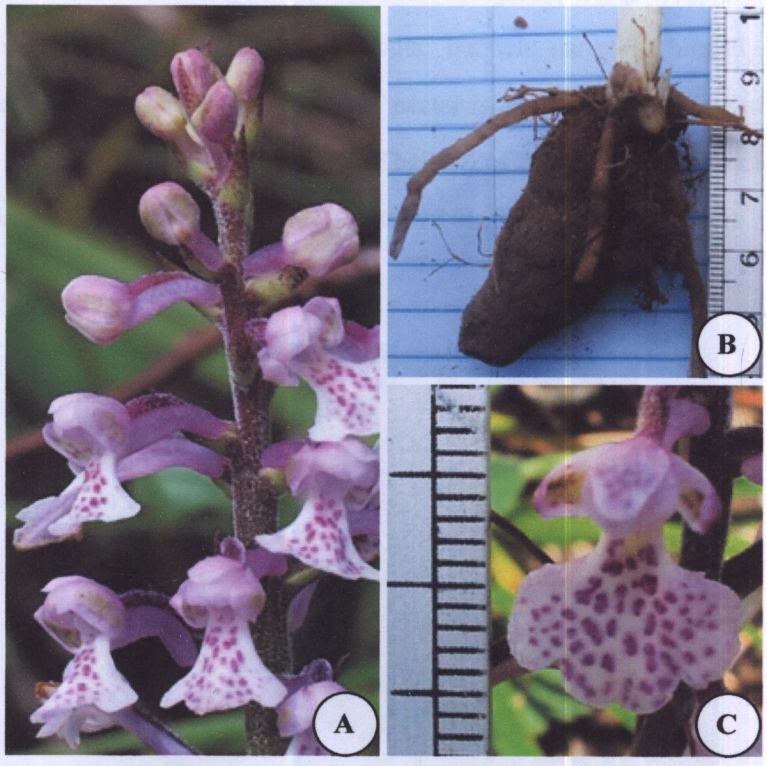

Figure 2.8 *S. monophylla* : (A) inflorescence (B) an ellipsoidal-shape tuber and (C) a flower.

***Sirindhornia pulchella* H. Æ Pedersen & Indhamusika**

S. pulchella is known in Thai name as Ueang Sri Chiang Dao. This orchid species is occurred above 1,800 m altitude on the southern and eastern ridges of Doi Chiang Dao. On this

Permian limestone massive, it grows among rock crevices in vegetation recognized as "open hill evergreen forest"

The heights of *S. pulchella* are between 5.0 - 25.6 cm. The inflorescences have flowers between 2 - 13 flowers (Figure 2.9 A). The tubers are ellipsoidal to terete (Figure 2.9 B). The stems are slender; including reddish to mottle purple/green, on its upper half. The floral size is approximate 1.5 cm in diameter. The sepals are spreading. The dorsal sepals are elliptic-oblong shape. Their colors are pink to rose-colored and usually with purple dots. The labellum is shallowly 3-lobed in distal part. Their colors are pink with purple spots (Figure 2.9 C). The pollinia are situating with separate viscidia; bursicle narrowly hinged, 2-loculate and ovary somewhat recurved. The concentration of nectar is approximately 7 mg/ml. Flowering period is between April - June. The distribution in Thailand of this species is restricted only at Doi Chiang Dao, Chiang Mai province, Thailand (Figure 2.10) (Pedersen *et al.*, 2002).

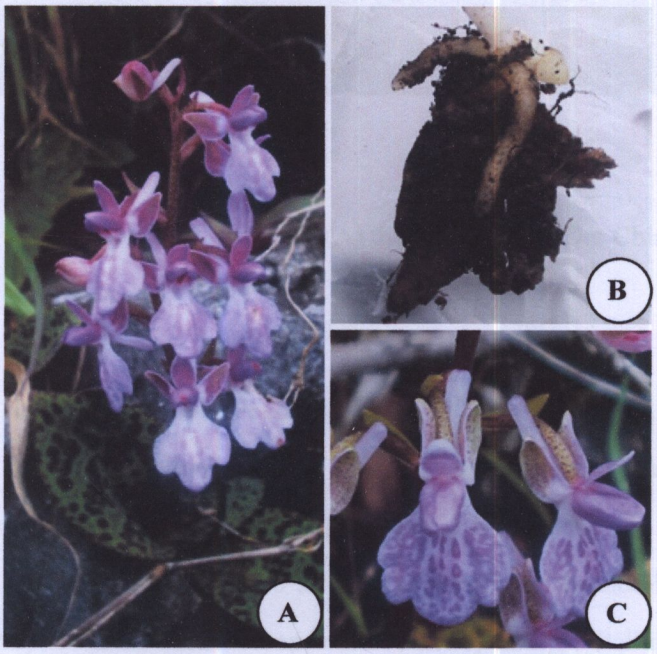

Figure 2.9 *S. pulchella*: (A) inflorescence (B) an ellipsoidal-shape tuber and (C) closed up flowers.

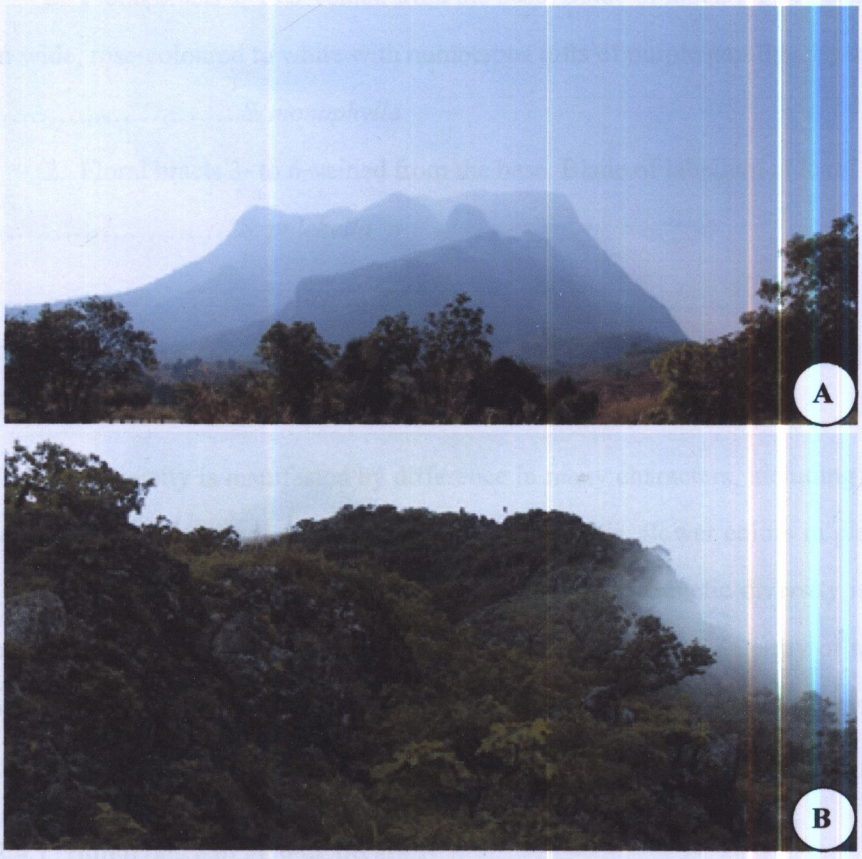

Figure 2.10 The habitats of *S. pulchella* (A) the over view of Doi Chiang Dao, (B) the area of study site.

2.3.2 Key to the species (Pedersen *et al.*, 2002)

1. Blade of labellum deeply 3-lobed in proximal part; the side-lobes green. Spur straight with downcurved and vertically inflated apex. Pollinia have one visidium……*S. mirabilis*

1. Blade of labellum less 3-lobed in its distal part; the side-lobes pink to white. Spur straight to slightly up- or downcurved; apex not vertically inflated. Pollinia with separate viscidia……2

2. Floral bracts 1- to 3-veined from the base. Blade of labellum less than 7 mm long and 9 mm wide, rose-coloured to white with numberous tufts of purple papillae. Spur less than 8 mm long.....*S. monophylla*

2. Floral bracts 3- to 6-veined from the base. Blade of labellum at least 11 mm long.....*S. pulchella*

2.4 Genetic diversity

Genetic diversity is manifested by difference in many characters, including eye, skin and hair color in human, colors and banding patterns of snail shells, flower colors in plant and in the proteins, enzymes and DNA sequences of almost all organisms. Genetic diversity is required for populations to adapt to environmental change. It is measured by using an array of molecular and quantitative methods. Large populations of naturally outbreeding species usually have extensive genetic diversity, but it is typically reduced in endangered species (Frankham, *et al.*, 2002; 2004).

2.4.1 Importance of genetic diversity

The International Union for Conservation of Nature (IUCN), the premier international conservation body, recognizes the need to conserve genetic diversity as one of the global conservation priorities. There are two major and interrelated issues. First, environmental change is a continuous process and genetic diversity is required for populations to evolve to adapt to such change. Second, loss of genetic diversity is usually associated with inbreeding and overall reduction in reproduction and survival (fitness) (Frankham *et al.*, 2004). Furthermore, it may provide disease resistance, adaptability to a changing climate, or some other trait necessary for the species to survive in the ever-changing world.

2.4.2 Assessing genetic diversity in natural populations

The assessment of genetic diversity range from quantification of morphological variation (including morphometrics and cladistic analysis) to protein (mostly alloenzymes) and DNA-based techniques, often collectively referred to as molecular techniques (Fay and Krauss, 2003).

The technique of gel electrophoresis enables us to measure genetic variation in a natural population. The frequency of heterozygotes for each gene is first determined and then the average heterozygosity for all the genes in the sample is calculated. A great deal of genetic variation is found in most natural populations of sexually reproducing organisms.

In addition to the enzyme or protein polymorphism, recent advances in biotechnology have enabled use of new types of polymorphisms discovered at the DNA level. Bacterial restriction endonucleases, which cleave DNA at sequence-specific sites, break down a very long DNA molecule into small fragments. If the variation in the DNA sequence at a particular locus is such that one of the variants is cleaved by a restriction enzyme and the other is not, then the variant that is not cleaved at that locus will be associated with a longer fragment of DNA. This kind of sequence variation is known as Restriction Fragment Length Polymorphism (RFLP). Such markers are found in the coding as well as non-coding regions of the DNA. They are usually biallelic and codominant so that they allow all the three genotypes to be discriminated. Another powerful technique for generating polymorphism is that of the Polymerase Chain Reaction (PCR) which requires much less DNA to produce a detectable band on the film. It amplifies a segment of DNA flanked by two specific sequences (Narain, 2000).

Early DNA-based techniques were mostly not applicable to conservation studies because the amount of DNA used required the destructive sampling of large amounts of plant tissue. However, since 1988, many molecular techniques based on PCR have been developed or improved, and DNA sequencing, random amplified polymorphic DNAs (RAPDs), PCR-retriction fragment length polymorphisms (PCR-RFLPs), amplified fragment length polymorphism (AFLP), nuclear microsatellites and plastid microsatellites have all been used in orchid conservation genetics (Fay and Krauss, 2003). Empirical studies in ecology and evolution often depend on accurate assessment of genetic diversity to address questions regarding genetic relatedness among individuals, population structure, phylogenetic relationships and mapping of quantitative trait loci. A series of techniques and genetic markers have been developed to estimate genetic diversity. However, no single technique is universally ideal; each available technique exhibits both strengths and weaknesses (Mueller and Wolfenbarger, 1999).

2.4.3 Amplified fragment length polymorphisms (AFLPs)

Among the available DNA molecular techniques, AFLP is a powerful technique for cultivar identification (Lombard *et al.*, 2000). It provides a large number of markers in a single analysis without requiring sequence information for their development (Vos *et al.*, 1995). AFLP technique, thus, provides a good general assessment of the variation in genomic DNA and also has a number of advantages over pre-existing techniques. As with RAPDs, only a small quantity of DNA is used because the technique is based on PCR, meaning that tiny quantities of plant material are required (Fay and Krauss, 2003).

AFLP methodology includes the first step of digestion total genomic DNA with two restriction enzymes endonucleases, which normally has only a few recognition sequences present in a given genome compared to the second endonuclease (e.g. a 6-bp cutter such as *EcoRI* and a 4-bp cutter such as *MseI*). The next step is ligation of adaptor (the short DNA oligonucleotides) at the ends of fragments. After that two PCR amplification are performed using a set of primer that incorporate a core sequence, the recognition sequences of the two endonucleases plus an additional base pair. This additional base pair adds a measure of selectivity to the amplification. This reduces the pool of fragments significantly from the original mixture. The second amplification step uses oligonucleotides that have three base pairs added to the 3' end to reduce the final pool to a manageable number (approximately 50-100 fragments) (Figure 2.10). The DNA fragments produced by this analysis normally are separated on denaturing polyacrylamide gels, generating DNA fingerprint. Alternatively, fluorescently labeled fragments can be characterized in an automated sequencer using software designed for that purpose (Vos *et al.*, 1995; Wolfe and Liston, 1998; Arnold and Emms, 1998).

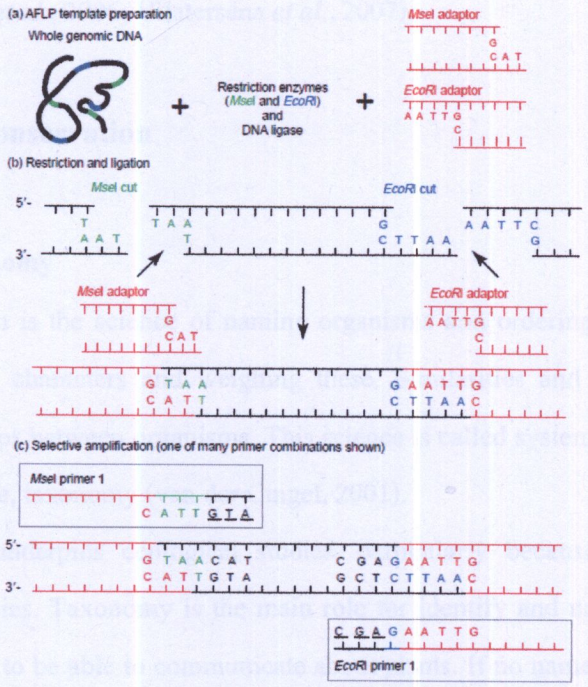

Figure 2.11 Overall steps of AFLP technique: (a) small amounts of DNA (~50 ng) are digested with two restriction enzymes, (b) AFLP adaptors are joined (ligated) to these ends and (c) selective amplification of a subset of these fragments, primers are extended into the unknown part of the fragments, usually one to three arbitrarily chosen bases beyond the restriction site (Mueller and Wolfenbarger, 1999).

2.4.4 Applications of AFLPs in orchids

AFLP markers have found the widest application in genetic variation of orchids below the species level, particularly in structure and differentiation among population including genetic variation within populations (Mueller and Wolfenbarger, 1999). For conservation genetics of orchids, AFLP markers can be applied to evaluate genetic structure and genetic variation at both species and population level, (Izawa *et al.*, 2007; Pillon *et al*, 2007; Li *et al.*, 2008; Jacquemyn *et al.*, 2009). Furthermore, the AFLP technique has been used successfully in other plant population genetic studies, especially for endangered species (e.g., Travis *et al.*, 1996; Palacios *et al.*, 1999;

Kim *et al.*, 2005; Ni *et al.*, 2006; Vilatersana *et al.*, 2007).

2.5. Science for conservation

2.5.1 Taxonomy

Classification is the science of naming organisms and ordering them in a system. It is based on comparing characters and weighing these. Similarities and differences are used to determine relationships between organisms. This science is called systematics or also, but usually with a less wide scope, taxonomy (van der Cingel, 2001).

Taxonomy underpins ecological studies, particularly because it allows for reliable identification of species. Taxonomy is the main role for identify and named orchids accurately. Names are necessary to be able to communicate about plants. If no name or wrong name is given then no useful information on a plant can be transmitted. Our knowledge of the diversity within genus or the geographic distribution of species depends upon good taxonomy. Since the advent of molecular approaches, the Orchidaceae has become the most intensively studied family of flowering plants in terms of their phylogenetic relationships. New classifications are now being published that have radically changed our understanding of generic delimitation and relationships (Cameron *et al.*, 1999; Cribb *et al.*, 2003; Cameron *et al.*, 2005).

Pillion and Chase (2006) used a checklist of orchids to compare the taxonomic treatment of orchids between Europe and neighboring areas to search for geographical patterns. Numbers of invalid, infraspecific, and hybrid names are significantly higher in Europe than in surrounding areas. Recognition of numerous and poorly circumscribed orchid taxa is a serious obstacle to their conservation because rare, poorly defined species may be prioritized for conservation over taxonomically "good" species. This phenomenon may be the result of the popularity of orchids in Europe. They suggested that more taxonomic effort should be made in other areas of the world (e.g., the tropics) and on less charismatic groups.

2.5.2 Distribution

Distribution is useful for knowing that a species which found within a country is not, in itself, useful to conservation. We need to know where it occurs and how common it is. In conservation point of view, endemic species which is narrow distribution, and rare species which found only few localities are most important concerning. However, in tropical region seems to be insufficient on natural orchid distribution due to limitation of exploration and orchidologist.

Recent developments in sophisticated computer based mapping such Geographic Information Systems (GIS) have begun to be used to predict where rare species might occur based upon information from known localities, vegetation maps, geological information, soil types and topography (Cribb *et al.*, 2003). This tool is another option for conservation program.

2.5.3 Demography

To conserve orchids in nature reserves, it needs to have some way of measuring the health of the population in the reserve. That can only be done if one already has an understanding of the population biology of the particular orchid species under natural conditions. Unfortunately, we have little information about those aspects of tropical orchid's life and certainly not enough to allow one to draw broad generalizations on which one can base actual conservation (Koopowitz, 2001). In the case of endangered plant species, population viability normally depends more on demographic features than on genetic ones. Therefore, demographic analyses, especially structured population models, are one of the most useful techniques to diagnose the health of populations (Marrero-Gomez *et al.*, 2003).

Demographic studies can provide information on the age structure of a population. A stable population typically has an age distribution with a characteristic ratio of juveniles, young adults, and older adults. An absence or low number of any age class, particular of juveniles, many indicate that the population is in danger of declining. Similarly, a large number of juveniles and young adults may indicate that the population is stable or even expanding. Careful analysis of long-term data, or of changes in the population over time, is often needed to distinguish short-term fluctuations from long-term trends (Primack, 2000).

2.5.4 Ecology

To preserved orchids population in the wild, it is necessary to manage, then we need to be able to understand them and their needs in some detail. It is necessary to examine their habitats and life processes as well as interactions within the family and with other plants, organisms, and the environment (Arditti, 1992). Much of the information that we need is of a simple nature, it is easy to collect and does not require sophisticated apparatus or facilities. Following populations for several years and counting their components is relatively easy to do and it can be carried out by nearly anyone with a minimum of training or instruction. Ecological studies of orchids are predominantly focused on temperate species, while the knowledge of tropical orchid ecology is limited (Koopowitz, 2001; Cribb *et al.*, 2003).

2.5.5 Genetics

Conservation genetics is the use of genetic theory and techniques which are called DNA markers for reduce the risk of extinction in threatened species. Conservation genetics is now a discrete discipline focusing on the consequences arising from reduction of once-large, outbreeding, populations to small units where stochastic factors and the effects of inbreeding are paramount. It is now accepted that ecological changes are likely to produce genetic effects. Conservation geneticists have attempted to minimize the consequences of the latter, principally by advocating rules about the size of minimum viable populations (Berry, 1995; Frankham *et al.*, 2004). The field of conservation genetics also includes the use of molecular genetic analyses to elucidate aspects of species biology relevant to conservation management. The major issues such as: the deleterious effects of inbreeding on reproduction and survival (inbreeding depression); loss of genetic diversity and ability to evolve in response to environmental change; fragmentation of populations and reduction in gene flow; random processes (genetic drift) overriding natural selection as the main evolutionary process; and accumulation and loss of deleterious mutations (Glover and Abbott, 1995; Usher, 1995; Gray, 1996; Chung and Chung, 2000; Hedrén *et al.*, 2001; Wallace, 2003; Frankham *et al.*, 2004; Chung and Chung, 2008; Leitch *et al.*, 2009; Chung, 2009; Neubig *et al.*, 2009)

2.6 Asymbiotic germination

A single orchid capsule contains millions of seeds, which are minute and do not have any endosperm. Orchid seeds can not utilize their own lipid reserves, break down starch, and photosynthesize. They are therefore dependent on an external source of nutrients throughout germination, until the first shoots and leaves are able to photosynthesis, enabling the plant to provide for its own energy needs (Arditti, 1992; Pathak *et al.*, 2001; Kalimuthu *et al.*, 2007). Previous researchers have suggested that all orchid seeds can be germinated as long as essential nutritional requirements are met, extending to undefined complex compounds that can be artificially supplied (Vacin and Went, 1949). However, no single solution applies to all orchids, with some requiring specific compounds too complicated for laboratory analysis or horticultural practicality (Arditti *et al.*, 1981).

Germination techniques are well established for epiphytic orchids based on the asymbiotic media developed by Knudson in the 1940s. Epiphytic orchid seedlings have been grown on many different media, often with undefined and complex additives from banana pulp, tomato juice or potato extract. For most epiphytic species, high levels of germination can be easily achieved using asymbiotic techniques. The media in regular use include half strength MS (Murashige and Skoog, 1962), VW (Vacin and Went, 1949) and Knudson C (Knudson, 1946) used as basal media or supplemented with homogenized banana (50 gL^{-1}) and or activated charcoal ($2gL^{-1}$) (Ramsay and Dixon, 2003).

However, most terrestrial orchid seed is difficult to germinate *in vitro* by using the nutrient media devised for epiphytic species (Ramsay and Dixon, 2003) due to specific nutrient and environmental requirements (Rasmussen, 1995; Vujanovic *et al.*, 2000; Thompson *et al.*, 2006). Rasmussen (1995) reported 40 media as being capable of sustaining terrestrial orchid germination *in vitro*. Beneficial modifications include reduced concentrations of inorganic salts and increased organic compounds (Harvais, 1972, 1973, 1974; Arditti *et al.*, 1981; Oliva and Arditti and Ernst, 1984; Rasmussen, 1995; Thomson *et al.*, 2006). Early elimination of mineral salts is advantageous (Arditti, 1982; Rasmussen, 1995) as a high water potential is desirable for

germination, but this does not sustain seedling development. Liquid substrates also ensure a high water potential, yet are not generally used in orchid germination as they offer little mechanical stability (Rasmussen, 1995).

Organic compounds in the form of carbohydrates, vitamins, amino acids and growth regulators may be incorporated into media directly, or through undefined additives such as coconut milk, banana homogenate or potato extract (Arditti, 1982; Arditti and Ernst, 1984; Vyas *et al.*, 2009) resulting in increasingly complex media in which no single factor can be identified as the germination-stimulating agent (Thompson *et al.*, 2006).

Optimal *in vitro* temperature and light parameters are subject to specific variation and/or differences in habitat. Optimum incubation temperatures for Orchidaceae of tropical origin were reported in the range 22–25 ^{0}C (Arditti, 1982; Oliva and Arditti, 1984; Rasmussen, 1995). Alternatively, Rasmussen *et al.* (1990) reported that temperate orchids required temperatures somewhat lower (< 20^{0}C) than those reported. Data are also available that have revealed the positive effects of cold and warm stratification seed treatments prior to long-term incubation (Miyoshi and Mii 1998). Many terrestrial species benefit from incubation in continuous darkness for a minimum defined period following sowing (Rasmussen *et al.*, 1990; Rasmussen, 1992, 1995). However, continuous low-light treatments and a range of low light dark photoperiods were included (Knudson, 1943; Stoutamire, 1963, 1964, 1974; Arditti, 1967, 1979; Harvais, 1972, 1973; Clements, 1986; Zettler and McInnis, 1994).

Seed maturity also has influences *in vitro* germination success. Immature seed (green pod) is widely used for germinating terrestrial orchids (Rasmussen, 1995; Lo *et al.*, 2004; Chen *et al.*, 2005; Hirano *et al.*, 2005; Yamazaki and Miyoshi, 2006). However, the appropriate developmental stage for excision must be assessed for each species.

2.7 Symbiotic germination

The history of germination of orchid seed is an interesting one. In the middle of 19th century commercial growers were germinating orchid seeds; their methods, kept as trade secrets,

probably simulated the natural jungle environments of the orchids. In the jungles, seedling orchids are found only rarely, usually at base of mature orchid plants. The difficulties of seed germination in their natural habitats can be appreciated after an examination of an orchid seeds. Not until the early of 20th century did botanist concern themselves with the problem of germinating orchid seeds when Burgeff in Germany and Bernard in France reported that orchid seeds possessed insufficient food for germination. Upon examination of germinating seed they found a fungus within the embryo, identified by Bernard as *Rhizoctonia*. Both authors postulated that asymbiotic condition existed (Vacin and Went, 1949).

Terrestrial orchids are generally regarded as one of most vulnerable groups of higher plants, because they are difficult to grow from seed in the absence of mycorrhizal fungi (Zettler, 2001). Unusually, orchids are consuming mycorrhizal fungi as an energy source (mycotrophy) in a parasitic symbiosis to initiate seed germination and seedling development (Rasmussen, 1995). Consequently, the establishment of orchids in restored habitats requires the presence of mycorrhizal fungi to recruit seedlings. One possible way to promote this process is by symbiotic seed germination (Clements *et al.*, 1986; Stewart and Zettler, 2002).

Epiphytic orchids rely on mycotrophy to a lesser extent because their above-ground habit provides them with greater access to light for photosynthesis (autotrophy) (Rasmussen, 1995). Terrestrial orchids appear to be absolute required for mycotrophy, particularly during seed germination and seedling (protocorms) development, processes that normally take place below ground in darkness. Fungal hyphae may be digested through adulthood, with mycotrophy supplementing photosynthetic nutrition (Warcup, 1971; 1973; 1981; Rasmussen, 1995; Zettler, 2001).

Growing terrestrial orchids from seed is often considered problematic; for some species it is very slow process. Then, before many orchid species become extinct, effort to develop effective protocols for artificial (seed) propagation should be a priority (Zettler, 2001).

McKendrick *et al.* (2000) studied the processes of symbiotic germination and seedling development in the mycoheterotrophic orchid *Corallorhiza trifida* Châtel. Seeds of which were buried in packets either adjacent to or at varying distances from adult plants in defined

communities of ectomycorrhizal tree species. Germination occurred within eight months of burial under *Betula-Alnus* and within seven months under *Salix repens* (L.). It was always associated with penetration of the suspensor by a clamp-forming mycorrhizal fungus. Four distinct developmental stages were defined and the rates of transition through these stages were plotted. There was no evidence of a relationship between extent of germination or rate of development and the presence of naturally distributed plants of *C. trifida* Châtel. at the spatial scale of 1.0 m. The best germination and the most rapid rate of development of *C. trifida* Châtel. seedlings occurred in a *Salix repens* (L.) community located at a considerable distance from any extant *C. trifida* Châtel. population.

Ochora *et al.* (2001) studied symbiotic germination of *Eulophia* Lindl. and *Polystachya* Hook. seeds from terrestrial and epiphytic habitats respectively. They were established cultures initiated from the seeds were grown on 2 % oats medium and germination medium, alone on oats medium supplemented with 10 $g.L^{-1}$ banana fruit homogenate and 0.2 $g.L^{-1}$activated charcoals. The highest germination/differentiation, which took place after 7 days of inoculation, was obtained on the 2% oats medium with glucose (2 $g.L^{-1}$) and $NH_4NO_3.4H_2$(10.0 $mM.L^{-1}$) as a source of inorganic nitrogen and supplemented with the additives. Development of protocorms in compatible orchid-fungus combination took place after the second subculture (30 days) from which seedling developed. Irrespective of the fact that the orchids showed possible specificity patterns at population level with their fungal partners in their natural habitats the *in vitro* symbiotic seed germination also showed significant tendency towards fungal specificity.

Shimura and Koda (2005) reported symbiotic germination of *Cypripedium macranthos* var. *rebunense* by isolated fungus from their roots and induced for germination. Cold treatment of the seeds at 4 ^0C prior to fungal inoculation was required for the symbiotic germination. Changing the timing of inoculation of the fungus to the seeds greatly improved germination frequency. Maximum germination was attained after seeds were inoculated just after the cold treatment for 12 weeks, and approximately 20% of the seeds developed into protocorms more than 1 mm long.

In addition, the successful of symbiotic germination technique of orchid seeds by *in vitro* methods have been reported more increasing (Terashita, 1985; Tsutsui and Tomita, 1986; Umata,

1995; Zettler and Hofer, 1998; McKendrick *et al.*, 2000; McKendrick *et al.*, 2002; Batty *et al.*, 2001; Stewart and Zettler, 2002; Leak *et al.*, 2004; Shimura and Koda, 2005; Diez, 2007; Shimura *et al.*, 2007; Stewart and Kane, 2006; Stewart and Kane, 2007; Yagame *et al.*, 2007).

2.8 Threats to natural orchid population

2.8.1 Changes of land use

The major threat to natural orchid population is habitat destruction. In some area, especially in tropical countries, the whole population is completely vanished. Thus many orchid species become rare or extinct. Logging and cultivation in most tropical countries are so widespread. For instance, in Sumatra, it is likely that logging on this island will removed all of the lowland forest within three years (Cribb *et al.*, 2003). All of orchid species found in the eastern Indonesian islands of Maluku was threatening from logging both illegal and legal logging for grow Palm oil industry (Hidayati, 2000).

In Thailand, as well, the forest has been reduced more than half within last 30 years cause of logging, cultivation and other land using such i.e. for dam and industry. In northern part there is serious shifting cultivation. The forest is burn every year until it can not control fire which consequently destroys the natural forest. It appears to be forest fragments.

The fragments of the forest that remain are rich in orchid, however species that require large stands of forest for reproductive success, and those that have restricted distribution, are on the edge of extinction (Cribb *et al.*, 2003; Garrison, 2005). Even for the more common species, such extreme habitat fragmentation causes the loss of vital pollinators and genetic erosion. The terrestrial orchid seems to be suffered directly from changing of land use and fire, especially the species thriving in evergreen forest and the species that require unique micro environment (Watthana, pers. Comm.).

2.8.2 Commercial Collecting

Commercial collecting is not illegal but in most countries a permit is necessary to collect orchid for any purpose. A common misconception is that implementation of the Convention on

International Trade in Endangered Species of Wild Fauna and Flora (CITES). However, it only controls the export of orchids across national borders but not controls the collection and over-collection orchids from the wild.

Over-collecting is often cited as the main cause of orchid extinction. However, it depends on the orchids targeted and the methods used. In horticulture, showy genera, such as *Cattleya, Laelia, Odontoglossum, Paphiopedilum, Phalaenopsis, Phragmipedium* and *Cymbidium*, cause most interest. Rare and attractive species are collected because of the potential rewards, especially for new or recently described species. Thus commercial collection can push an orchid towards extinction, particularly if the taxon is naturally rare and when the horticultural or ethnobotanical demand is high. For instant, the recently described *Paphiopedilum helenae, P. vietnamense* and *Renanthera citrina,* narrow endemics in northern Vietnam, are already threatened with extinction in their known localities (Averyanov *et al.*, 2000; 2003). In Thailand, the recently described *P. thaianum* has been collected from natural habitat until few individual remaining (Pumikong, per. comm.).

The quantities collected for medicinal purpose can be large, sometimes in tens of kilograms. The repeated collection from the same localities is common practice. Averyanov *et al.* (2000) commented on the quantities of *Anoectochilus* spp., *Cymbidium aloifolium*, *Dendrobium chrysanthum, D. fimbriatum, Goodyera hispida* and *Nervilia punctata* being collected in northern Vietnam for the Chinese medicinal plant market. Some species used in Chinese herbal medicine, such as *Dendrobium officinale*, are now very rare in the wild.

Commercial collecting seldom benefits local communities or the countries where the orchids are native. The villagers who are the main collectors get very little reward for their work. The most immediate profit is made by the middlemen: often nurserymen or dealers. The country of origin continues to suffer loss of potential revenue many years after the original plant was removed. Many countries are understandably aggrieved at such losses to their economies. The Convention on Biological Diversity (CBD) attempts to ascribe benefit-sharing as a key component of the international trade in species (Cribb *et al.*, 2003).

2.8.3 Environment changing

Today, global warming may also reduce available orchid habitat, as has been postulated for *Calypso bulbosa* in North America (Keenan, 1998). The increasing frequency of El Niño has greatly reduced orchid population in the Malay Archipelago. The populations seem to be disappeared due to drought. The issue of the Intergovernmental Panel on Climate Change (IPCC) informed that approximately 20 - 30% of plant and animals species are likely to be at increased risk of extinction if increases in global average temperature exceed 1.5 - 2.5 °C (Parry *et al.*, 2007).

Judging from preliminary climate model in Thailand found that some conserve area seem to be higher temperature (Boonpragob and Santisirisomboon, 1996). However, there is no information of the impact to natural orchid population in relation to climate change, which is quite important for conservation point of view.

2.9 Orchid conservation

Many orchid species are threatened by mainly human activities such as habitat destruction, degradation, fragmentation and over-collecting throughout their range. Thus it needs to manage to conserve the natural population, with requiring the orchid conservation strategy plans. To ensure that strategies will be the most effective for provided orchid conservation it needs the basic information. Knowledge of the causal factors of orchid rarity to whether research outcomes and management plans are being converted to successes in the orchid conservation strategies (Koopowitz *et al.*, 2003; Dixon and Phillips, 2007). There is currently being recommended as a conservation strategy to help preserve the biological and genetics diversity (Primack, 2000). In general, genetic and species diversity can be maintained as *in situ* and *ex situ* conservation. However, both combination of these practices and participation of communities shall be more effective.

2.9.1 *In situ* conservation

The best way for the long-term protection of biological diversity is the preservation of natural communities and populations in the wild, known as *in situ* or on-site preservation (Primack, 2000). *In situ* conservation is not conserves only a variety's genetic diversity but also the evolutionary interactions. Orchid conservation *in situ* and habitat preservation are the first line of defense for safeguarding orchid species for the future. Orchids currently appear to be in the midst of an active evolutionary radiation, with the production of large numbers of new species. One of the goals of a preserve should be to allow these processes to continue naturally. As soon as man starts to interfere with the natural evolutionary processes, they lose their integrity and are tainted by aspects of artificial selection (Koopowitz, 2001).

The number of nature reserves and conservation areas that have been established specifically for orchids demonstrates their powerful role in habitat conservation (Cribb *et al.*, 2003). There are many strategies for management of orchid conservation such as management plan, manipulating population, reintroductions or recovery plan or even artificial pollination. However, for some species, active interference and management may be the only way that the plants can be saved from extinction (Koopowitz, 2001).

2.9.2 *Ex situ* conservation

Ex situ conservation is the conservation of plants outside their natural habitats. This may include activities such as growing the plants in pots or garden beds, or storing seed in a seed bank. However, Hamilton (1994) argued that *ex situ* collections may be ineffective at preserving genetic diversity and the evolutionary potential of populations for adaptive or neutral evolution. The collection of genetic variation for seed banks as a problem in efficient sampling of genetic polymorphism is a limited view of the types and organization of genetic variation present in wild plant species. Furthermore, *ex situ* conservation is still difficult for terrestrial orchids than for other plant species due to the manner by the seeds germination (Primack, 2000).

Ex situ preservation outside natural environments is often envisioned as maintaining rare plants in arboretums, botanical gardens, wildlife preserves, etc. These have been a long-term mission of many conservation organizations (Rivero, 2001; Whitlow, 2001). However, such

organizations, whose commitment with the change of administration or by financial crises have not been developed. Then many challenges the value of private collections has the advantage of being curetted by collectors at no cost to the public. Furthermore, the goal of individual collectors are not financial, they focus on species which are often not found in general cultivation, and which are usually considered difficult to grow and to propagate. It is under the hand of such individuals that many of the 'significant' species, when extinct in the wild may be found. There is too often a rift between the 'expert' in institutions with good 'book' knowledge and the collector with practical knowledge. What we need is a multilateral approach to *ex situ* conservation (Whitlow, 2001).

In addition, some of the habitat where orchids grow naturally is being converted to farms or plantations by simply cutting and burning forests. Orchids live in the trees that are being cut and burned. They are being destroyed by the thousands this way at an unbelievable rate around the world. Although the important goal of *ex situ* conservation is to make rare and new plants immediately available to the propagators, so that they can produce large numbers of *in vitro* propagated plants as soon as possible (Withlow, 2001). In Western Australia, many rare flora are now in the *ex situ* collection maintained by Kings Park and Botanic Garden. The macro-and micropropagation procedures are used including conventional cutting and seed propagation and in the *in vitro* programs whole seeds (asymbiotic and symbiotic germination), excised seed embryos, shoot apices and inflorescence sections. Results of these programs will guide future efforts in conserving and recovering rare Australian species including strategy conservation (Dixon, 1994). Finally, the education is the most valuable and effective tool for conservation of native species.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

Observations were made during the period from June 2006 to June 2008 at two separated sites. The first site was natural habitats of *S. mirabilis* and *S. monophylla* on Doi Hua Mot, Umphang Wildlife Sanctuary. This site consists of limestone cliffs, scrub forest and grassy slopes in the deciduous forest zone (800 – 1,000 m altitude). *S. mirabilis* primarily grows on limestone crevices, whereas *S. monophylla* are mainly found on open grassy slopes and in scrub forest with the canopy reaching 10 m or less.

S. pulchella was studied at the second site in the southwestern part of Doi Chiang Dao (i.e. on the ridge called Doi Luang), Chiang Dao Wildlife Sanctuary. This site consists of exposed limestone cliffs in the upper montane scrub zone (1,800 – 2,225 m altitude).

Plots 50 × 50 m^2 were demarcated for studying each species in details. Due to the particularly scattered occurrence of *S. monophylla*, we included three different plots for this species, for the sake of convenience; they are referred to as one plot.

3.2 Soil property analysis

3.2.1 Soil collection

Soil samples were collected from the top 10 cm depth in each study plot. Then, they were stored in plastic bags, transported to the laboratory and stored at 4 ^{0}C until further analysis.

3.2.2 Soil preparation

Approximately 100 g of each soil sample was air-dried by spreading on a tray and kept in a dry and dust free place at room temperature for 3 days. The samples were considered to be dry if the weight of soil between the third and fourth day did not change more than 5%. The dried samples were sieved through a 2 mm pore size sieve. The samples left on the sieve were crushed by an agate mortar until the particle sizes of the samples were less than 2 mm. The sieved samples were mixed for 30 minutes in a 1,500 ml beaker with spatula and then poured onto a clean plastic tray in a cone shape. The cone was flatten and formed a circular layer of soil. This layer was divided into four even parts by clean spatula and discarded the two opposite quarters. The two remained parts were combined. The coning and quartering were repeated until 10 g of soil samples were obtained.

3.2.3 Soil analysis

The soil pH was defined as the activity of the hydronium ion in the soil samples by using a pH meter. The organic matter in soil was analyzed according to the protocol modified from Walkley-Black method (1934). Total nitrogen was analysed by Kjeldahl's method (Pansu and Gautheyrou, 2006). The soil available phosphorous was analyzed by soil survey standard test method (Bray No. 2) modified from Bray and Kurtz method (1945). The soil available potassium, calcium and magnesium were analyzed by Atomic Absorption Spectroscopy method (AAS). A one-way ANOVA was performed to determine the significant differences between soil samples followed by Duncan test.

3.3 Morphological size correlation and population structure

All *Sirindhornia* individuals that were visible above ground in the study plots were counted in 2006 (*S. mirabilis*, *S. monophylla*) and 2007 (*S. pulchella*). In all detected individuals of these species, the length of the longest leaf and the length (from apex to lowermost flower node) of the inflorescences (if any) were measured. The numbers of flowers in each inflorescence were recorded. The correlations between leaf length on the one hand and inflorescence length and

number of flowers per inflorescence on the other were tested with the Pearson's correlation coefficient.

The length of the longest leaf was used as a general indicator of the plant size - as supported by the positive correlations that found between leaf length and inflorescence length (in all three species) and between leaf length and number of flowers per inflorescence (in *S. mirabilis* and *S. monophylla* only). Assuming a positive correlation between the size and age of individuals within each species, all individuals were arbitrarily defined species-specific size classes to reveal the current demographic structure of each population sample. In order to assess the proportion of flowering individuals in each size class, every individual as vegetative or flowering were classified.

3.4 Flowering and flowering phenology

All flowering individuals consistently one inflorescence only and the number of flowers per inflorescence were obtained from each study plot. The number of individuals blooming in each plot were calculated the proportion of flowering individuals in that year.

For the flowering phenology was established from weekly observation of 31 inflorescences of *S. mirabilis* (in 2006), 24 of *S. monophylla* (in 2006) and 29 of *S. pulchella* (in 2008) during the study period. In each inflorescence (always one per individual), the numbers of floral buds, fresh flowers and capsules plus withered flowers ten weeks (*S. mirabilis*) and six weeks (*S. monophylla, S. pulchella*) were counted.

3.5 Breeding system experiments

In 2006, six plants of *S. mirabilis* and four plants of *S. monophylla* with altogether 179 and 56 flower buds, respectively from their natural habitat were transplanted to a site nearby the resort of Umphang and covered with fine-meshed nylon net. Additionally, 25 individuals of *S. pulchella* with altogether 124 flower buds, remaining in their natural habitat on Doi Chiang Dao,

were covered with nylon net in 2007. To test for autogamy through spontaneous self-pollination (and for apomixis) in the caged individuals, 96 flowers of *S. mirabilis*, 30 flowers of *S. monophylla*, and 72 flowers of *S. pulchella* were left untouched. To test for self-compatibility, 83 flowers of *S. mirabilis*, 26 flower of *S. monophylla* and 52 flowers of *S. pulchella* were manually self-pollinated.

3.6 Male reproductive success

To determine the male reproductive success in the population, 20 and 13 inflorescences of *S. mirabilis* and *S. monophylla*, respectively (a subset of those examined for flowering phenology) were checked for pollinarium removal in 2006. The total number of flowers on each inflorescence were recorded together with the number of flowers from which the pollinarium was removed (no flowers were observed in which only one hemipollinarium was removed). A t-test was performed using the program SigmaStat to test for difference in pollinarium removal between *S. mirabilis* and *S. monophylla*. The SPSS program was used to calculate Pearson's correlation coefficient to test for correlation between the number of pollinaria removed and the number of flowers per inflorescence.

The patterns of pollinarium removal within inflorescences by comparing removal rates between the basal, middle and apical parts of each inflorescence were studied, generally using the same sequence of statistical tests as indicated patterns of fruit set. However, as the data set for *S. mirabilis* passed the normality test, a one-way ANOVA (rather than a one-way ANOVA on ranks) was applied for this species.

Mann-Whitney rank sum tests (as the data sets failed the normality test) were performed using the SigmaStat program in order to test for differences between relative fruit set and proportion of pollinaria removed in *S. mirabilis* and *S. monophylla*.

3.7 Female reproductive success

The number of inflorescences and the number of flowers per inflorescence in each study plot in 2006, 2007 (*S. pulchella* only) and 2008 were counted. At the end of flowering each year, in order to determine the female reproductive success in each inflorescence, the fruit setting was assessed. Using the program SigmaStat network version 1.01, we performed a one-way ANOVA on ranks (as the data failed the normality test) to test for differences in relative fruit setting success (pooled over the entire study period) between the three species. Subsequently, Dunn's test was used to mutually different in this respect. Using the program SPSS for Windows 16, Pearson's correlation coefficient test for correlation between fruit set and number of flowers per inflorescence were calculated.

To study the internal patterns of capsule production in inflorescences of *S. mirabilis* (N = 14), *S. monophylla* (N = 11) and *S. pulchella* (N = 17) in 2008, a basal, a middle, and an apical part were defined in each inflorescence (each part comprising the same percentage of flowers), and the fruit set was characterized accordingly. The program SigmaStat were performed a one-way ANOVA on ranks (as all data sets failed the normality test) to test for differences in relative fruit set between the three inflorescence parts in each species. Whenever a statistically significant difference was found, Student-Newman-Keul's multiple comparison method was used to reveal which parts of the inflorescence that mutually different in this respect.

Furthermore, at the end of flowering each year (2006 - 2008), Lorenz curves (Weiner & Solbrig, 1984, Calvo, 1990) were constructed for each population sample to visualize the relative annual contributions of flowering individuals to the capsule pool. Individuals were sorted in ascending order by the number of capsules they produced, and the cumulative percent of capsules was plotted against that of individuals. A diagonal line from the lower left to the upper right corner of the diagram would indicate equal individual contributions, whereas curves deviating from this diagonal line would indicate inequality.

3.8 Seed productivity

Seeds from dehisced capsules of *S. mirabilis*, *S. monophylla* and *S. pulchella* (30 capsules each) were collected and dried in silica gel. To complete the observation, this study was carried out in the study site of *Sirindhornia* species as the same time with the pollination experiment. The survey of seed productivity of *S. mirabilis* and *S. monophylla* was carried out in 2006. For the seed productivity of *S. pulchella* was collected in 2007. The number of seeds per capsule was assessed by counting under a low-power binocular microscope. For each species and study year, the mean number of seeds per inflorescence was calculated as the product of (1) the mean number of seeds per capsule and (2) the mean number of capsules per inflorescence. Furthermore, the number of seeds produced per individual (flowering or vegetative) in the population sample of each species was estimated (for the year with known proportion of flowering individuals) as the product of (1) the mean number of seeds per inflorescence and (2) the proportion of flowering individuals. Provided that this rate (as well as the population size) was sufficiently stable over several years, it can be used to measure the efficiency of recruitment: the fewer seeds produced per individual (i.e. the fewer seeds required for recruitment of one new adult individual), the higher efficiency.

3.9 Pollinarium movements

In most genera under subtribe Orchidinae, pollinia just extracted from the flower are positioned on the insect so as to strike the anther rather than the stigma of the same or another flower. However, in 30 – 80 seconds (depending on the species), differential drying of the caudicles will result in bending or twisting that changes the orientation of the pollinia so that they will strike the stigma when the pollinator later visits another flower – an adaptation that increases the rate of allogamy to auto- or geitonogamy (Darwin, 1862). To test for this adaptation in *Sirindhornia* spp., three pollinaria from each species were experimentally extracted and observed for subsequent movements during the next 10 minutes.

3.10 Pollinator observation of *S. mirabilis*

Only pollinators of *S. mirabilis* were assessed by observing pollinator behavior varies time from 7.00 am to 6.00 pm local time (GMT+7 hr). The observation stopped when raining. Flower watching session was carried out for a total 157 man-hours.

3.11 Pollen and seed morphology of *Sirindhornia*

Dried mature pollen from fresh flower and seeds from dehisced capsules were collected and placed in silica gel bags. Then, they were studied by scanning electron microscopy (SEM), after the previous coating of the samples with gold (1450 VP/LEO electron microscope).

3.12 Orchid mycorrhiza

3.12.1 Collection of root material and isolation of DNA

Without damaging orchid individual, the fresh roots of the adult plants of *S. mirabilis, S. monophylla* and *S. pulchella* were collected from their natural habitats. Then, they were washed by water to eliminate all soil particles, cleaned with 70 % of ethanol and kept in silica gel. DNA extraction was performed by using DNeasy Plant Mini Kit (Qiagen, Germany).

3.12.2 Molecular investigations

Total DNA was recovered in 40 µl deionized distilled water and kept at –80 °C. PCR amplification of fungal internal transcribed spacer (ITS) of ribosomal DNA was performed as described by Selosse *et al.* (2002).

The fungal ITS sequence (encompassing the ITS1, 5.8S and ITS2 sequences) was amplify using two sets of primers: ITS1F + ITS4 and ITS1F + ITS4B (Gardes and Bruns, 1993). PCR was carried out in a 50 µL, reaction with final concentrations of 66 µM for each dNTP, 0.6

μM for each of the primers (Invitrogen, USA), 10 mM TrisHCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 $mgmL^{-1}$ gelatin, 0.1% (v/v) Triton X100, 5% (v/v) dimethyl sulfoxide and 1.5 units of Taq DNA polymerase (Invitrogen, USA) and 2.5 μL of the DNA samples. The reactions were performed in a TRIO-Thermoblock (Biometra, Germany) under the following thermoprofile: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 second, annealing at 55 °C for 30 second and extension at 72 °C for 30 second. 2.0 μL of the products were separated on 1.5% agarose gel in 0.5X Tris–borate–EDTA (TBE) buffer to ensure the success of the amplification.

To validate the uniqueness of fugal by ITS amplification, longer rDNA stretches were amplified from orchids roots from cores A1-01, N1-01, L1-01, L6-07 and P1-01, using ITS1F with backward primers located at various positions within the 28S sequence, namely starting from its 5' end: LR21 (5'-CTTCAAGCGTTTCCCTTT-3'), C2 (5'-GAACTCTCTCTTCAAAGTTCT TTTC-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Selosse *et al.*, 2002; O'Donnell, 1993). Sequencing and sequence editing was performed as in Roy *et al.* (2009) and corrected sequences (or consensus sequences for similar clones) will be deposited in GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov).

3.12.3 Fungal identification

In order to identify fungi, searching for similar fungal sequences was conducted by Blast against GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). The sequences in Genbank were downloaded and aligned together with our using ClustalW (Roy *et al.*, 2009; Thompson *et al.*, 1994) and then corrected by manually.

3.13 Seed germination in the field

3.13.1 Seed collection

Seed germination in field of *S. mirabilis* and *S. monophylla* was observed in natural habitat at Umphang Wildlife Sanctuary, Tak province, Thailand. Mature capsules were collected in early of August 2006. The seeds of each capsule were thoroughly mixed before used.

3.13.2 Sowing experiment *in situ*

The seeds were sown in 40x60 mm rectangles of plankton net and constructed from 2x23x36 mm plastic slide mounts. The pore size of plankton nets were 35 μm to retain the seeds while allowing minor soil organisms, bacteria, and fungal hyphae to pass through. Average 200 seeds (50 – 300 seeds) were transferred onto the net that was folded once and affixed in the slide mount. The seed packets were placed approximately 6 - 10 cm depth and away from mature plants up to 1 m. Sets of 40 packets for each species were buried in August 2006. After 1.5, 3, 6, 9, 12 and 18 months, seed packets were took to investigate seed viability and % seed germination.

Seed viability, germination and seedling development were assessed under a microscope. Developmental growth stages were scored as Zettler and Hofer (1998) (Figure 3.1). Seed germination percentages were based on number of developed seeds, i.e. those containing distinct, rounded and hyaline embryos, in relation of total seeds in a packet. The number of packets which collected for checking seed germination was varies between 5-13 packets.

3.13.3 Viability testing

The seeds in plankton net packet were collected. Then, they rinsed with tap water and consequently soaked in triphenol tetrazolium chloride (TTC) solution (1 g in 100 ml phosphate buffer, pH 6.5-7.0) for 48 hours in darkness at room temperature. TTC solution was removed by rinsed with five times in sterile distilled water. Seeds were mounted in wet condition on slide before studied under stereo microscope. The number of viable seeds was assessed. Embryos with completely pink to red colored were considered viable, whilst seeds with embryos partially colored, white, yellow or brown were assumed not viable (Huynh and Coates, 1999).

3.14 *In vitro* seed germination

3.14.1 General preparation of capsule for various media

Mature capsules of *S. mirabilis, S. monophylla* and *S. pulchella* were collected from their natural habitats, placed in small paper envelopes and allowed them to dry at room temperature. The capsules were treated with 5 % sodium hypochlorite for 10 minutes and washed thoroughly 3 times in sterile distilled water. Seeds were removed from the capsules under aseptically conditions and placed on various seed germination media which are Vacin & Went Modified Orchid Medium, Murashige and Skoog medium (MS), ½ MS, ¼ MS, and Knudson C.

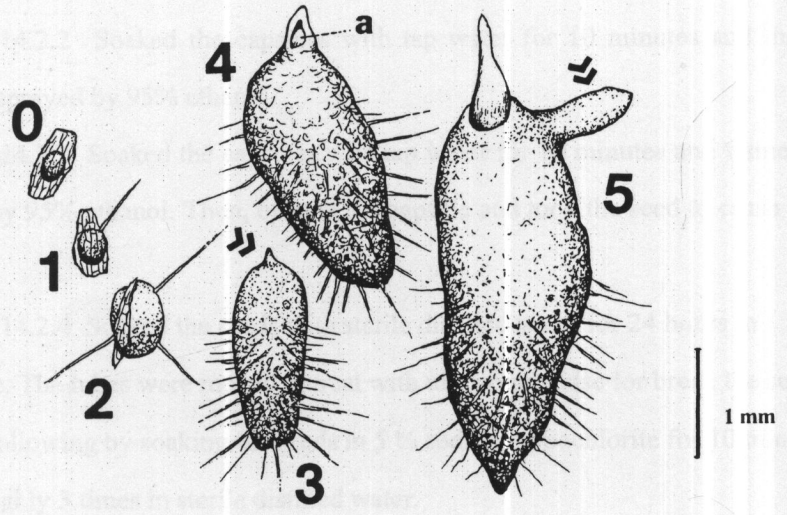

Figure 3.1 Growth stages used to determine germination and development of *Sirindhornia.* Stage: 0, no germination; 1, production of rhizoids (germination); 2, rupture of the testa by enlarged embryo; 3, appearance of promeristem, denoted by arrow; 4, appearance of first true leaf (a); and 5, elongation of true leaf and formation of branch root, denoted by arrow (Zettler and Hofer, 1998).

The media pH was adjusted to 5.8-6.0 before gelling with 8 gL^{-1} agar. Twenty milliliters of the media were dispensed into 8 onz glass culture bottle (4 cm high x 8 cm diameter) and autoclaved at 121 ^{0}C for 20 minutes. All of the cultures were incubated at 25 $\pm$2 ^{0}C under cool white fluorescence tubes providing 40 µmol $m^{-2}S^{-1}$ with 16- h photoperiod. A randomized complete block design was used for the experiments, with 20 replicates (culture bottles).

3.14.2 Effects of different surface sterile methods

To gain the most efficient sterilized preparation before sowing seed on the media 5 different methods were conducted with the same culture media,Vacin & Went modified orchid medium agar, which are;

3.14.2.1 Soaked the capsules with 5 % sodium hypochlorite for 10 minutes and then washed thoroughly 3 times in sterile distilled water.

3.14.2.2 Soaked the capsules with tap water for 10 minutes and then flamed the capsules after sprayed by 95% ethanol.

3.14.2.3 Soaked the capsules with tap water for 10 minutes and flamed the capsules after sprayed by 95% ethanol. Then, opened the capsule and took the seed to crush with sterilized sand.

3.14.2.4 Soaked the seeds with sterile distilled water for 24 hours in 1.5 ml micro centrifuge tube. The tubes were moved to treat with ultrasonic pulse for break the seeds testa for 10 minutes. Following by soaking the seeds in 5 % sodium hypochlorite for 10 minutes, then washed thoroughly 3 times in sterile distilled water.

3.14.2.5 Kept the capsule at -20^{0}C for 2 weeks before disinfected with 5 % sodium hypochlorite for 10 minutes and then washed thoroughly 3 times in sterile distilled water.

3.14.3 Effects of light and dark incubations

Twenty replicates (culture bottles) were covered with aluminum foil to keep them in the dark condition and another 20 replicates were not covered by using Vacin & Went Modified Orchid Medium (VW) as media with capsule preparation as the general method (see 3.14.1). A randomized complete block design was used for the experiments.

3.14.4 Effect of hormones

In addition to test the effect of hormones Auxin and Cykinin, Vacin & Went modified orchid medium with capsule preparation as the general method (see 3.14.1) were supplemented with different levels of indole-3-acetic acid; IAA (0.5, 1.0, 2.0 and 5.0 ppm), N_6 – benzyladenine; BA, (0.5, 1.0, 2.0 and 5.0 ppm) either alone or combination. For the effect of gibberellic acid (GA), 1 ppm of GA was added in Vacin & Went modified orchid medium, Murashige and Skoog medium (MS), 1/2MS, 1/4MS strength and Knudson C.

3.14.5 Effects of capsule ages

The flowers of *S. mirabilis* and *S. monophylla* were hand-pollinated under glasshouse conditions at Mae Fah Luang University. The capsules were collected at 4, 5, 6, 7, 8 and 10 weeks after the pollination. The seeds derived from different capsule ages were cultured in the same media, Vacin & Went modified orchid medium with capsule preparation as the general method (see 3.14.1). A randomized complete block design was used for the experiments, with 20 replicates (culture bottles).

3.15 Genetic diversity of the genus *Sirindhornia*

3.15.1 Plant material and isolation of genomic DNA

From intensive survey, it can be defined as 2 subpopulations for each species. The distance between subpopulations of *S. mirabilis*, *S. monophylla* and *S. pulchella* are 7 km, 15 km and 4 km, respectively.

All plant materials were collected from each habitat of *Sirindhornia* populations. Fresh young leaves were cut off and kept in an ice box. The samples are comprised 40 samples from 2 subpopulations in each species. The DNA extraction was followed by DNA extraction kits method (Qiagen, Germany). The AFLP analysis was performed as AFLP® Core Reagent Kit and an AFLP® Primer Kit (Invitrogen, USA).

3.15.2 Electrophoresis and visualization

For silver staining, the long glass plate was pre-treated with Silane® A-174 (Sigma, USA) in order to bind the gel to the glass plate for easy handling. For this procedure, the long plate was well washed, dried and coated with a 1 ml ethanol solution containing 0.5% acetic acid and 3.5 µl silane. This mixture was applied with a lint-free tissue and left to dry for 5 min. The resulting white film was removed with 3 to 4 rinses in 100% ethanol and by wiping. 0.4 mm gels were pre-run at 60 W for 20-30 min in 1x TBE.

Before loading, an equal volume (5 µl) of formamide dye (98% formamide, 10 mM EDTA, 1 $mgmL^{-1}$ of each bromophenol blue and xylene cyanol) was added to the reaction mixture. The reaction was heated for 5 min at 95°C and immediately placed on ice. Then, 6 µl of denatured selectively amplified DNA was loaded and the gel was run at 300 volt for 6 hours. The 25 kb ladder was used as a size marker. For non-radioactive visualization, it used the improved photochemical derived procedures for protein silver staining proposed by Bassam *et al.* (1991) with some modification. All procedures were performed at room temperature. Constant agitation of the bath during all the staining steps is essential. The gel bound to the long plate was fixed in a stop solution, (10% acetic acid) for 20 min then rinsed 3 times in deionised water.

The staining was performed for 30 min in the coloring solution: $AgNO_3$ (1 gL^{-1}) (Merck, Germany), 1.5 mL 37% formaldehyde. The plate was then removed, vertically drained, rinsed 8 second in deionised water and immediately submerged into the developing bath. In case of a delay exceeding 20 second between rinse and developer, the coloring step was repeated. The developer consisted of sodium carbonate, formaldehyde and thiosulfate (30 g Na_2CO_3/L, 1.5 mL 37% formaldehyde, 2 mg $Na_2S_2O_3.5H_2O$/L). The sodium carbonate solution was prepared and added just before use to the formaldehyde and thiosulfate. This increased the time to achieve proper staining, allowing repeatable image development. When the optimal staining was reached (5 to 10 min), the reaction was stopped with the same stop solution used in the first step (5 to 10 min until stopped). The gel was rinsed twice in water over 10 min and left to dry vertically overnight. The dried gel was either preserved at room temperature, a copy made with scanned using the Adobe Photoshop™ CS3 program (Adobe Systems, USA).

3.15.3 Data Analysis

AFLP fragments were visually scored as present (1) or absent (0) to create the binary data set. The data were entered into a binary data matrix as discrete variables. The resulting binary data matrix was first analyzed using POPGENE v.1.31 (32-bit) (Yeh *et al.*, 1999), assuming Hardy-Weinberg equilibrium. The following parameter were used to estimate genetic diversity at a subpopulation level: the percentage of polymorphic fragment (*P*%), the genetic diversity (H_E), and Shannon's information index of diversity (I_S). Genetic parameters (*P*, H_E and *I*s) were also calculated at the species level. Nei's unbiased genetic identity (I) between populations and genetic differentiation among the populations (G_{ST}) was estimated by Nei's gene diversity statistics (Nei, 1973) as well as the effective number of migrants per generation ($Nm = 0.5(1-G_{ST})/G_{ST}$), which is an indirect estimate of gene flow between the two populations. A UPGMA clustering of all the individuals, based on a genetic similarity index calculated with Dice's coefficient, was constructed using NTSYS-pc software (Rohlf, 1997) to show the relationships of the populations.

CHAPTER 4

RESULTS

4.1 Soil property analysis

The chemical properties such as soil pH, organic matter, total nitrogen (N), available phosphorus (P), available potassium (K), available calcium (Ca) and available magnesium (Mg) were studied to determined characteristic of soil from the study sites. The results revealed no significant different all soil study sites in pH, organic matter, total N, available K and available Mg. The different of properties in all soil sites were available P and available Ca (Table 4.1). Available P of soil from *S. mirabilis*'s sites was the highest, while available Ca of soil is the lowest.

4.2 Morphological size correlation and population structure

The study of all individuals visible above ground in the study plots revealed 105 individuals of *S. mirabilis*, 120 of *S. monophylla* (both counted in 2006) and 274 of *S. pulchella* (counted in 2007). Inflorescence length was positively correlated with leaf length in *S. mirabilis* ($r = 0.62$; $P < 0.01$), *S. monophylla* ($r = 0.75$; $P < 0.01$), and *S. pulchella* ($r = 0.67$; $P < 0.01$). The number of flowers per inflorescence was positively correlated with leaf length in *S. mirabilis* ($r = 0.49$; $P < 0.01$) and *S. monophylla* ($r = 0.59$; $P < 0.01$), but not in *S. pulchella*.

For each study species, the relative distribution of individuals among size classes and the proportion of flowering individuals in each size class are shown in table 4.2 and figure 4.1.

Distribution among size classes was rather uniform for all three species. In *S. mirabilis* (Figure 4.1 A), distribution of plants among size classes resembled a normal distribution, whereas the distribution was skewed to the left in *S. monophylla* (Figure 4.1 B) and particularly in *S. pulchella* (Figure 4.1 C). *S. monophylla* had very low numbers in size class 1 (Figure 4.1 B).

Table 4.1 Characteristic of soil from study site of *Sirindhornia* species. All values are given as mean ± SD.

Properties	*S. mirabilis*	*S. monophylla*	*S. pulchella*
pH	6.81(±0.15)	6.33(±0.57)	6.79(±0.36)
Organic matter ($g.kg^{-1}$)	68.00(±14.99)	52.07(±13.23)	55.05(±3.32)
Total N ($g.kg^{-1}$)	3.67(±0.46)	3.12(±0.28)	3.10(±0.00)
Available P ($mg.kg^{-1}$)	53.67(±5.51)a	46.17(±3.81)ab	38.5(±0.70)b
Available K ($mg.kg^{-1}$)	252.33(±129.16)	185.83(±112.07)	203.0(±168.29)
Available Ca ($mg.kg^{-1}$)	3,025.33(±610.03)b	4,442.66(±326.23)a	4,201.5(±132.22)a
Available Mg ($mg.kg^{-1}$)	905.67(±352.20)	897.50(±78.90)	859.00(±5.65)

The smallest flowering plants of *S. mirabilis* had a leaf length of 8.5 cm, whereas *S. monophylla* and *S. pulchella* could start flowering when the leaf length exceeded 3.5 cm and 5.0 cm, respectively. The proportion of flowering plants generally increased with plant size in each species, although this trend was not completely consistent.

Table 4.2 Definitions of species-specific size classes for *S. mirabilis*, *S. monophylla* and *S. pulchella*, using length of the longest leaf (cm) as general indicator of plant size.

Size class	*S. mirabilis*	*S. monophylla*	*S. pulchella*
1	0.1–5.0	0.1–2.0	0.1–3.0
2	5.1–10.0	2.1–4.0	3.1–6.0
3	10.1–15.0	4.1–6.0	6.1–9.0
4	15.1–20.0	6.1–8.0	9.1–12.0
5	20.1–25.0	8.1–10.0	12.1–15.0
6	25.1–30.0	10.1–12.0	15.1–18.0
7	30.1–35.0	12.1–14.0	18.1–21.0
8	35.1–40.0	14.1–16.0	21.1–24.0

4.3 Flowering and flowering phenology

Thirty-six individuals of *S. mirabilis* in the study plot bloomed in 2006 (corresponding to 34.3 flowering), whereas 25 individuals (20.8% flowering) of *S. monophylla* in 2006 were bloom. All flowering *Sirindhornia* individuals consistently produced one inflorescence only. In all three species, flowering started from the proximal part of the inflorescence (for surveys of overall progression in flowering) (Table 4.3 - 4.5; Figure 4.2 - 4.4). The longevity of individual flowers was approximately six weeks in *S. mirabilis* and three weeks in *S. monophylla* and *S. pulchella.* However, it could be observed in all three species that the flowers would wither in less than one week if they were pollinated.

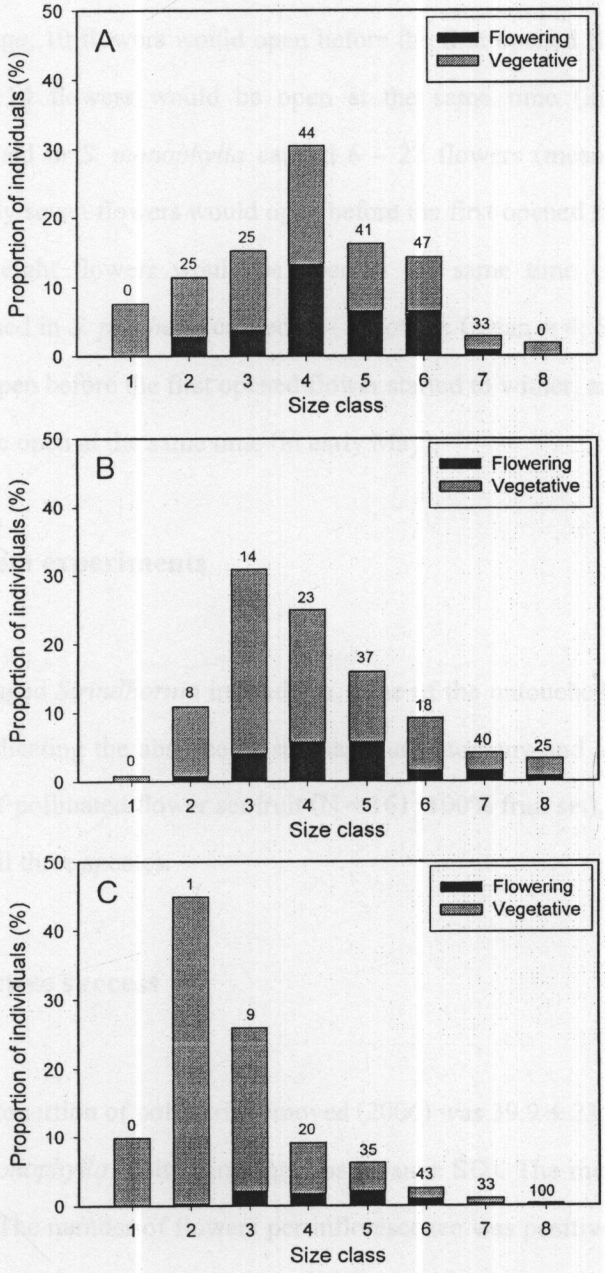

Figure 4.1 Distribution of flowering and vegetative individuals among size classes in the study plots for A: *S. mirabilis* (2006), B: *S. monophylla* (2006) and C: *S. pulchella* (2007). Numbers on top of columns indicate the proportion of flowering individuals within each size class (%).

Thirty-one inflorescences examined in *S. mirabilis* carried 8 – 48 flowers (mean = 23; SD = 10.9). On average, 10 flowers would open before the first opened flower started to wither, and a maximum of 14 flowers would be open at the same time (in early May). The 24 inflorescences examined in *S. monophylla* carried 6 – 27 flowers (mean = 15; SD = 6.1). On average, approximately seven flowers would open before the first opened flower started to wither, and a maximum of eight flowers would be open at the same time (in late May). The 29 inflorescences examined in *S. pulchella* carried 2 – 9 flowers (mean = 4; SD = 1.9). On average, three flowers would open before the first opened flower started to wither, and a maximum of three flowers would be open at the same time (in early May).

4.4 Breeding System experiments

Among the caged *Sirindhornia* individuals, none of the untouched flowers set fruit (N = 198; 0% fruit set), indicating the absence of spontaneous autogamy and apomixis. On the other hand, all manually self-pollinated flower set fruit (N = 161; 100% fruit set), indicating genetic self-compatibility in all three species.

4.5 Male reproductive success

The overall proportion of pollinaria removed (2006) was 39.9 ± 23.1% in *S. mirabilis* and 91.2 ± 10.4% in *S. monophylla* (values indicated as mean ± SD). The means were significantly different ($P < 0.001$). The number of flowers per inflorescence was positively correlated with the number of pollinaria removed in both and *S. mirabilis* ($r = 0.67$; $P < 0.01$) (Figure 4.5) and *S. monophylla* ($r = 0.96$; $P < 0.01$ (Figure 4.6).

The male reproductive success depended on position of flower in both species examined that is *S. mirabilis* and *S. monophylla* ($P < 0.001$) (Table 4.6). The male reproductive success of *S. mirabilis* was tend to decreasing from basal part to apical part. The position of flower in the inflorescence was significantly different ($P < 0.001$). The male reproductive success of *S.*

monophylla was the most successful at basal part and middle part (100%) but different from the apical part ($P < 0.001$), which slightly decrease.

The progression of flowering and pollinarium removal in relation to progression of flowering in *S. mirabilis* was carried out from April – June 2006. The flower buds were higher than fresh flowers, capsules or withered flowers and pollinaria removed (Table 4.3). The withered flowers were not found. After 10 weeks, the withered flower plus capsules were highest. While the flowers buds did not exist. However, the proportion of pollinaria removed was not changed since 7 weeks of the observation.

Table 4.3 Progression of flowering and pollinarium removal in *S. mirabilis* throughout the study period. All values are given as mean ± SD.

Date	Flower buds	Fresh flowers	Capsules plus withered flowers	Proportion of pollinaria removed (%)
17 April	21.6 ± 11.2	1.1 ± 4.2	0.0 ± 0.0	2.9 ± 12.8
24 April	17.9 ± 10.4	4.6 ± 6.8	0.3 ± 1.3	5.8 ± 16.0
1 May	11.8 ± 10.2	9.8 ± 11.1	1.1 ± 5.1	13.0 ± 19.7
8 May	5.4 ± 7.2	13.9 ± 11.5	3.5 ± 7.6	20.0 ± 21.9
15 May	2.0 ± 3.5	12.7 ± 12.7	8.0 ± 10.3	31.4 ± 22.3
22 May	0.6 ± 1.6	7.5 ± 12.8	14.6 ± 10.9	36.4 ± 26.5
29 May	0.2 ± 0.8	5.2 ± 9.5	17.4 ± 11.0	39.9 ± 23.1
5 June	0.0 ± 0.0	2.3 ± 5.5	20.5 ± 10.0	39.9 ± 23.1
12 June	0.0 ± 0.0	0.4 ± 1.6	22.3 ± 11.8	39.9 ± 23.1
19 June	0.0 ± 0.0	0.2 ± 0.8	22.6 ± 11.5	39.9 ± 23.1

Table 4.4 Progression of flowering and pollinarium removal in *S. monophylla* throughout the study period. All values are given as mean ± SD.

Date	Flower buds	Fresh flowers	Capsules plus withered flowers	Proportion of pollinaria removed (%)
20 May	5.7 ± 4.4	6.4 ± 4.9	2.0 ± 3.2	26.7 ± 30.4
27 May	1.0 ± 2.8	6.7 ± 4.4	6.4 ± 6.8	55.1 ± 27.9
2 June	1.0 ± 2.3	3.1 ± 3.5	10.0 ± 7.8	85.1 ± 17.1
10 June	0.2 ± 0.5	1.0 ± 2.3	12.9 ± 6.6	90.1 ± 11.2
17 June	0.0 ± 0.0	0.6 ± 1.8	13.4 ± 6.1	91.2 ± 10.4
24 June	0.0 ± 0.0	0.1 ± 0.4	14.0 ± 6.0	91.2 ± 10.4

Table 4.5 Phenological progression in *S. pulchella* throughout the study period. All values are given as mean ± SD.

Date	Flower buds	Fresh flowers	Capsules plus withered flowers
27 April	3.2 ± 1.5	0.8 ± 0.9	0.0 ± 0.0
4 May	1.1 ± 1.1	2.9 ± 1.8	0.1 ± 0.4
12 May	0.0 ± 0.2	1.9 ± 1.6	2.1 ± 2.0
18 May	0.0 ± 0.0	0.8 ± 1.2	3.3 ± 2.1
26 May	0.0 ± 0.0	0.1 ± 0.4	4.0 ± 1.6
1 June	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 1.6

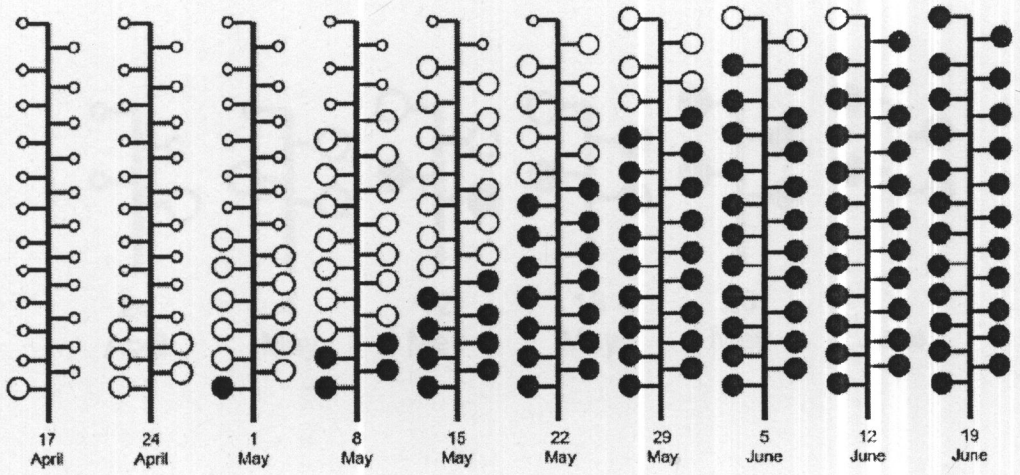

Figure 4.2 Phenological progression in *S. mirabilis* during the periods of observation. Small white circle: bud. Large white circle: fresh flower. Large black circle: capsule or withered flower.

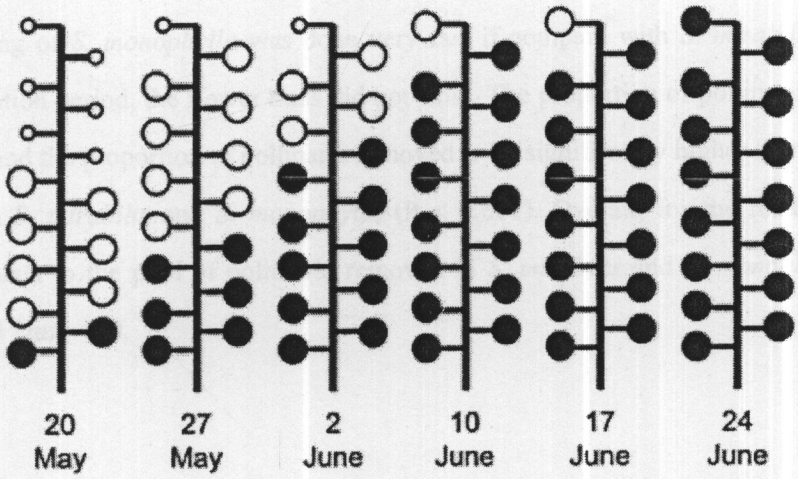

Figure 4.3 Phenological progression in *S. monophylla* during the periods of observation. Small white circle: bud. Large white circle: fresh flower. Large black circle: capsule or withered flower.

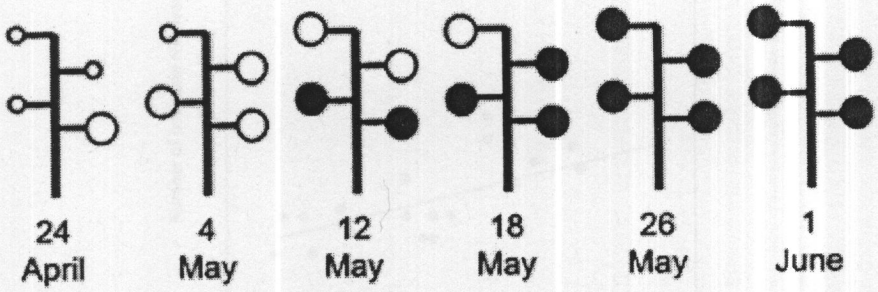

Figure 4.4 Phenological progression in *S. pulchella* during the periods of observation. Small white circle: bud. Large white circle: fresh flower. Large black circle: capsule or withered flower.

The progression of flowering and pollinarium removal in relation to progression of flowering in *S. monophylla* were carried out May – June 2006 (Table 4.4). The flower buds and fresh flowers were high. The capsules or withered flowers and pollinaria removed still less. The flowering of *S. monophylla* was done very fast if compare with *S. mirabilis*. Only 4 weeks of observation period, the flower buds did not exist. The proportion of pollinaria removed was high. It is found the proportion of pollinaria removed to be significantly higher than the relative fruit set in both *S. mirabilis* and *S. monophylla* ($P < 0.001$). Overall, for the relative contributions of individuals to the pool of pollinaria removed in *S. mirabilis* and *S. monophylla* see the Lorenz curve (Figure 4.7).

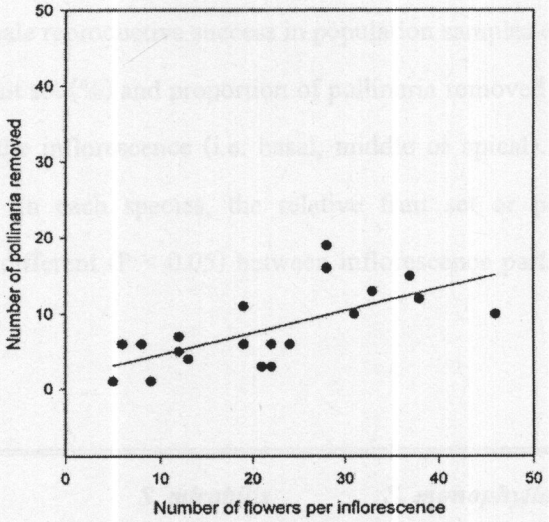

Figure 4.5 Scatter plot with regression line illustrating positive correlation between male reproductive success (measured indirectly as number of pollinaria removed) and the number of flowers per inflorescence in *S. mirabilis*.

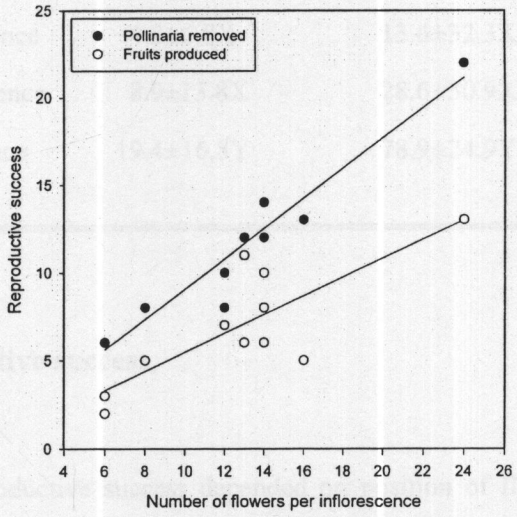

Figure 4.6 Scatter plot with regression lines illustrating positive correlation between numbers of capsules produced and number of pollinaria removed on the one hand and the number of flowers per inflorescence in *S. monophylla* on the other.

Table 4.6 Male and female reproductive success in population samples of *Sirindhornia*: surveys of relative fruit set (%) and proportion of pollinaria removed (%) in relation to flower positions in the inflorescence (i.e. basal, middle or apical). All values are given as mean ± SD. In each species, the relative fruit set or pollinarium removal was significantly different ($P < 0.05$) between inflorescence parts designated by different letters.

	S. mirabilis	*S. monophylla*	*S. pulchella*
Male reproductive success			
Apical part of inflorescence	14.3±19.5X	74.2±31.4X	-
Middle part of inflorescence	33.4±23.2Y	100.0±0.0Y	-
Basal part of inflorescence	60.5±26.7Z	100.0±0.0Y	-
Female reproductive success			
Apical part of inflorescence	4.8±6.8X	13.6±32.3X	0.0±0.0X
Middle part of inflorescence	8.9±13.8X	28.6±30.9X	47.2±46.9Y
Basal part of inflorescence	19.4±16.8Y	78.9±24.9Y	70.4±42.2Y

4.6 Female reproductive success

The female reproductive success depended on position of flower in *S. mirabilis* ($P < 0.05$) as well as in *S. monophylla* and *S. pulchella* (both $P < 0.001$) (Table 4.6) was carried out in 2008. The female reproductive success at basal part of inflorescence in *S. mirabilis* was higher than middle and apical part of inflorescence which not different ($P < 0.05$). This phenomenon was found also in *S. monophylla*. The female reproductive success only in *S. pulchella* that the basal

and middle part of inflorescence was not different ($P < 0.05$). Surprisingly, the female reproductive success at apical part of *S. pulchella* was not found.

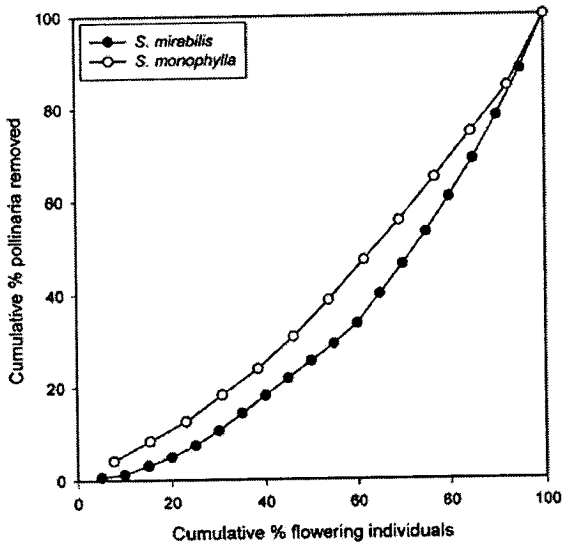

Figure 4.7 Lorenz curves for *S. mirabilis* and *S. monophylla* for each species illustrating the relative contributions of individuals to the pool of pollinaria removed in 2006.

For annual values of overall relative fruit set in each study species was carried out from 2006 – 2008 (Table 4.7). Overall relative fruit set depended on the species ($P < 0.001$) and was significantly different ($P < 0.05$) between *S. monophylla* on the one hand and *S. mirabilis* and *S. pulchella* on the other, but not between *S. mirabilis* and *S. pulchella*. The only fruit set of *S. monophylla* was correlated with the number of flowers per inflorescence ($r = 0.52$; $P < 0.01$).

A Lorenz curve is a graphical tool used to measure balance (inequality) of some variable with respect to some other variable. In this case, the Lorenz curve was used to determine the reproductive system of *Sirindhornia* species if larger inflorescence increased the probability to producing at least one fruit. To evaluate the relative contributions of flowering individuals to the capsule pool of all *Sirindhornia* species in 2006 – 2008, the Lorenz curves was constructed for *S. mirabilis*, *S. monophylla* and *S. pulchella* (Figure 4.8 – 4.10). A Lorenz curve of *S. mirabilis*

(Figure 4.8) and *S. pulchella* (Figure 4.10) showed that the distribution of fruits among flowering individuals were being skewed to the right. Which indicates that *S. mirabilis* and *S. pulchella* were non-autogamous species. This result was confirmed with the study of pollination experiments which concluded that all *Sirindhornia* were absence of spontaneous autogamy and apomixis. In the other hand, a Lorenz curve of *S. monophylla* (Figure 4.9) seem more likely the autogamous species because the distribution of fruits among individuals were skewed to the equality. But information from the pollination experiment was confirmed that *S. monophylla* is non-autogamous species. Although, it is assumed that the inflorescence size did not have any effect on the probability that a plant set at least one fruit in *S. monophylla.*

Table 4.7 Survey of the number of inflorescences in each study plot, together with the overall relative fruit set (indicated as mean ± SD) for each study year.

Species	Year	N	Relative fruit set (%)
S. mirabilis	2006	36	9.6 ± 12.3
	2008	34	4.5 ± 7.2
S. monophylla	2006	25	51.6 ± 31.2
	2008	12	38.0 ± 20.0
S. pulchella	2006	13	17.3 ± 20.8
	2007	23	25.9 ± 31.0
	2008	62	11.4 ± 19.7

Note. number of inflorescences.

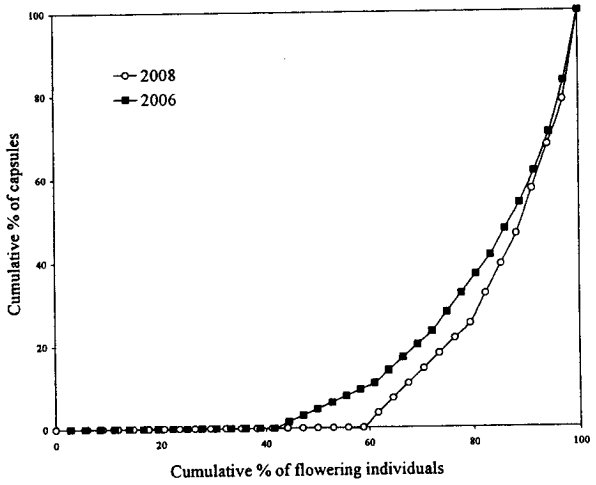

Figure 4.8 Lorenz curves for *S. mirabilis* illustrating the relative contributions of individuals to the capsule pool in 2006 and 2008.

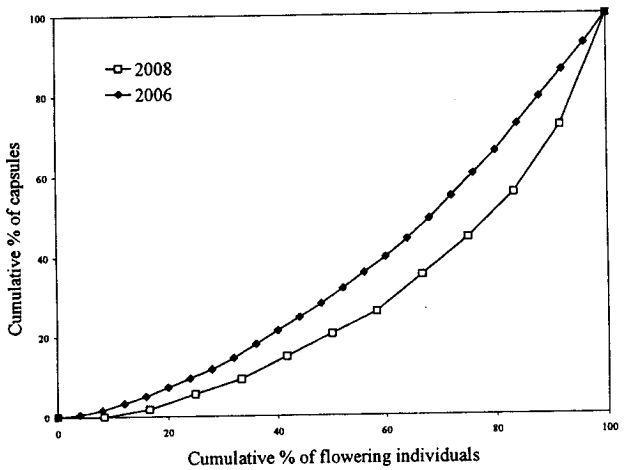

Figure 4.9 Lorenz curves for *S. monophylla* illustrating the relative contributions of individuals to the capsule pool in 2006 and 2008.

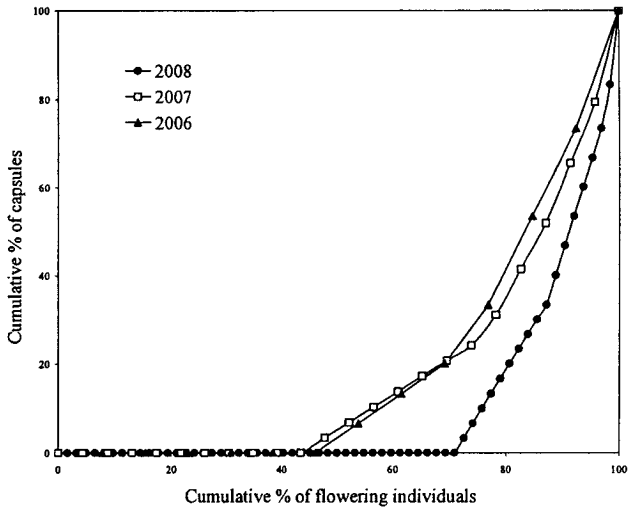

Figure 4.10 Lorenz curves for *S. pulchella* illustrating the relative contributions of individuals to the capsule pool in 2006 and 2008.

4.7 Seed productivity

The number of seeds produced per capsule was 5,911 ± 1,613 in *S. mirabilis*, 1,974 ± 902 in *S. monophylla*, and 3,075 ± 1,134 in *S. pulchella* (indicated as mean ± standard deviation). The mean number of seeds produced per inflorescence each study year is given for each species in table 4.8, together with the number of seeds produced per individual (flowering or vegetative) in each study population in the year with known proportion of flowering individuals.

Table 4.8 Survey of the number of *Sirindhornia* inflorescences in each study plot, together with the overall relative fruit set (%) indicated as mean ± standard deviation, the mean number of capsules per inflorescence and the mean number of seeds per inflorescence for each study year -and the approximate number of seeds produced per individual (flowering or vegetative) in the population for the study year with known proportion of flowering individuals.

Species	Year	N	Relative fruit set (%)	Capsules per inflorescence	Seeds per inflorescence	Proportion of flowering plants	Seeds per individual
S. mirabilis	2006	36	9.6 ± 12.3	1.806	10,675	0.343	3,662
S. monophylla	2006	25	51.6 ± 31.2	7.160	14,134	0.208	2,940
S. pulchella	2007	23	25.9 ± 31.0	1.261	3,878	0.084	326

Note. number of inflorescences.

4.8 Pollinarium movements

No pollinarium bend down or movement could be observed during the experimental periods.

4.9 Pollinator observation of *S. mirabilis*

There were many types of bees (Hymenopteras) found during pollinator observation at the site of *S. mirabilis*. Overall, there were insects in superfamily Apoidea such as Family Apidae,

Halictidae, Andrenidae and Xylocopinae. Most of the visitors were *Trigona collina*, *T. apicalis*, *T. terminata*, *T. leaviceps*, *T. forgdeniformi*, *Braunsapis hewitti*, *Auregilla* spp. (Apidae), sweat bees *Pachyhalictus burmanus* (Halictidae), including hoverflies, *Eumerus* spp. (Calliphoridae), *Isomyia* spp. (Calliphoridae), and various unidentified beetles.

Until now, only small carpenter bee (*Ceratina* sp.) was found to be pollinators of *S. mirabilis* (Figure 4.11). The visiting frequency of this pollinator was very low. However, their behavior can be distinguishable from other insects. Apparently, the visitors were found that they spent in a short time for searching their food in flowers of *S. mirabilis*. Whereas, the pollinators were found that they stayed longer on each flower for searching their food and visited many flowers in the same inflorescence. Sometime they would come over several inflorescences per each time.

The behavior of the visitors was variable. The wingless ants and small insects seemed to visit the inflorescence for some specific purposes. They were moving around the inflorescence among the flowers (e.g. on the rachis, outer side of sepal and petal or labellum) in random directions. The members of Hymenoptera such as ants were searching for the nectar inside the flowers. However, they did not spend time long enough at each flower. After a few minutes, they departed from the flower. The member of Diptera such as flies or true flies were found flying around the inflorescence and sometimes settling at the labellum for a few seconds. The members of stingless bee have the body size similar to the pollinators of *S. mirabilis*. So far, they have not been recorded as the pollinator.

During the observation time, the pollinarium removal was observed by *Ceratina* sp. (Xylocopinae) entered the flowers. They took a long time (5 – 30 min.) to forage the flowers. The pollinator was face up during seeking for the bursicle. Whenever the bursicle was touch, suddenly, they retreated quickly with a pollinarium sticking "V" like on the frontal part of its head (Figure 4.11) and then it flew off to visit other flowers.

Figure 4.11 The pollinator of *S. mirabilis* is visiting the flower showing a pollinarium stuck on its head.

4.10 Pollen and seed morphology

The SEM observation of pollen and seed morphology of the genus *Sirindhornia* was shown a substantial uniformity in the genus with a characteristic of pollinium and mussulae morphology and seed morphology.

The pollinium of the genus *Sirindhornia* comprises several sectiled mussulae, typically found in Orchidinae. The mussulae morphology of *S. mirabilis*, *S. monophylla* and *S. pulchella* are triangle shape. Comparative among the species base on the pollen morphology did not find any significant differentiation. In average, the mussulae of *S. mirabilis* were 274.4 $\pm$ 26.51 μm long and 193.07 $\pm$ 31.95 μm wide. The exine sculturing is rugulate (Figure 4.12 A & 4.12 B). The mussulae of *S. monophylla* were approximately 180.63 $\pm$ 10.90 μm long and 110.34 $\pm$ 49.18 μm

wide. The sculpturing is regulate (Figure 4.12 C & 4.12 D). The mussulae of *S. pulchella* were 221.50 ± 30.63 μm long and 139.82 ± 28.79 μm wide. Its sculpturing is regulate (Figure 4.12 E & 4.12 F). There is not significant different size of mussulae among the species. The mussulae length of *S. mirabilis* was longest in this genus. While, the mussulae of *S. monophylla* and *S. pulchella* were more or less same size.

The seed morphological characteristics of *Sirindhornia* show the testa surface are generally reticulated. The testa cells are pentagonal or hexagonal with marginal ridges (Figure 4.13). The seeds size of *S. mirabilis*, *S. monophylla and S. pulchella* were 331.22 ± 63.88, 327.60 ± 36.80 and 406.54 ± 49.05 μm long and 161.46 ± 34.15, 133.08 ± 25.95 and 146.10 ± 27.49 wide, respectively. Comparative seed length of *S. pulchella* has longer than of *S. mirabilis* and of *S. monophylla* ($P < 0.05$). However, it was not found any significant difference between their widths ($P < 0.05$).

4.11 Orchid mycorrhiza

The roots of adults and flowering plants of *Sirindhornia* spp. were collected to identify orchid mycorrhiza by using fungal internal transcribed spacer (ITS) of ribosomal DNA. In *S. mirabilis* roots were found *Epulorhiza* sp., *Terfezia* sp. including unidentified species of Tulasnellaceae and other unidentified species (basidiomycete isolate) (Table 4.9). The orchid mycorrhiza from *S. monophylla* roots were also found *Terfezia* sp., *Ceratobasidium* sp. and other identified species (basidiomycete). While, orchid mycorrhiza from *S. pulchella* roots were found fungi more than other two species. These fungi are *Sebacinales*, *Epulorhiza* sp., *Peziza* sp., *Ceratobasidium* sp., *Fusarium solani*, *Amauroascus aureus*, *Resinicium mutabile* and *Exophiala salmonis* (Table 4.9).

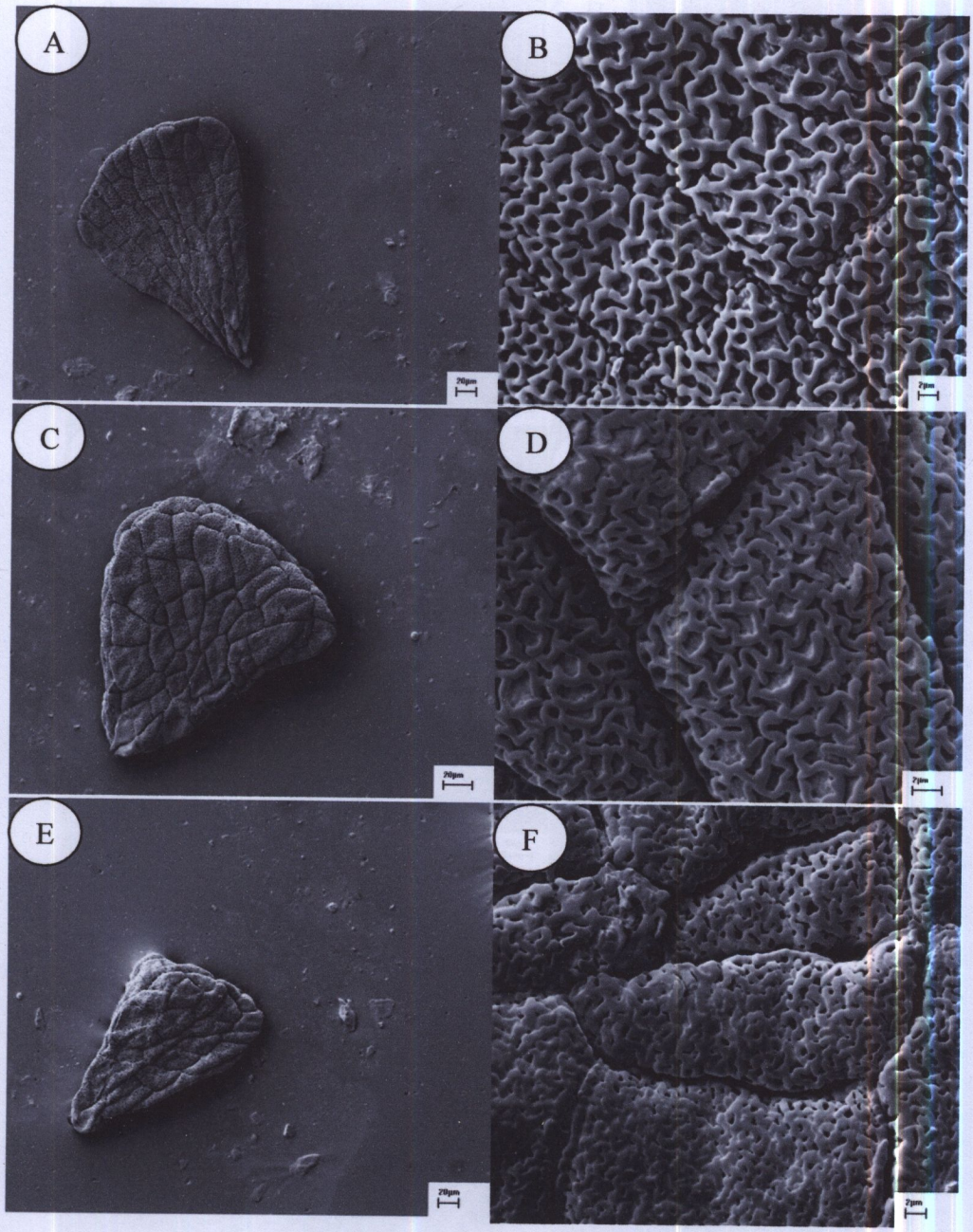

Figure 4.12 A) A massulae morphology of *S. mirabilis* (2Kx), B) Exine micromorphology of *S. mirabilis* (2Kx), C) A massulae morphology of *S. monophylla* (200x), D) Exine micromorphology of *S. monophylla* (3Kx), E) A massulae of *S. pulchella* (200x), F) Exine micromorphology of *S. pulchella* (2Kx).

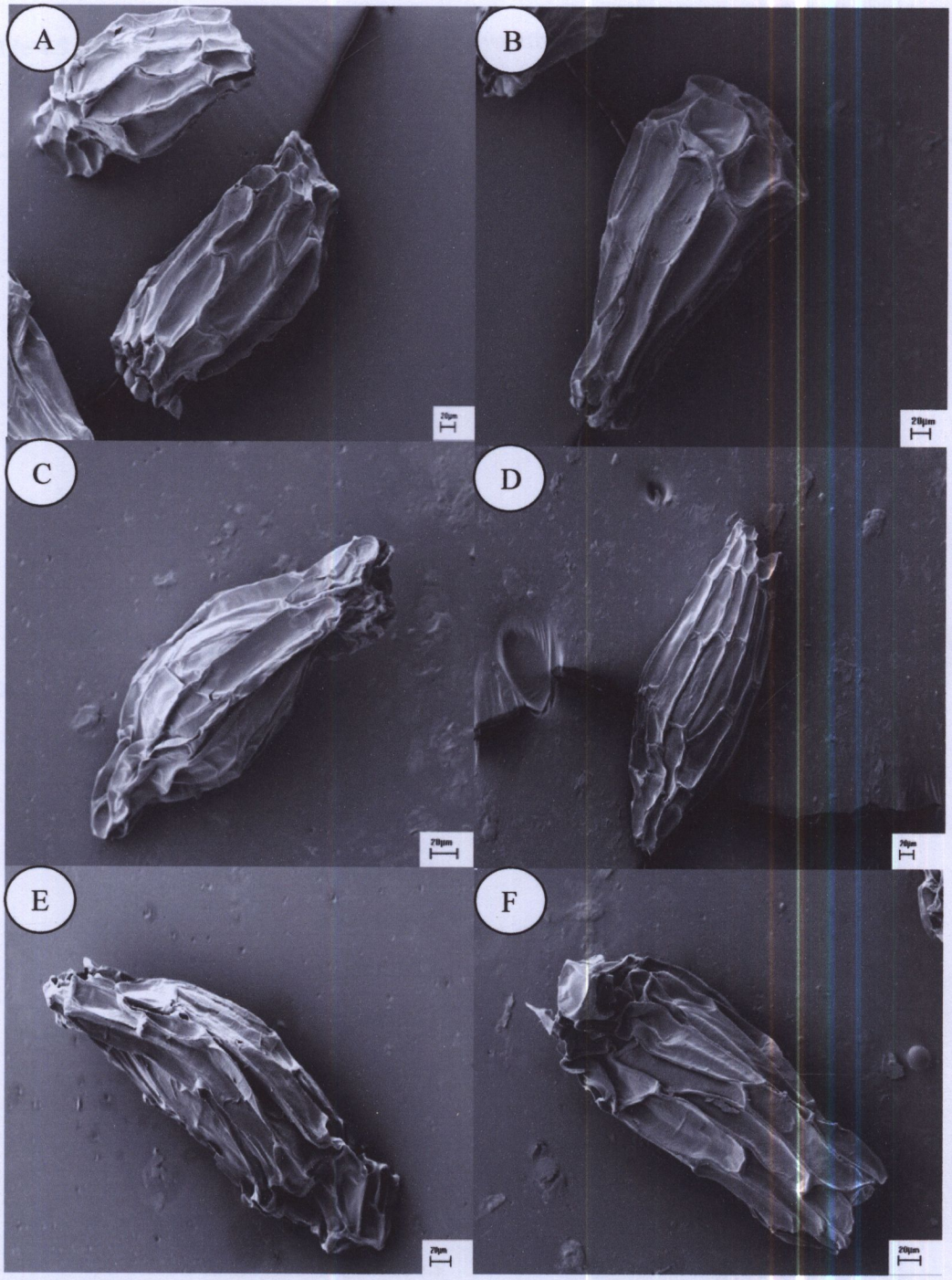

Figure 4.13 Seed morphology of *Sirindhornia* genus; A & B) *S. mirabilis* (180x), C & D) *S. monophylla* (250x) and E & F) *S. pulchella* (220x).

Table 4.9 Summary of identification of orchid mycorrhiza in *Sirindhornia* genus.

Species	Reference of the best BLAST	Identity of the best BLAST
S. mirabilis	EU490419.1	Uncultured basidiomycete isolate
	AM711607.1	Uncultured mycorrhizal fungus
	EU583714.1	Uncultured Tulasnellaceae
	EU583714.1	Uncultured Tulasnellaceae
	AJ313458.1	*Epulorhiza* sp.
	DQ061109.1	*Terfezia* sp.
	DQ061109.1	*Terfezia* sp.
S. monophylla	DQ061109.1	*Terfezia* sp.
	DQ061109.1	*Terfezia* sp.
	FM866384.1	Uncultured Ceratobasidium
	DQ672268.1	Uncultured soil basidiomycete clone
	AB219145.1	*Ceratobasidium* sp.
	AB444653.1	Uncultured Thelephoraceae
	AF472285.1	*Ceratobasidium* sp.
	DQ061109.1	*Terfezia* sp.
S. pulchella	EU214559.1	*Fusarium solani*
	AJ271431.1	*Amauroascus aureus*
	DQ826556.1	*Resinicium mutabile*
	AB369937.1	*Epulorhiza* sp.
	DQ421308.1	Uncultured soil fungus
	DQ826556.1	*Resinicium mutabile*
	FJ475812.1	Uncultured Sebacinales
	AF050274.1	*Exophiala salmonis*
	EU571229.1	*Peziza* sp. cf. badioconfusa RH52806
	EU480008.1	Uncultured soil fungus

4.12 Seed germination in the field

The total viable seeds of *S. mirabilis* in a packet were approximately 200 at the beginning. After 1.5, 3, 6, 9, 12 and 18 months of incubation, they tend to decrease (Table 4.10). Seed germination occurred after 12 months of sowing (1.94%). Then, the seed germination increased to 2.78% after 18 months.

After 12 months of incubation, the testa ruptured due to enlargement of the embryo. The seeds developed into protocorms (growth 2nd stage), while a few roots appeared (Figure 4.14). After 18 months of incubation, it finally found that the seeds developed to stage 5th. Their protocorms produced root-like organs and the first leaf elongated and displayed opposite geotropism by penetrating the plankton net (Figure 4.15).

Table 4.10 Seed germination *in situ* of *S. mirabilis*, all values are given as average.

Incubation time (months)	Total seeds	Viable seeds (%)	Germination seeds (%)
1.5	979	61.49	0.0
3	1,708	70.67	0.0
6	903	61.79	0.0
9	767	60.89	0.0
12	1,446	67.35	1.94
18	359	30.64	2.78

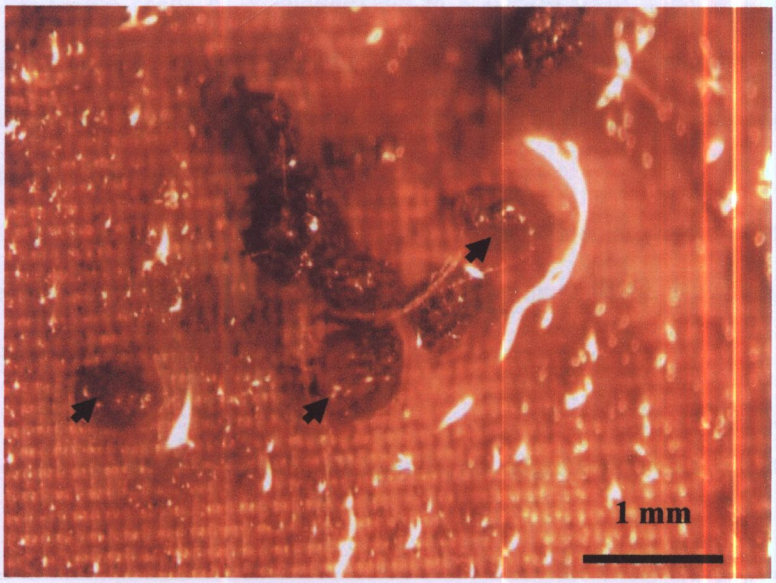

Figure 4.14 Development of seed germination in field experiments of *S. mirabilis* at 12 months protocorms: arrow showed the enlargement of embryos and root development.

Figure 4.15 Development of seedling of *S. mirabilis* at 18 months in natural habitat.

The total viable seeds of *S. monophylla* in a packet were approximately 200 at the beginning. After 1.5, 3, 6, 9, 12 and 18 months of incubation, viable seeds tend to decrease (Table 4.11). Seed germination occurred after 12 months of sowing (0.82%). Then the seed germination increased to 1.08% after 18 months.

After 12 months of incubation, the testa ruptured due to enlargement of the embryo, and the seeds developed into protocorms (growth 2nd stage) (Figure 4.16). However, there was no further progression of development.

Table 4.11 Seed germination *in situ* of *S. monophylla*, all values are given as average.

Incubation time (months)	Total seeds	Viable seeds (%)	Germination seeds (%)
1.5	710	43.80	0.0
3	1,083	63.25	0.0
6	449	73.49	0.0
9	783	50.06	0.0
12	368	30.16	0.82
18	461	20.61	1.08

4.13 *In vitro* seed germination

All of the experiments with 5 different methods of surface sterile, light and dark incubations and the effects of hormones on seed germination *in vitro* of *Sirindhornia* spp. were not success. Only the capsule age effects to seed germination was success. The capsules age at least 5 weeks after pollination of *S. mirabilis* were capable to germinate (Table 4.12) within milk-

white and epidermal hairs protocorms. The protocorms developed into growth stage 5 with first leave after 3 months (Figure 4.17). Then they developed to completely seedling (Figure 4.18) only in 5 months and found only 2 replications.

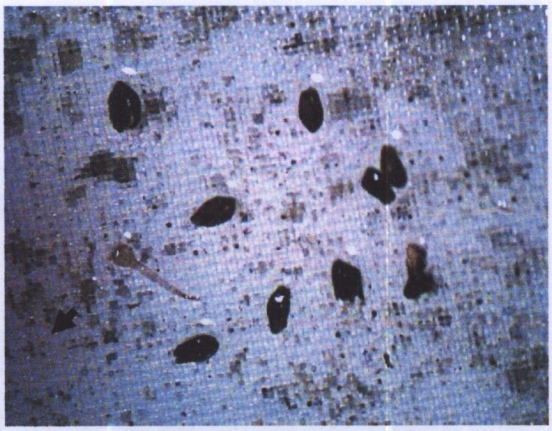

Figure 4.16 Seed germination in field experiments of *S. monophylla*; arrow showed the enlargement and elongation of the embryo.

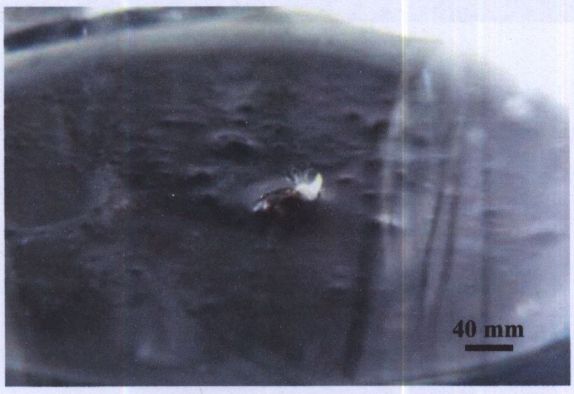

Figure 4.17 The development of protocorms from 6 weeks of capsules age in *S. mirabilis* at 3 months of culture in ½ MS.

The germination of immature capsule of *S. monophylla* at 4, 5, 6, 7 and 8 weeks after pollination was unsuccessful (Table 4.12). Only the mature capsule (10 weeks after pollination) of *S. monophylla* began to germinate after 16 months of culture in VW medium and found only 4 replications (Table 4.12). The coloration of protocorms was milk-white. Some protocorms were developed the first leave. While some were only produced more epidermal hair (Figure 4.19).

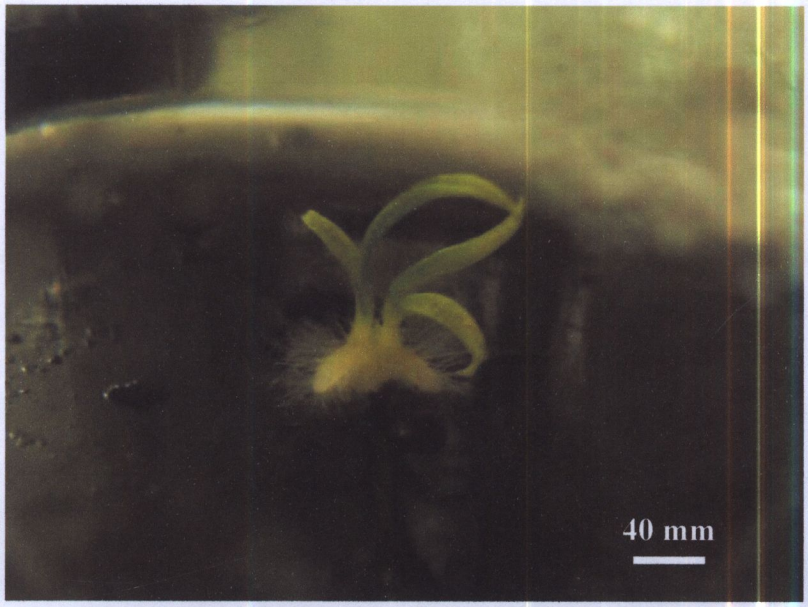

Figure 4.18 The development of protocorms from 6 weeks of capsule age in *S. mirabilis* at 5 months of culture in ½ MS.

Table 4.12 The effect of capsules age of *Sirindhornia* on seed germination *in vitro*

Capsules age (weeks)	germination (replication)	
	S. mirabilis	*S. monophylla*
4	0	0
5	2	0
6	2	0
7	2	0
8	2	0
10	0	4

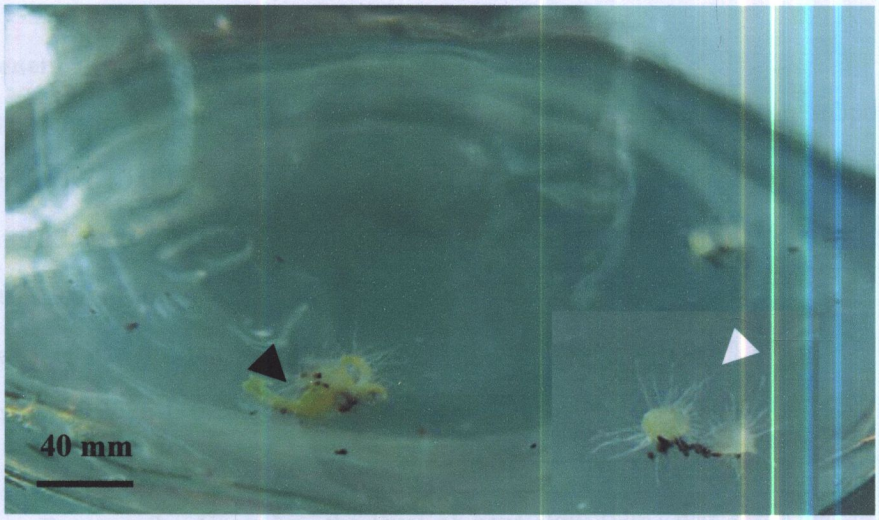

Figure 4.19 The development of protocorms from 6 weeks of capsule age in *S. monophylla* at 16 months of culture in VW medium: white arrow showing the protocorms at growth stage 4 and black arrow showing protocorms at growth stage 5.

4.14 Genetic diversity of *Sirindhornia* spp.

Two subpopulations of each species of *Sirindhornia* were analyzed genetic diversity by using AFLP, with totally 64 primer combinations, *Eco*RI+3 and *Mse*I+3 selective primer combinations. The number and type of primer pairs that showed banding pattern with very high reproducibility and clear band resolution are different for the species.

The AFLP analysis of *S. mirabilis* was generated with 4 primer combinations. There are E-AAC/M-CAA, E-ACC/M-CTC, E-AGG/M-CAA and E-AGG/M-CAC with total of 97 interpretable bands and average at 24 bands per primer pair. While the AFLP analysis of *S. monophylla* was generated with 3 primer combinations. There are E-AAC/M-CAA, E-ACG/M-CTC and E-AGG/M-CAA with total of 60 interpretable bands and average at 20 bands per primer pair. The AFLP analysis of *S. pulchella* was generated with 6 primer combinations. There is E-AAC/M-CAA, E-ACC/M-CTC E-ACC/M-CTT E-ACG/ M-CAC E-ACG/M-CTC and E-AGG/M-CAA with total of 148 interpretable bands and average at 25 bands per primer pair.

Genetic diversity within subpopulation and species

The genetic diversity value at subpopulation level of *S. mirabilis* in subpopulation 1 (gene diversity (H_E) =0.1303 (±0.1874); 40.21 % polymorphic loci (P); Shannon's information index (I_S) =0.1969 (±0.2691)) is higher than population 2 (H_E =0.0837(±0.1469); 34.02 % P; I_S =0.1324 (±0.2209) (Table 4.13).

Total genetic diversity (H_T) at species level of *S. mirabilis* species was 0.1284(±0.1693); 49.48 %P; I_S = 0.2026 (±0.2474). The coefficient of genetic differentiation among population (G_{ST}) was 0.167 as estimated by portioning the total gene diversity, assuming Hardy-Weinberg Equilibrium. Base on the G_{ST} value, the level of gene flow (Nm) was estimated to be 2.4944 among populations (Table 4.14). The UPGMA was used to derive a dendrogram to summarize the interrelationship conserved among the 40 individuals from 2 subpopulations see figure 4.20.

Table 4.13 The genetic diversity within and between populations of three species of *Sirindhornia*.% P = percentage of polymorphic fragments; H_E = Nei's (1973) gene diversity; *I*s = Shannon's information index.

Species	Sub population	Sample size	Loci	% *P*	H_E (±SD)	I_S (±SD)
S. mirabilis	1	20	97	40.21	0.1303 (±0.1874)	0.1969 (±0.2691)
	2	20	97	34.02	0.0837 (±0.1469)	0.1324 (±0.2209)
S. monophylla	1	20	60	63.33	0.1795 (±0.1803)	0.2787 (±0.2621)
	2	20	60	35.00	0.1080 (±0.1764)	0.1637 (±0.2556)
S. pulchella	1	20	148	33.78	0.1214 (±0.1899)	0.1794 (±0.2729)
	2	20	148	23.65	0.0741 (±0.1535)	0.1125 (±0.2237)

Table 4.14 The genetic diversity between subpopulations of three species of *Sirindhornia*. % *P* = percentage of polymorphic fragments; H_T = Nei's (1973) gene diversity; *I*s = Shannon's information index; G_{ST} = coefficient of genetic differentiation among subpopulations; Nm = gene flow among subpopulation [$0.5(G_{ST} - 1)/ G_{ST}$].

Species	%*P*	*I*s (±SD)	H_T (±SD)	G_{ST}	Nm
S. mirabilis	49.48	0.2026 (±0.2474)	0.1284 (±0.1693)	0.167	2.4944
S. monophylla	65.00	0.2442 (±0.2425)	0.1524 (±0.1662)	0.0565	8.3541
S. pulchella	38.51	0.1738 (±0.2471)	0.1122 (±0.1671)	0.1286	3.3880

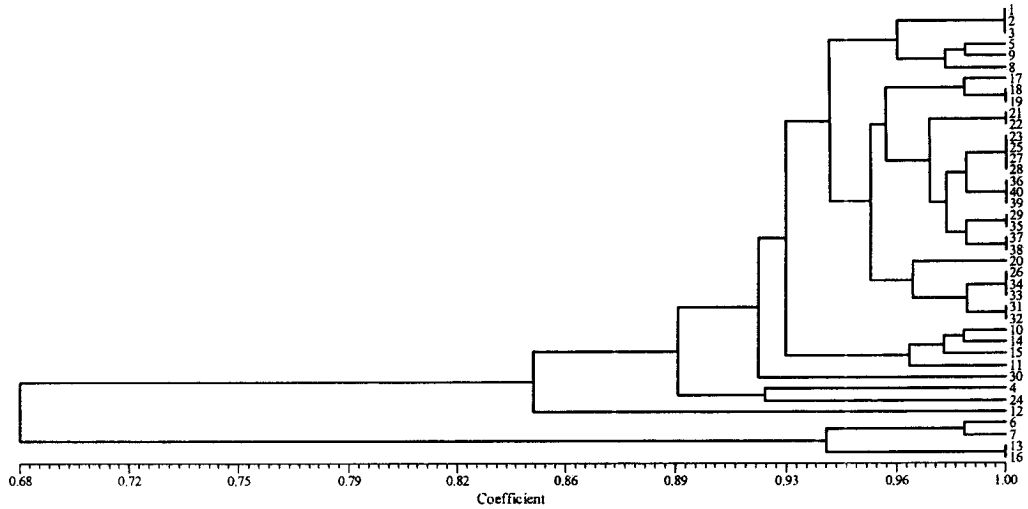

Figure 4.20 Dendrogram of 40 individuals of *S. mirabilis*, number 1-20 are individuals from subpopulation 1 and 21-40 are individuals from subpopulation 2.

The genetic diversity value at population level of *S. monophylla* in subpopulation 1 gene diversity (H_E) =0.1795 (±0.1803); 63.33 % polymorphic loci (*P*); Shannon's information index (I_S) =0.2787 (±0.2621)) is higher than subpopulation 2 (H_E =0.1080 (±0.1764); 35.00 % *P*; I_S =0.1637 (±0.2556) (Table 4.13).

Total genetic diversity (H_T) at species level was 0.1524 (±0.1662); 65.00 %P; I_S = 0.2442 (±0.2425). The coefficient of genetic differentiation among subpopulation (G_{ST}) was 0.0565 as estimated by portioning the total gene diversity, assuming Hardy-Weinberg Equilibrium. Base on the G_{ST} value, the level of gene flow (Nm) was estimated to be 8.3541 among subpopulations (Table 4.14). The UPGMA was used to derive a dendrogram to summarize the interrelationship conserved among the 40 individuals from 2 subpopulations see figure 4.21.

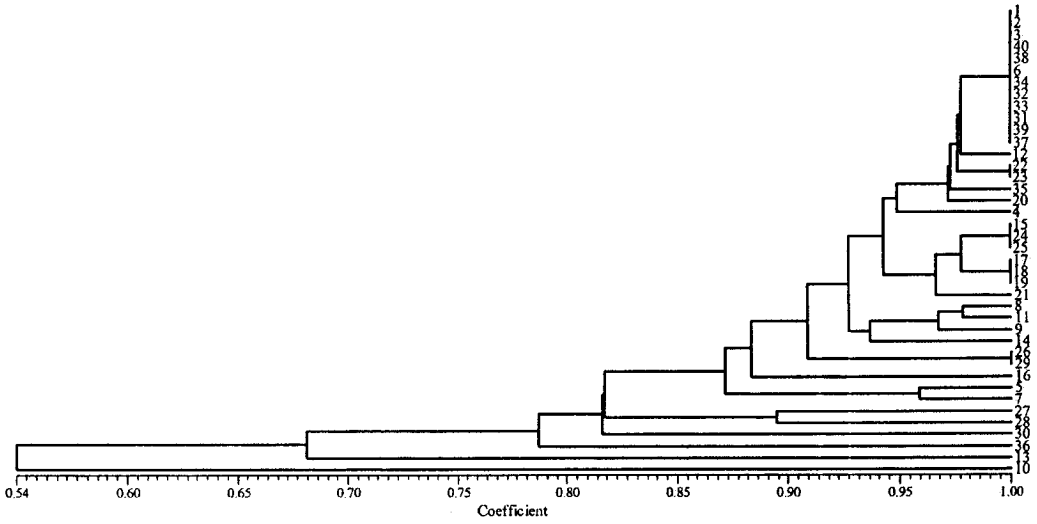

Figure 4.21 Dendrogram of 40 individuals of *S. monophylla*, number 1-20 are individuals from subpopulation 1 and 21-40 are individuals from subpopulation 2.

The genetic diversity value at subpopulation level of *S. pulchella* in subpopulation 1 (gene diversity (H_E) =0.1214 (±0.1899); 33.78 % polymorphic loci (*P*); Shannon's information index (I_S) =0.1794 (±0.2729) is higher than subpopulation 2 (H_E =0.0741 (±0.1535); 23.65 % *P*; I_S =0.1125 (±0.2237) (Table 4.13).

Total genetic diversity (H_T) at species level was 0.1122 (±0.1671); 38.51 %P; I_S = 0.1738 (±0.2471). The coefficient of genetic differentiation among subpopulation (G_{ST}) was 0.1286 as estimated by portioning the total gene diversity, assuming Hardy-Weinberg Equilibrium. Base on the G_{ST} value, the level of gene flow (Nm) was estimated to be 3.3880 among populations (Table 4.14). The UPGMA was used to derive a dendrogram to summarize the interrelationship conserved among the 40 individuals from 2 subpopulations see figure 4.22.

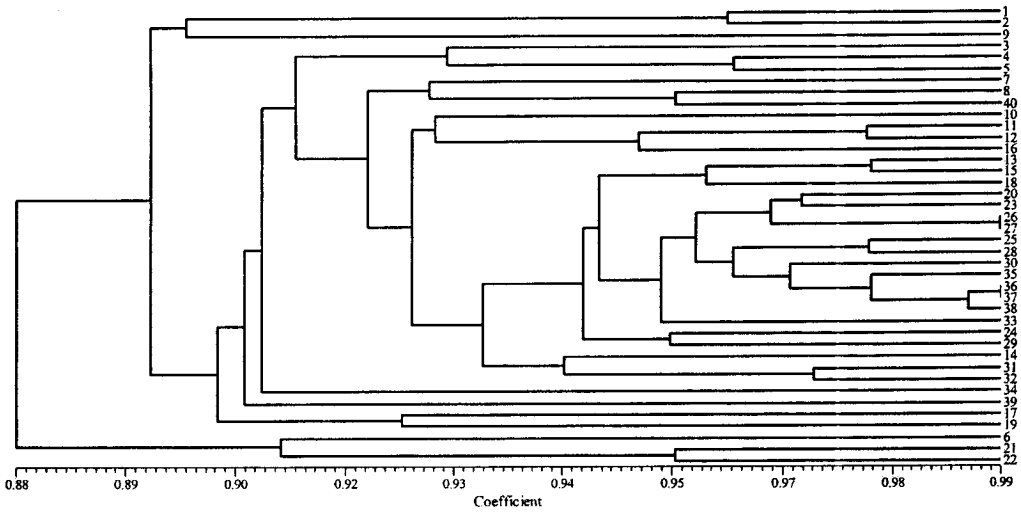

Figure 4.22 Dendrogram of 40 individuals of *S. pulchella*, number 1-20 are individuals from subpopulation 1 and 21-40 are individuals from subpopulation 2.

CHAPTER 5

DISCUSSION

5.1 Quality of soil from *Sirindhornia* habitats

The available phosphorous in the site of *S. mirabilis* was the highest. In contrast, the amount of available calcium in soil of *S. mirabilis* was the lowest. It might effect on plant size because among 3 species *S. mirabilis* is the largest. It is difficult to explain the relation of the amount of phosphorus and calcium available in soil and the amount of the orchid use, there is no information on phosphorus and calcium in the orchids. However, in general, deficiency of phosphorus usually shows symptoms as stunted growth. Whereas, deficiency of calcium usually causes of the death of root and shoot tips (Pansu and Gautheyrou, 2006).

The sites of *S. mirabilis*, *S. monophylla* (Doi Hua Mot) and *S. pulchella* (Doi Chiang Dao) are Limestone rock bed. Basically, it should be alkaline soil with high pH, i.e. 8.0. But from the results, all of soil samples were a little acidity or more or less neutral, due to humus or organic matter in soil.

The preliminary study of soil properties of three studied sites, representative for each species, revealed not much differentiation. However, this information showed the environment property in the habitat of this rare species. It can be applied for reintroduction or transplant program for long term conservation.

5.2 Demography

The relative distribution of individuals among size classes was rather similar in the populations of *S. mirabilis*, *S. monophylla* and *S. pulchella* (Figure 4.1), all being characterized by their low numbers of small and large individuals. Likewise, it was characteristic of all three species (irrespective of the species-specific arbitrary size class definitions) that only size class 1 did not contain any flowering plants, and that the proportion of flowering plants in size class 2 was low. Thus, it can be assumed that size class 1 in each species consists of juvenile plants only, and that size class 2 probably comprises a mixture of juvenile and adult (i.e. potentially flowering) plants, whereas the higher size classes consist entirely of adult plants (partly flowering, partly vegetative).

Against this background, the age structure of all three populations seems heavily dominated by adult plants. This can be due to recently decreased levels of reproduction by seed, or it can be due to the adult stage of the plant lasting much longer than the juvenile stage. Judging from the strong variation in age spectra found in populations of numerous terrestrial orchid species (Vakhrameeva and Tatarenko, 1998), all studied in undisturbed habitats, and noting the similar spectra that we found on Doi Hua Mot and Doi Chiang Dao approximately 360 km apart. It tends to think that the latter explanation is the more likely, and that a long-lasting adult stage is characteristic of *Sirindhornia*. Vakhrameeva and Tatarenko (1998) studied nine temperate to subtropical orchid species representing the same life form as *Sirindhornia*. Among these, the study populations of *Anacamptis pyramidalis* (L.) Rich., *Neottianthe cucullata* (L.) Schltr., *Orchis mascula* (L.) L. and *Traunsteinera globosa* (L.) Rchb. were likewise dominated by adult plants.

Observing the distribution of individuals among size classes 3–8 (i.e. the classes presumed to consist of adult plants only), a consistent decline according to size class is evident in *S. monophylla* and *S. pulchella* (Figure 4.1 B- 4.1 C), probably reflecting an age related curve of accumulated mortality that is already well known from a range of other orchid species (Zotz, 1998; Winkler and Hietz, 2001; Watthana, 2005; Watthana *et al.*, 2006; Watthana and Pedersen,

2008). In comparison, it is puzzling that size class 3 in *S. mirabilis* contains fewer individuals than size class 4 (Figure 4.1 A).). However, such a relatively minor deviation from a consistent decline might well be explained by the circumstance that cohorts of juvenile individuals vary in size between years due to natural variation in growth and reproductive conditions.

Orchids are known to vary widely in flowering frequency, both between species, between populations and between years in one and the same population. Thoroughly studied examples of the latter phenomenon in species with root/stem tuberoids and annual renewal of all organs (as in *Sirindhornia*) include populations of *Anacamptis morio* (L.) R. M. Bateman *et al.* (Wells *et al.*, 1998 ; Jersáková *et al.*, 2002), *Coeloglossum viride* (Willems and Melser, 1998), *Dactylorhiza incarnata* (L.) Soó and *D.sambucina* (L.) Soó (Tamm, 1972), *D. majalis* (Rchb.) P. F. Hunt & Summerh. subsp. *lapponica* (Hartm.) H. Sund. (Øien and Moen, 2002), *Gymnadenia conopsea* (L.) R. Br. (Øien and Moen 2002, Gustafsson, 2007), *Herminium monorchis* (L.) R. Br. (Wells, 1994, Wells *et al.*, 1998), *Neotinea ustulata* (L.) R. M. Bateman *et al.* (Tali, 2002), *Ophrys apifera* Huds. (Wells and Cox, 1989, 1991), *O. insectifera* L. (Dorland and Willems, 2002), *O. sphegodes* Mill. (Hutchings, 1987a, 1987b), *Orchis mascula* (Tamm, 1972), *O. militaris* L. (Hutchings *et al.*, 1998; Waite and Farrell, 1998) and *O. purpurea* Huds. (Jacquemyn *et al.*, 2002). The above references together propose several factors that might explain the observed flowering dynamics - including resource limitation, temperature, precipitation and light environment. In *S. mirabilis*, 36 individuals bloomed in the study plot in 2006 and 34 did so in 2008; in *S. monophylla*, the number of flowering individuals decreased from 25 to 12 between 2006 and 2008; in *S. pulchella*, the corresponding number increased from 13 in 2006 to 23 in 2007 and 62 in 2008.

Judging from the population structure as mapped in 2006 (*S. mirabilis*, *S. monophylla*) and 2007 (*S. pulchella*), (Figure 4.1), the proportion of flowering plants increased with increasing plant size in all species of *Sirindhornia*. However, this trend was not completely consistent, but due to the low number of individuals in the highest size classes. It is not possible to decide if the divergent patterns in especially size classes 7-8 are characteristic or fortuitous. Perhaps, a senility

factor causes a generally decreased probability of flowering among the oldest individuals. Monitoring of individual plants over several years would be needed to decide whether flowering dynamics in the three species of *Sirindhornia* are governed primarily by climate, resources or age.

5.3 Individual contributions of progeny

The Lorenz curve based on pollinarium removal in *S. mirabilis* (Figure 4.7) is skewed to the right, indicating unequal contribution of individuals to the pool of pollinaria removed. It differs from the corresponding Lorenz curve for *S. monophylla* (Figure 4.7), which forms a nearly diagonal line from the lower left to the upper right corner, indicating almost equal individual contributions.

The Lorenz curves based on fruit set in *S. mirabilis* and *S. pulchella* (Figure 4.8 and 4.10) are strongly skewed to the right and much more similar to the curves that Calvo (1990), Pedersen *et al.* (2004), Watthana *et al.* (2006) and Watthana and Pedersen (2008) provided for allogamous orchid species than to the curve that Calvo (1990) provided for the autogamous *Oeceoclades maculata* Lindl. In contrast, the Lorenz curves based on fruit set in *S. monophylla* (Figure 4.9) are less skewed to the right, so they indicate more equal contributions of individuals to the capsule pool. If compared to the curves provided by Calvo (1990), *S. monophylla* could be assumed to be autogamous. However, the pollination experiments demonstrated that this is not the case; the only slightly skewed condition of the Lorenz curves simply reveals a consistently high pollination success in an insect-pollinated species.

The unequal distribution of capsules (Figure 4.8 – 4.10) among individuals, particularly in the populations of *S. mirabilis* and *S. pulchella*, accounts for differences in the female genetic contribution of progeny. If the same individuals continue to be the most important contributors during a number of years, this might influence the genetic composition of the populations. The almost identical curves that we obtained for each species during two or three years (Figure 4.8 – 4.10) suggest that the degree of (in)equality is fairly constant and partly species-specific in

Sirindhornia, but our data do not reveal whether the same individuals have been the most important contributors every year.

A possible high rate of full sibness (resulting from one pollinarium fertilizing all ovules in an ovary) might account for a correspondingly unequal male genetic contribution, especially when taking into account the unequal contributions of pollinaria among individuals of particularly *S. mirabilis* (Figure 4.7). However, it should be remembered that the pollinia of *Sirindhornia* are sectile and consist of fairly loosely assembled massulae. Each pollinarium can probably pollinate several flowers by leaving minor clumps of massulae rather than entire pollinia on individual stigmas (Neiland and Wilcock, 1995; Singer and Cocucci, 1997; Singer, 2001). By reducing pollen carry over from one individual to another, geitonogamy can reduce the male fitness of a plant. As we found no bending mechanism in experimentally extracted pollinaria from *Sirindhornia* flowers, the species of this genus must be more prone to geitonogamy (Johnson and Nilsson, 1999). Consequently, the low levels of heterozygosity and genetic variation in the populations of all three species was expected (see detail in genetic diversity of *Sirindhornia* spp.).

5.4 Relations between fruit set, seed production and recruitment

Low relative fruit set is commonly found in allogamous orchid species (Calvo, 1990; Neiland and Wilcock, 1998; Huda and Wilcock, 2008). In *Sirindhornia*, we generally found the narrowly endemic *S. mirabilis* and *S. pulchella* to have lower relative fruit set than the more widely distributed *S. monophylla* (Table 4.7). However, the number of seeds per capsule seems to be negatively correlated with the (species-specific) relative fruit set in *Sirindhornia*. Thus, *S. monophylla* exhibited the highest relative fruit set (38.0-51.6%), but had the lowest number of seeds per capsule (1,972 ± 902), whereas *S. mirabilis* exhibited the lowest fruit set (4.5-9.6%), but produced the highest number of seeds per capsule (5,911 ± 1,613). As proposed by Calvo (1990), a high number of seeds per capsule may help to counterbalance low fruit set. This is clearly seen in *Sirindhornia* where the mean number of capsules produced per inflorescence in *S. mirabilis*

was 12-19% of the level in *S. monophylla* in 2006–2008, whereas the corresponding figure for seeds per inflorescence was 55-76% in the same period.

Although, one *Sirindhornia* fruit typically contains from 2,000 - 6,000 seeds, depending on the species, successful germination must rely heavily on environmental factors like in other orchids (Rasmussen, 1995). We found the relation between total estimated seed production and population size (above ground) was widely different between the three *Sirindhornia* species (Table 4.2); in *S. pulchella* only 326 seeds were produced per plant in the study population (in 2007), whereas the corresponding rates for *S. mirabilis* and *S. monophylla* (in 2006) were 3,662 and 2,940, respectively. Thus, recruitment appears to be more efficient in *S. pulchella*. At least four possible explanations exist. First, a considerably higher proportion of the dispersed seeds may be lost without germinating in *S. mirabilis* and *S. monophylla*, either as a result of fundamentally different soil seed-bank dynamics (Batty *et al.*, 2000; Whigham *et al.*, 2006) or because of lower densities of unsaturated microsites suitable for germination (Eriksson and Ehrlén, 1992; Jersáková and Malinová, 2007). Second, mortality of the youngest, completely subterranean stages may be much higher in *S. mirabilis* and *S. monophylla*. Third, a much higher proportion of individuals in the two latter species (relative to *S. pulchella*) may be subterranean at any one time (in which case, population sizes are more strongly underestimated for these species than for *S. pulchella*). Fourth, the study populations may not be stable; if the population of *S. pulchella* is declining rapidly, and/or if the populations of *S. mirabilis* and *S. monophylla* are growing rapidly, the calculated figures simply give an erroneous impression of the magnitude of recruitment of adult individuals in relation to seed production. Long term monitoring of the populations (Whigham and Willems, 2003) combined with *in situ* germination experiments (Rasmussen and Whigham, 1993, 1998) are needed to achieve a better understanding of the phenomenon.

5.5 Breeding system and pollination

None of the untouched *Sirindhornia* flowers on caged individuals set fruit, whereas all of the manually self-pollinated flowers set fruit. This demonstrates that *S. mirabilis*, *S. monophylla* and *S. pulchella* are genetically self-compatible, non-autogamous species, and that no apomixis occurs. Consequently, seed production in all three species is completely dependent on pollinators.

With their zygomorphic, mainly white to purple, more or less horizontal flowers (Figure 2.6 C; 2.8 C and 2.9 C) that exude nectar hidden in a spur and offer an enlarged labellum as a landing platform (provided with markings or tufts of colored papillae that serve as nectar guides), the flowers of all three *Sirindhornia* species fit the bee pollination syndrome of van der Pijl and Dodson (1966). Indeed, the observation of a pollination-related insect visit to *Sirindhornia* involved small carpenter bees (*Ceratina* sp., Apidae) that, retreating from an *S. mirabilis* flower where it had probed the spur for nectar, extracted a pollinarium that was attached to the frontal part of its head (Figure 4.11).

Most insects settling on the *S. mirabilis* flowers were evidently not pollinators. Based on their body size, which was not compatible with flowers. They always come to visited flowers for exploring the flowers very short time because the *Sirindhornia* flowers not have the food that they want. Sometimes they probed several flowers, but also perched on the inflorescences without exploring the flowers. Different from the behavior of pollinator which is always take longer time. From the personal observation seemed that the nectar in the spur of *Sirindhornia* for the others insects. The pollinators seemed does not want nectar but prefer pollen. The pollination happen when the pollinarium that was attached to the frontal part of the head when they face up for break into the bursicle. After that they flying with the pollinarium at the head part to visited another flowers in inflorescence including another nearly inflorescence.

The flowering seasons of the sympatric study populations of *S. mirabilis* and *S. monophylla* overlap in May and June (Tables 4.3 – 4.4 and Figure 4.2 – 4.3). The recent discovery of the hybrid *S. mirabilis* × *monophylla* in this colony (Pedersen and Ormerod, 2009) and personal observation demonstrated that one pollinator species must be shared between the

two, at least occasionally.

5.6 Reproductive success in relation to floral display

The longevity of individual flowers in *S. mirabilis* was found to be about six weeks, were only three weeks in *S. monophylla* and *S. pulchella*. However, if a flower in any of the three species was pollinated, it would wither in less than one week. Prolonged longevity of flowers in *S. mirabilis* may have evolved in response to low visitation rates (personal observation) in line with the findings of Sugiura *et al.* (2001) for *Cypripedium macranthos* Sw. and the same explanation might well apply to the generally higher number of flowers per inflorescence in *S. mirabilis* (Figure 4.2). This hypothesis is congruent with the level of pollinarium removal in *S. mirabilis* (which was significantly lower than in the other examined species, *S. monophylla*) and partly congruent with the level of natural fruit set in *S. mirabilis* (which was significantly lower than in *S. monophylla* but not significantly different from the level in *S. pulchella*, see also Table 4.7).

Pollinarium removal in *S. monophylla* and *S. mirabilis* positively correlated with the number of flowers per inflorescence (Figure 4.5 - 4.6). It was found a positive correlation between fruit set and number of flowers per inflorescence in *S. monophylla* only (Figure 4.6). Similar correlations between reproductive success and inflorescence size are known from several other orchid species, both rewarding (Schemske, 1980; Inoue, 1986; Piper and Waite, 1988; Waite *et al.*, 1991; Murren and Ellison, 1996; Jersáková and Kindlmann, 2004; Kindlmann and Jersáková, 2006) and non-rewarding (Nilsson, 1983; Firmage and Cole, 1988; Fritz, 1990; Waite *et al.*, 1991; Jersáková and Kindlmann, 2004; Kindlmann and Jersáková, 2006; Watthana *et al.*, 2006). Thus, we would expect directional selection towards an increase of inflorescence size through male function in *S. mirabilis* and through both male and female function in *S. monophylla* (although it should be remembered that pollinarium removal is only an indirect measure of male reproductive success; many pollinaria were evidently lost).

As noted by Calvo (1990), the potential for pollinator-mediated selection on floral display may be limited by the often low overall pollination level in orchids. Positive correlations between fruit set and floral display are sometimes limited to certain populations (Kindlmann and Jersáková, 2005) and may be limited to favorable years because pollinators otherwise occur less predictably in time or space (Sutherland, 1986; Jersáková and Kindlmann, 2004). Furthermore, increased fruit set in orchids may reduce future growth and/or reproduction (Montalvo and Ackerman, 1987; Ackerman, 1989; Snow and Whigham, 1989; Zimmerman and Aide, 1989; Ackerman and Montalvo, 1990; Mattila and Kuitunen, 2000; Meléndez-Ackerman *et al.*, 2000; Primack, 2002). Consequently, directional selection for increased floral display in *S. mirabilis* and *S. monophylla* may be counterbalanced by stabilizing selection for a sustainable annual fruit set that maximizes the lifetime reproductive success of the individual.

The consistently higher male reproductive success (as compared to female success) in *S. mirabilis* and *S. monophylla*, together with the fact that only pollinarium removal was found to be positively correlated with the number of flowers per inflorescence in *S. mirabilis*, suggest that large inflorescence size primarily evolved in response to selection on male reproductive success in accordance with the so-called male function hypothesis (Willson and Rathcke, 1974; Willson and Price, 1977; Burd, 1994). However, it should still be remembered that pollinarium removal is an indirect and over-estimating measure of male reproductive success (Nilsson *et al.*, 1986; Snow, 1989) and that orchid reproduction is more limited by pollination than by pollinarium removal (Nilsson, 1992). For example, if the magnitude of pollinarium removal from *S. mirabilis* is compared to fruit set in the same population in 2006, it appears that approximately 76% of the removed pollinaria were lost. This is a high loss compared to the corresponding figure for *S. monophylla* (43%). It may indicate lower pollinator specificity in *S. mirabilis*, as the risk of pollen wastage during transport generally increases with the taxonomic diversity of the pollinator fauna (Wilcock and Neiland, 2002; Tremblay *et al.*, 2005). The consistently lower female reproductive success in *S. mirabilis* and *S. monophylla* (as compared to male success) may be due to resource limitation (Willson and Price, 1977; Stanton *et al.*, 1986). Limited support for this hypothesis in orchids has been provided for *Caularthron bilamellatum* (Rchb.f.) R. E. Schult.

(Fisher, 1992), *Dactylorhiza maculata* (L.) Soó (Vallius, 2000) and *Platanthera bifolia* (L.) Rich. (Mattila and Kuitunen, 2000). However, corresponding studies of several other orchid species (Tremblay *et al.*, 2005) did not give consistently similar results.

5.7 Reproductive success in relation to position of flower

Pollinarium removal in *S. mirabilis* and *S. monophylla* decreased markedly from the basal part to the apical part of the inflorescence (Table 4.6). This is surprising as the rewarding condition of the *Sirindhornia* flowers would be expected to induce pollinators to visit more flowers during each visit to an inflorescence (Johnson and Nilsson, 1999). In *S. monophylla* (contrary to *S. mirabilis*, Table 4.3), pollinaria were generally removed soon after the flowers had opened (Table 4.4).

Relative fruit set decreased on average 75% from the basal to the apical part of the inflorescence in *S. mirabilis*, by 83% in *S. monophylla* and 100% in *S. pulchella* (Table 4.6). This consistent trend of decrease matches previously reported observations of fruit set (or pollen deposition) in the non-rewarding, mainly bumblebee-pollinated *Anacamptis morio* (L.) R. M. Bateman *et al.* (Nilsson, 1984; Jersáková and Kindlmann, 1998), *Dactylorhiza sambucina* (L.) Soó (Nilsson, 1980), *Orchis mascula* (L.) L. (Nilsson, 1983; Johnson and Nilsson, 1999) and *O. spitzelii* Saut. ex W. D. J. Koch (Fritz, 1990). Experimental pollinations performed by Fritz (1990) demonstrated that all flowers in inflorescences of *O. spitzelii* were capable of producing fruits – and in all the non-rewarding species above, differential fruit set was attributed to the pollinating bumble bees always starting their visit on one of the lowermost flowers and only probing a few flowers (in vain) before leaving the inflorescence. However, *Sirindhornia* flowers exude nectar in their spur, and similar patterns of differential pollination are known from the rewarding *Myrosmodes cochleare* Garay (Berry and Calvo, 1991), *Platanthera blephariglottis* (Willd.) Lindl. (Cole and Firmage, 1984) and *P. chlorantha* (Custer) Rchb. (Johnson and Nilsson, 1999). In these species, like in *Sirindhornia*, pollinators searching for nectar may likewise, at least in some cases, start their visit on one of the lowermost flowers, but due to the nectar reward they

have no reason to leave the inflorescence soon after – and indeed, Johnson and Nilsson (1999) demonstrated that pollinators stayed longer on each flower and visited more flowers per inflorescence when artificial nectar was added to the flowers of an otherwise non-rewarding species.

The propose of hypothesis that in rewarding orchids, differential pollination is often due to a combination of (1) the flowers opening in sequence from the base towards the apex of the inflorescence with all flowers being simultaneously open for only a short period, and (2) the importance of floral display for attracting pollinators. At the beginning of flowering, only the flowers in the basal (to middle) part of the inflorescence are open and accessible to pollinators, but the colored buds in the (middle to) apical part contribute to the floral display. Towards the end of flowering, the uppermost flowers are open and accessible to pollinators, but at this time the lowermost flowers are withered and do no longer contribute to the floral display. Furthermore, the lowermost flowers are usually larger than the uppermost (personal observation), meaning that the floral advertisement consisting of open flowers only also differs between the basal and the apical part of the inflorescence. Admittedly, this description does not fit *Myrosmodes cochleare* in which the flowers open in the opposite sequence, but for this species Berry and Calvo (1991) revealed experimentally that the uppermost flowers were unable to produce fruits. Based on the consistently higher male than female reproductive success in all parts of the inflorescence in both *Sirindhornia* species examined, resource limitation also needs to be taken into account when explaining differential fruit set. Additional factors that can lead to differential pollination in orchids include (1) that a pollinator moving up the inflorescence may become satiated with the resources and leave before visiting the upper flowers (Tremblay *et al.,* 2005), and (2) that pollinators are sometimes ineffective at depositing pollinia in the smaller upper flowers (Tremblay, 2006).

5.8 Orchid mycorrhizal fungi of *Sirindhornia* spp.

The most of fungi which isolated from *Sirindhornia* genus were quite reliable as orchid mycorrhiza. The abundant mycorrhizal fungi in the orchid genus *Sirindhornia* were *Tulasnella* sp., *Terfezia* sp. *Ceratobasidium* sp., *Peziza* sp. and *Epulorhiza* sp.

Orchid mycorrhiza in *Sirindhornia* spp. i.e. *Tulasnella*, *Epulorhiza* sp., *Ceratobasidium* and *Sebacinales* were reported as orchid mycorrhiza in other terrestrial orchids (Beyrle *et al.*, 1991; Currah *et al.*, 1987, 1990; Hadley and Purves, 1974; Hadley and Ong, 1978; Warcup, 1971, 1973; 1981; 1985; Warcup and Talbot, 1967, 1971, 1980; Rasmussen, 1995; Shan *et al.*, 2002; Dearnaley, 2007). While, *Terfezia* sp., unidentified species (Thelephoraceae) and *Peziza* sp. were known as ectomycorrhiza fungi which are associated with roots of Fagaceae and Dipterocarpaceae tree species (Selosse *et al.*, 2002; McKendrick *et al.*, 2002; Taylor *et al.*, 2003; Smith and Read, 2008). However, the role of other fungi that found in *S. pulchella* roots such as *Fusarium solani* used to be reported as pathogen fungi (Smith and Read, 2008) including another fungi species are still unknown.

In common, these mycorrhizal fungi are difficult to classify due to the scarcity of sexual sporulation (necessary to define teleomorphic genera) and the morphological similarity of anamorphic genera in this group (Bonnardeaux *et al.*, 2007). Consequently, molecular methods which have done become more efficiency method for identified this fungi group (Taylor *et al.*, 2003; McCormick *et al.*, 2004; Selosse *et al.*, 2002; Shefferson *et al.*, 2005; Roy *et al.*, 2009). These results showed that many different fungi type exist in variety of associations with *Sirindhornia* roots (Table 4.9). It may have ability to produce enzymes such as cellulase and polyphenol oxidase that enable them to break down soil organic matter to simple sugars that may be utilized by both partners in the symbiosis. If necessary these fungi could potentially survive as saprophytes in the soil, and act as viable sources of inoculums for developing orchid's protocorms (Peterson *et al.*, 2004). This is congruent with Sneh *et al.* (1991) recommend that a single root can be infected with a number of symbiotic fungi representing several taxa and also in the each growth stage of life of orchid may be associated with different mycorrhiza.

From this study, it seems that *Sirindhornia* species have ecology close to most terrestrial orchids, but also associated with different fungi including cosmopolitan soil saprophytes (Table 4.9). It is possible that *Sirindhornia* may be connected to both orchid endophytes (i.e., the Rhizoctonia-forming fungi) and to basidiomycete-forming ectomycorrhizal in root trees. This may explain the fact that all *Sirindhornia* species does not grow in pot easily if without ectomycorrhizal from those trees.

Dearnaley (2007) emphasized protection of orchid populations and orchid-associated fungi are important in maintaining global biodiversity, and it also has implications for overall ecosystem health. From the results of seed germination in the field, it was found that both seeds of *S. mirabilis* and *S. monophylla* were very difficult to germinate. Thus the further investigation would be determined the specificity of mycorrhizal association in *Sirindhornia* species to understand that which exactly mycorrhiza have ability to promoted seed germination and protocorms development.

5.9 Seed germination *in situ*

It can be concluded that *S. mirabilis* and *S. monophylla* have low germination rate in natural condition. This congruent with many terrestrial orchid species reported i.e. *Dactylorhiza lapponica* (Laest. Ex Hartm.) Soó (Øien *et al.*, 2008), *Cypripedium macranthos*, (Miyoshi and Mii, 1998), *Epipactis helleborine* (L.) Crantz (van der Kinderen, 1995). However, after 12 months of incubation revealed that only one packet had seed germination. It might be assumed that there was an appropriated fungus to promote the germination in that place. This could be explained that low number of seedling was detected in the study site. Unfortunately, some packets were damage by fire and tourists (personal observation). More investigation needs to be done to confirm the seed germination.

5.10 Asymbiotic seed germination

Although, several experiments had been conducted such as surface sterile techniques, variation of hormone and capsule age yielded unsatisfied results. In contrast, those experiments seem to be success to induce in vitro seed germination of tropical epiphytic orchids. It was found that *S. mirabilis* and *S. monophylla* have also low seed germination *in vitro*. This phenomenon was also congruent with other terrestrial orchids (Rasmussen, 1995; Miyoshi and Mii, 1998).

However, it was found that seed propagation *in vitro* required different factors for each species such as *S. mirabilis* could be germinated better when used immature seeds. While, *S. monophylla* could be germinated better when used mature seeds. The further works need to be conducted to achieve beside of the experiments from this studies.

5.11 AFLP analysis for genetic diversity of *Sirindhornia* spp.

5.11.1 Genetic diversity within species

Over all, most of genetic diversity is estimated in other AFLP studies for other endangered species have ranged only from 0.02-0.20 (Travis *et al.*, 1996; Palacios *et al.*, 1999; Drummond *et al.*, 2000; Gaudeul *et al.*, 2000; Ni *et al.*, 2006). Some critical endanger species such as *Dendrobium officinale* Kimura *et* Migo, endemic to China has high level of genetic diversity (H_T = 0.269) (Li *et al.*, 2008), at least higher than common range over all endangered species above. Likewise, the endangered and endemic plant to Japan, *Cypripedium macranthos* var. *rebunense* has relatively high genetic diversity (H_T = 0.187) (Izawa *et al.*, 2007). Comparing to the same genus, the genetic variation in *C. macranthos* from South Korea revealed rather high (H_T = 0.185) but *C. japonica* revealed no variation either within or among population. The latter species is indicated having very low genetic diversity. Therefore, *Sirindhornia* species appears to have moderate genetic diversity ranged from 0.11-0.15.

5.11.2 Genetic diversity among subpopulation

Previous studies revealed G_{ST} range of 0.103-0.538 for endangered species by AFLP markers. (Travis *et al.*, 1996; Palacios *et al.*, 1999; Guadeul *et al.*, 2000). Comparing to this G_{ST} range value, it can be concluded that the G_{ST} value of 0.167 for *S. mirabilis*, 0.129 for *S. pulchella* and 0.0565 for *S. monophylla* is rather low. This means that each species has rather similar genetic variation.

Absence of strong genetic differentiation between populations can be explained by high gene flow (pollen-mediated and seed mediated) or by the history of populations, such as recent colonization or continue connection between population in the past as a large population (Kim *et al.*, 2006; Chung *et al.*, 2009). For instance, the recent new colonization has been occurred because their dust seeds are supposed to long distantly dispersal. In addition, 2 subpopulations of each species located in the same unique environment and habitat. Thus, it might be recently one large population in the past.

Comparing to all species of the genus, *S. monophylla* has the lowest genetic differentiation. This can be explained that it has the highest gene flow (Nm=8.3541). *S. pulchella* and *S. mirabilis* have gene flow value as 2.4944 and 3.3880, respectively. Lower gene flow value of the latter two species is congruent to their lower reproductive success than of *S. monophylla.*

5.11.3 Genetic diversity within subpopulation

The breeding system is expected influence the genetic structure of plant population (Hamrick and Godt, 1996). For instance, comparative studied of autogamous and allogamous of *Epipactis* species revealed that there are no genetic variation in *E. phyllanthes* which is autogamous species but rather high polymorphism in *E. helleborine* subsp. *helleborine* (mean H_E=0.27), which is allogamous species (Ehlers and Pedersen, 2000). This is congruent with other studied (Chung and Park, 2008; Chung, 2009).

Allogamous *Sirindhornia* spp. expected to have genetic variation value more or less similar to other allogamous species, but they have lower value than of those allogamous species (see above). The cause of reducing of genetic variation in the genus *Sirindhornia* can be

explained by sharing male gamete from the same plant, due to their characteristic of pollinarium and behavior of the pollinator. The sectile pollinia can be separated into several massulae (Figure 4.12). This can be confirmed by our experiment during the studied. Apparently, using a pollinarium touched on several stigmas revealing 100% fruit setting. Connecting to pollinator observation in *S. mirabilis*, it was found that very few pollinators. So far, only one species found to be pollinator which was the small carpenter bee (*Ceratina* sp.). After the pollinarium firmly attached on the frontal part of its head, it still visited the neighboring flowers both from same inflorescence or other inflorescence of different plant.

The population size of *Sirindhornia* species are very small, thus genetic drift is inevitable, which has major impacts on the evolution (Frankham, 2002). Consequently, some specific alleles of the population may be gone, meaning the genetic variation loss. This could be another reason why the genetic diversity of *Sirindhornia* species is rather low. However, it is difficult to know how large population size of each species in the past, but there are some relevant evidences to show that the number of individuals seem to be reduced due to low seed germination and small number of seedling detected.

5.11.4 Comparative genetic diversity of wider distribution vs. local endemics

Rare, endemic, and otherwise geographically restricted species tend to have lower level of genetic diversity than more wide-spread species (Hamrick & Godt, 1990; Ellstrand and Elam, 1993; Frankham, 2002; Cole, 2003; Ni *et al.*, 2006; Gibson *et al.* 2008). Consistent with this trend, there are lower genetic diversity in both *S. mirabilis* (polymorphism = 49.48%; H_T = 0.1285; I_S = 0. 2026) and *S. pulchella* (polymorphism = 38.51%; H_T = 0.1122; I_S = 0. 1738), which are endemic species, than *S. monophylla* (polymorphism = 65.00%; H_T = 0.1524; I_S = 0.2442), which is found in North Myanmar, South China and North Thailand. These differences are a direct result of the interacting processes of selection, genetic drift, mutation and migration (Frankham, 2002).

5.11.5 Conservation implications

One of the global conservation priorities is to conserve genetic diversity, according to the International Union for Conservation of Nature (IUCN), so that the natural living population could have more adaptability to environment changing and disease. Genetic diversity plays a very important role in species survival and adaptability. Increase in genetic diversity is also essential for an organism to evolve. The species that have very little genetic variation are at a great risk for lacking of adaptation to environment changing and disease. Consequently, they might lead to lack of disease resistant or be the cause of extinction. The low value of genetic variation and the loss of genetic diversity may result from inbreeding, genetic drift or genetic bottleneck (Falk and Holsinger, 1991).

The result from this study reveals that all species of the genus *Sirindhornia* has rather low genetic variation. Moreover, there are few number of individual in population and restrict to the specific geographic habitat. Including, low ability of germination and low number seedling were found at least in the 3 years study (Figure 4.1). Thus, three species of the genus *Sirindhornia* are vulnerable species which need special care to conserve genetic and natural population. Furthermore, *S. monophylla* can also be found in Myanmar but there is no information available about their status. However, it is still a vulnerable species for Thailand.

To conserve the natural populations and genetic diversity, it needs to well protect the habitat. Moreover, conserving the mature individuals in natural populations need to be concerned, because they can produce flower for reproduction and distribute gene through the progeny.

The population size of this genus has been found small. Taking off any an individual and seed should be avoided due to genetic drift which led to loss of genetic diversity of the population. Moreover, the preliminary study on *in vitro* propagation does not seem to be easy. So, further propagation or program should be concern. It needs special technique, especially seed physiology. Recently, many orchid conservation projects have been succeeded on symbiosis germination. However, the suitable mycorrhiza for promote seed germination needs to be more investigated.

Due to low genetic diversity of these orchid species, increasing of genetic diversity needs to be consulted with scientific study. It should be studies dynamic population in the long term permanent plots. One possibility is cross hand pollination to increase genetic variation in their population and compare with the non treatment plot. The further result will inform us whether this performance is appropriate or not.

In Thailand, none studies concern with endanger species permanent plots have been initiated. To achieve the threatened species, the conservation point needs to be integrated among researcher, forest staff and local people. The forest staff is one who can help about collecting data in the natural permanent plot after training from the researcher. Finally, local people should be informed the knowledge base on this study for public awareness.

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APPENDIX

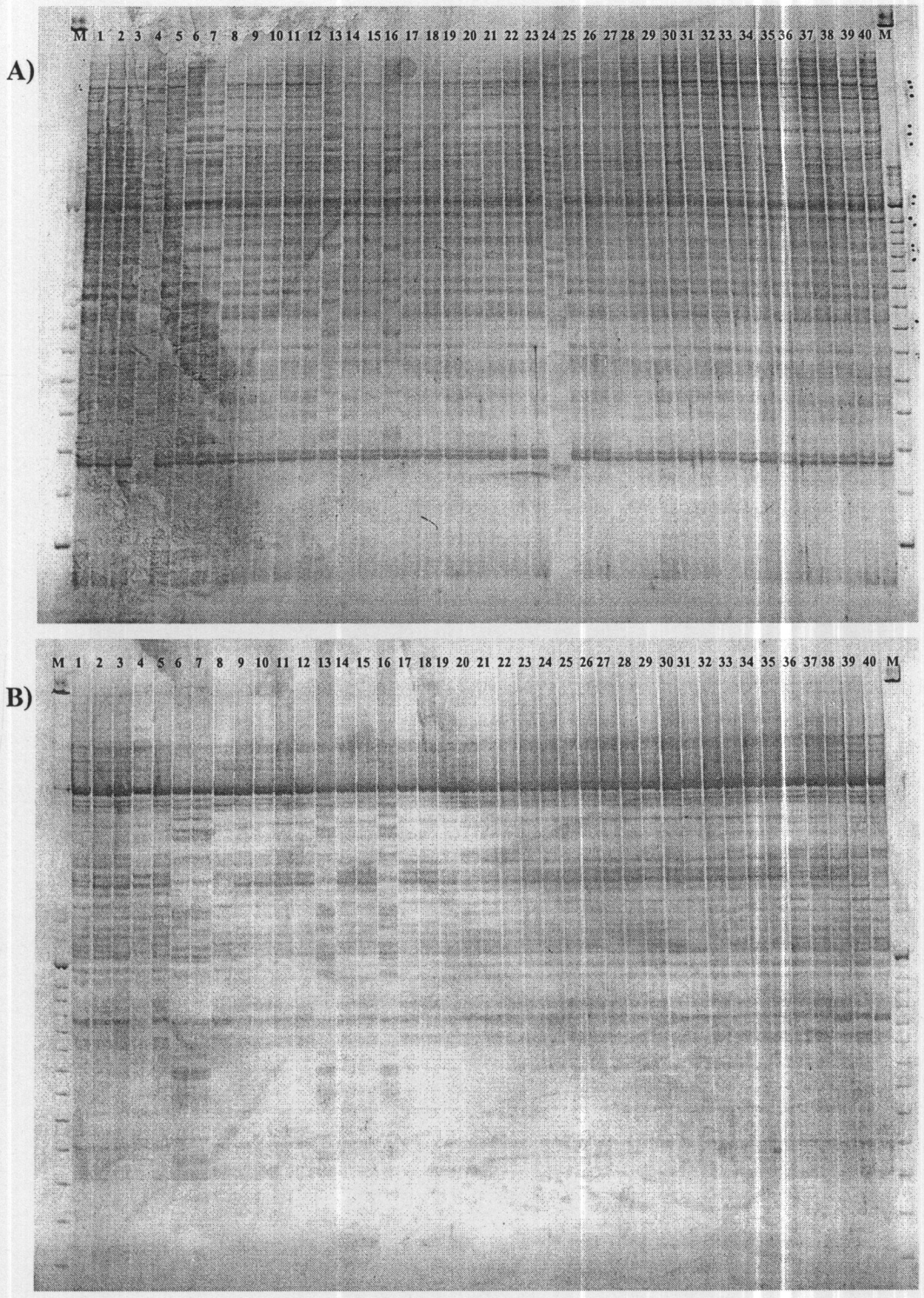

Figure 1 Amplified Fragment Length Polymorphism profiles of *S. mirabilis* all 40 individual with primer pair E-AAC/M-CAA (A) and E-ACC/M-CTC (B), Lane M: DNA marker.

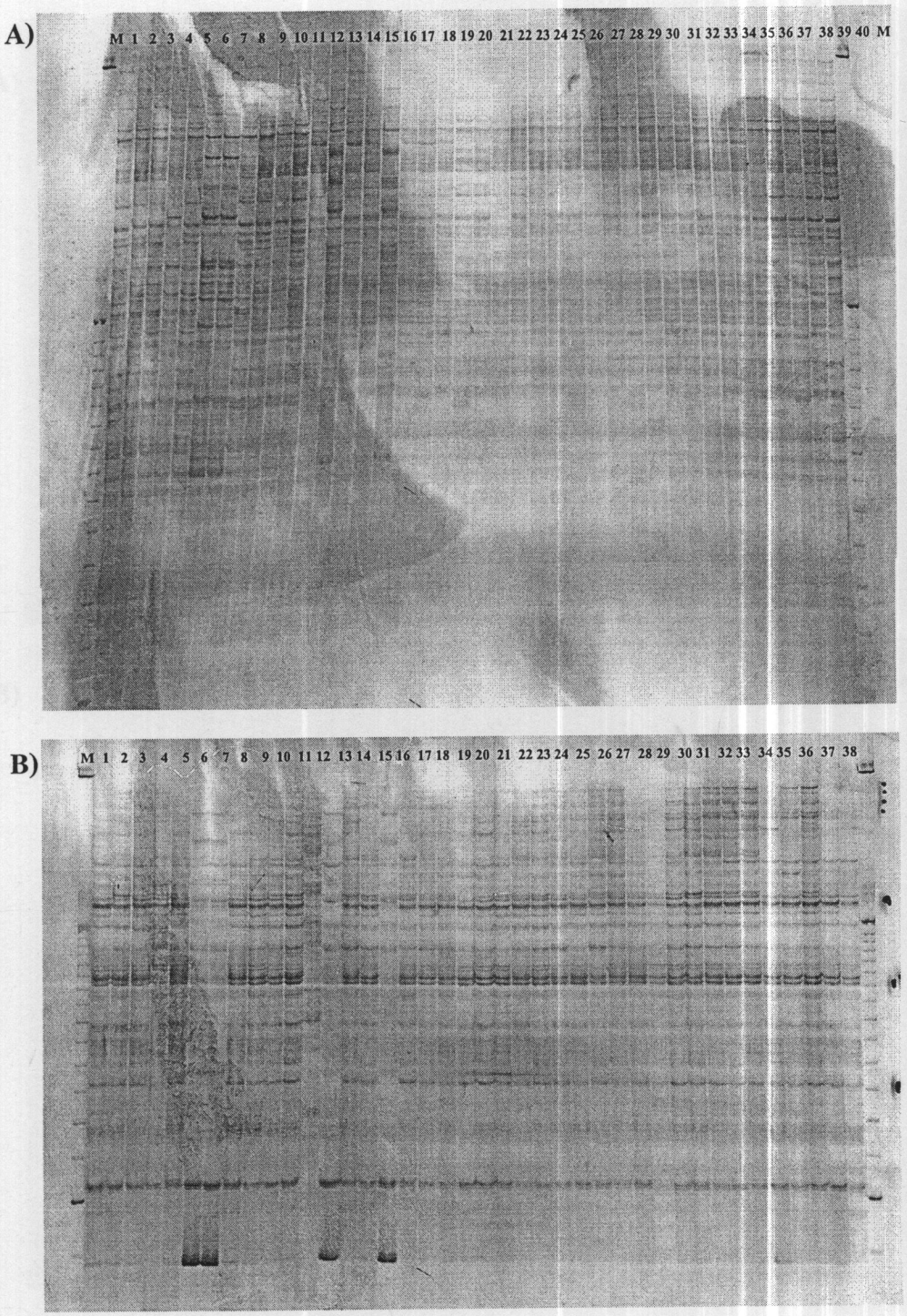

Figure 2 Amplified Fragment Length Polymorphism profiles of *S. mirabilis* all 40 individual with primer pair E-AGG/M-CAA (A) and E-AGG/M-CAC (B), Lane M: DNA marker.

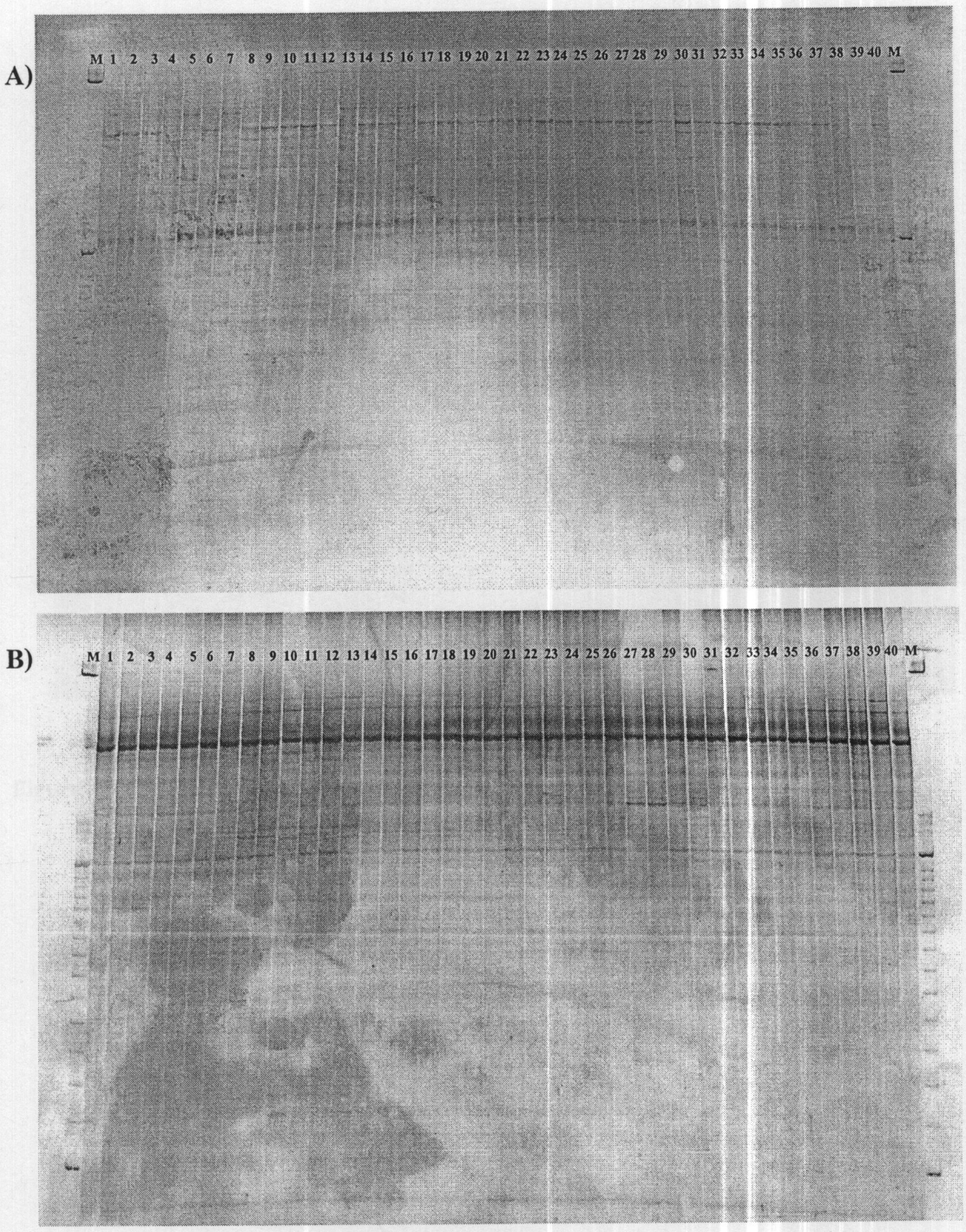

Figure 3 Amplified Fragment Length Polymorphism profiles of *S. monophylla* all 40 individual with primer pair E-AAC/M-CAA (A) and E-ACG/M-CTC (B), Lane M: DNA marker.

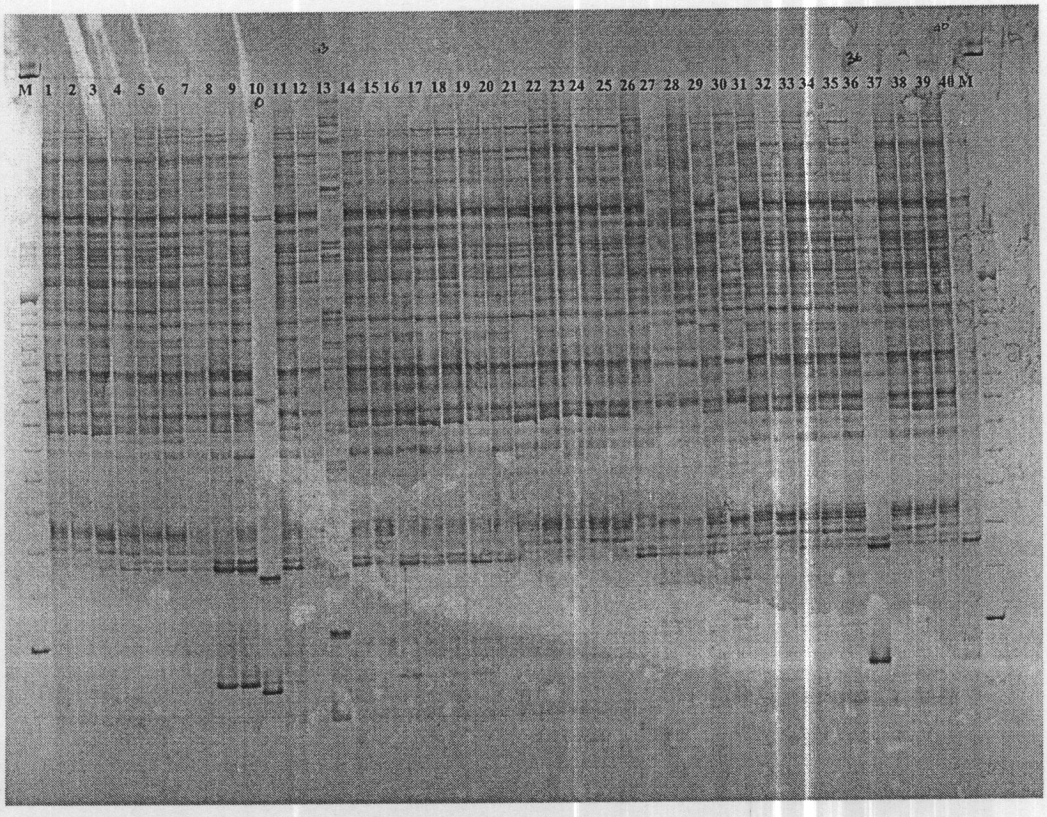

Figure 4 Amplified Fragment Length Polymorphism profiles of *S. monophylla* all 40 individual with primer pair E-AGG/M-CAA, Lane M: DNA marker.

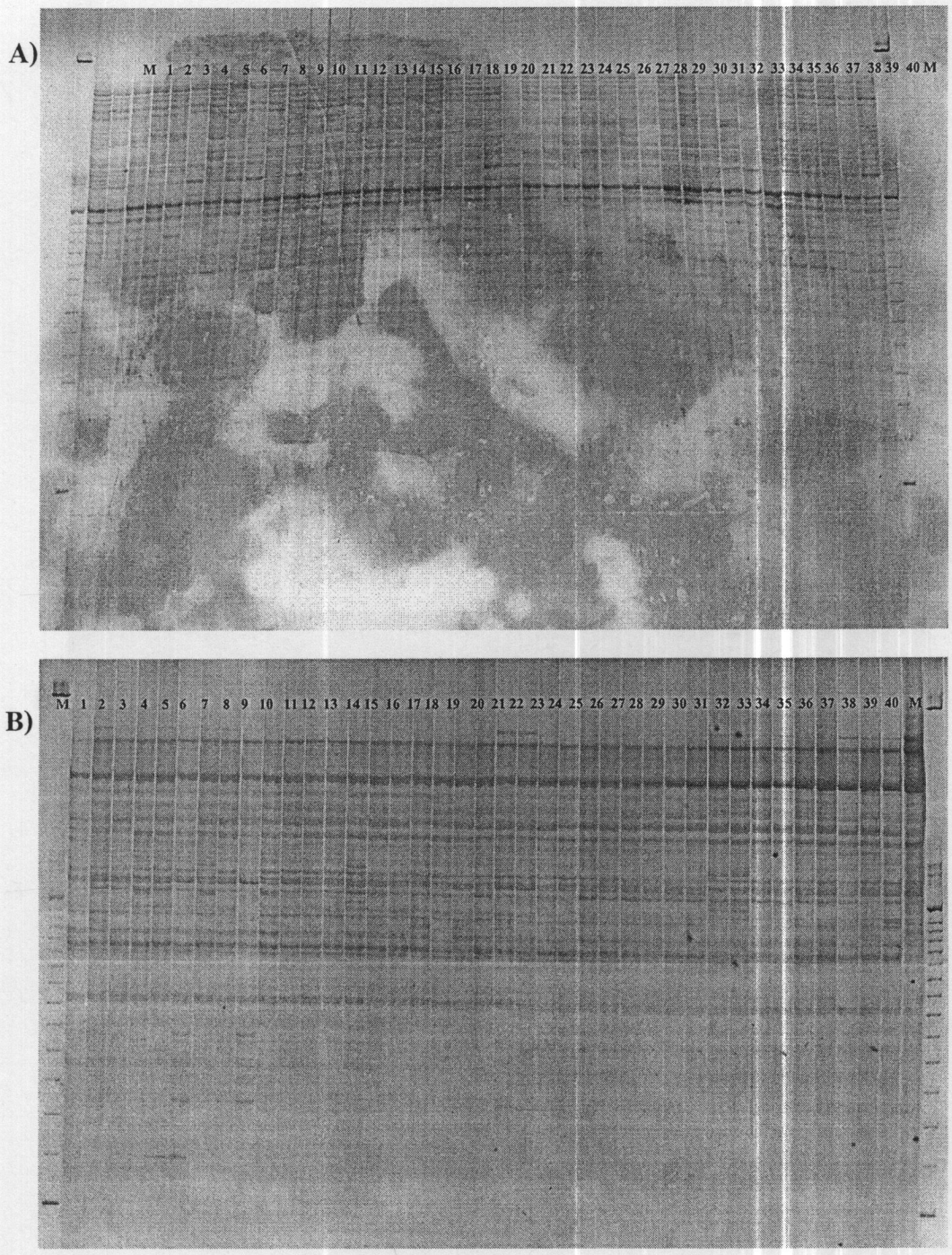

Figure 5 Amplified Fragment Length Polymorphism profiles of *S. pulchella* all 40 individual with primer pair E-AAC/M-CAA (A) and E-ACC/M-CTC (B), Lane M: DNA marker.

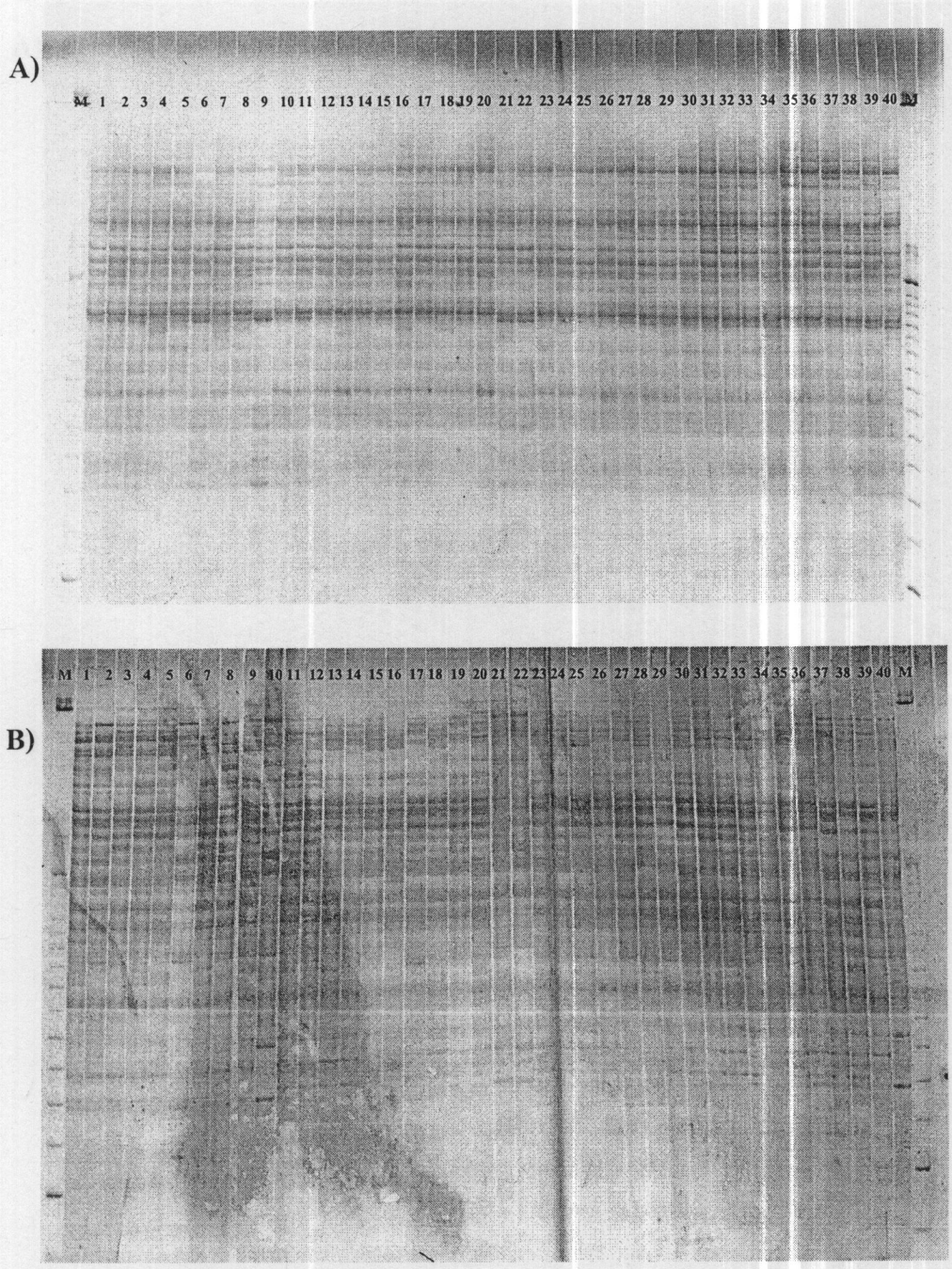

Figure 6 Amplified Fragment Length Polymorphism profiles of *S. pulchella* all 40 individual with primer pair E-ACC/M-CTT (A) and E-ACG/ M-CAC (B), Lane M: DNA marker.

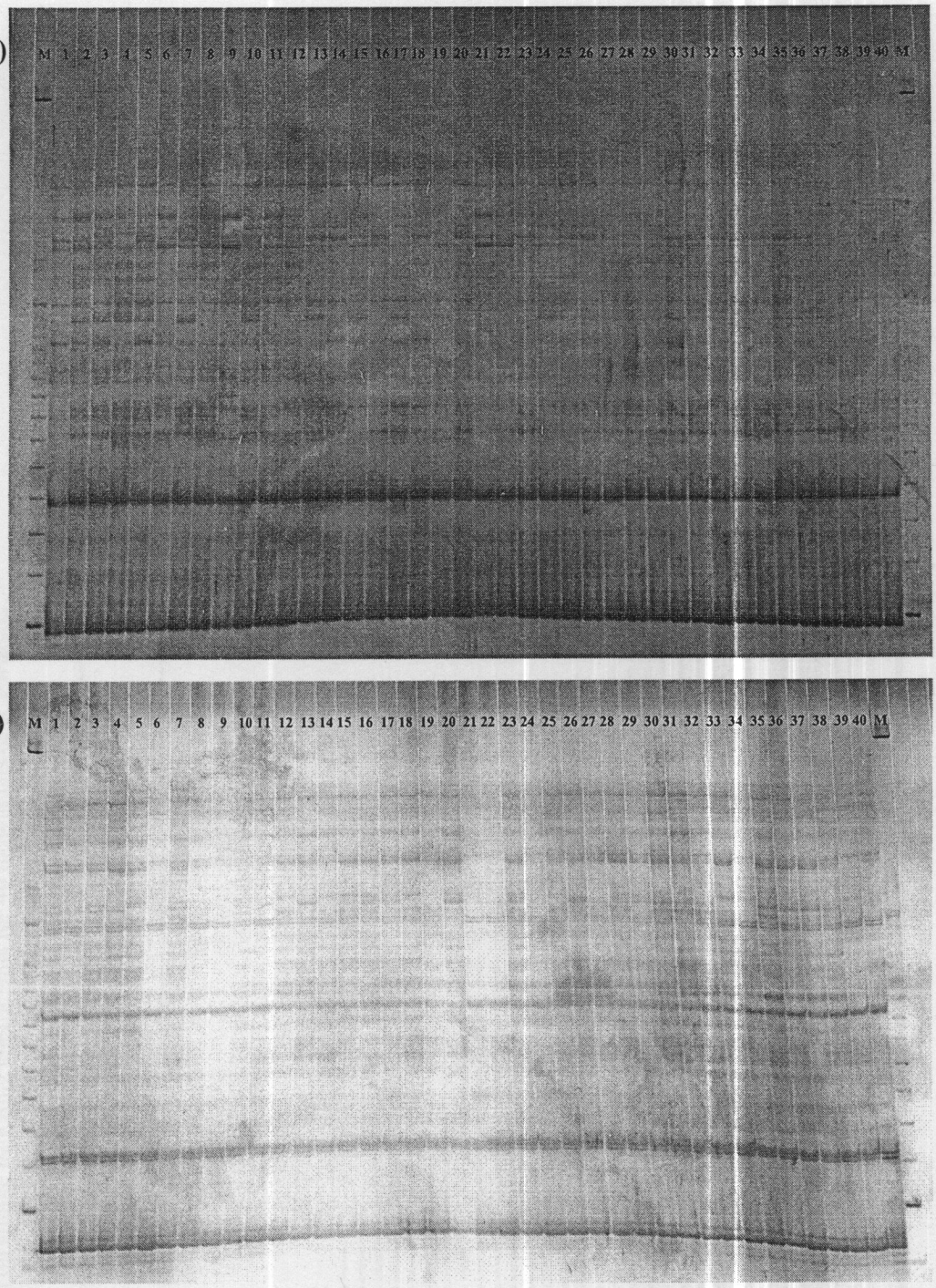

Figure 7 Amplified Fragment Length Polymorphism profiles of *S. pulchella* all 40 individual with primer pair E-ACG/M-CTC (A) and E-AGG/M-CAA (B), Lane M: DNA marker.

Table 1 Similarity matrix based on simple matching coefficients for 40 individuals of *S. mirabilis*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	********																																							
2	1.0000000	********																																						
3	1.0000000	1.0000000	********																																					
4	0.8974359	0.8974359	0.8974359	********																																				
5	0.9740260	0.9740260	0.9740260	0.8750000	********																																			
6	0.6666667	0.6666667	0.6666667	0.6444444	0.6521739	********																																		
7	0.6593407	0.6593407	0.6593407	0.6371628	0.6451613	0.9868421	********																																	
8	0.9610390	0.9610390	0.9610390	0.8625000	0.9870130	0.6593407	0.6521739	********																																
9	0.9615385	0.9615385	0.9615385	0.8641975	0.9871795	0.6451613	0.6382979	0.9743590	********																															
10	0.9605263	0.9605263	0.9605263	0.9090909	0.9358974	0.6555556	0.6483516	0.9230769	0.9487179	********																														
11	0.9605263	0.9605263	0.9605263	0.9090909	0.9358974	0.6555556	0.6483516	0.9230769	0.9487179	0.9733333	********																														
12	0.8684211	0.8684211	0.8684211	0.8181818	0.8461538	0.6705882	0.6627907	0.8333333	0.8589744	0.8800000	0.9054054	********																												
13	0.6966292	0.6966292	0.6966292	0.6741573	0.6813187	0.9358974	0.9487179	0.6703297	0.6739130	0.6853933	0.6853933	0.7023810	********																										
14	0.9480519	0.9480519	0.9480519	0.8974359	0.9240506	0.6483516	0.6413043	0.9113924	0.9367089	0.9866667	0.9605263	0.8684211	0.6777778	********																										
15	0.9350649	0.9350649	0.9350649	0.9090909	0.9113924	0.6555556	0.6483516	0.8987342	0.9240506	0.9733333	0.9733333	0.8800000	0.6853933	0.9866667	********																										
16	0.6966292	0.6966292	0.6966292	0.6741573	0.6813187	0.9358974	0.9487179	0.6703297	0.6739130	0.6853933	0.6853933	0.7023810	1.0000000	0.6777778	0.6853933	********																										
17	0.9230769	0.9230769	0.9230769	0.8974359	0.9000000	0.6853933	0.6777778	0.8875000	0.9125000	0.9605263	0.9350649	0.8641558	0.7159091	0.9480519	0.9350649	0.7159091	********																										
18	0.9358974	0.9358974	0.9358974	0.8860759	0.9125000	0.6777778	0.6703297	0.9000000	0.9250000	0.9480519	0.9480519	0.8571429	0.7078652	0.9358974	0.9230769	0.7078652	0.9868421	********																										
19	0.9358974	0.9358974	0.9358974	0.8860759	0.9125000	0.6777778	0.6703297	0.9000000	0.9250000	0.9480519	0.9480519	0.8571429	0.7078652	0.9358974	0.9230769	0.7078652	0.9868421	1.0000000	********																									
20	0.9367089	0.9367089	0.9367089	0.8641975	0.9620253	0.6813187	0.6739130	0.9493671	0.9500000	0.9000000	0.9000000	0.8125000	0.7111111	0.8888889	0.8765432	0.7111111	0.9367089	0.9493671	0.9493671	********																								
21	0.9610390	0.9610390	0.9610390	0.8860759	0.9367089	0.6966292	0.6888889	0.9240506	0.9250000	0.9230769	0.9230769	0.8333333	0.7272727	0.9113924	0.8987342	0.7272727	0.9610390	0.9740260	0.9740260	0.9743590	********																								
22	0.9610390	0.9610390	0.9610390	0.8860759	0.9367089	0.6966292	0.6888889	0.9240506	0.9250000	0.9230769	0.9230769	0.8333333	0.7272727	0.9113924	0.8987342	0.7272727	0.9610390	0.9740260	0.9740260	0.9743590	1.0000000	********																							
23	0.9615385	0.9615385	0.9615385	0.8875000	0.9375000	0.6813187	0.6739130	0.9250000	0.9500000	0.9487179	0.9487179	0.8589744	0.7111111	0.9367089	0.9240506	0.7111111	0.9615385	0.9743590	0.9743590	0.9500000	0.9743590	0.9743590	********																							
24	0.9000000	0.9000000	0.9000000	0.9230769	0.8780488	0.6344086	0.6276596	0.8658537	0.8902439	0.8875000	0.8875000	0.8000000	0.6630435	0.8765432	0.8641975	0.6630435	0.9000000	0.9125000	0.9125000	0.8902439	0.9125000	0.9125000	0.9375000	********																						
25	0.9615385	0.9615385	0.9615385	0.8875000	0.9375000	0.6813187	0.6739130	0.9250000	0.9500000	0.9487179	0.9487179	0.8589744	0.7111111	0.9367089	0.9240506	0.7111111	0.9615385	0.9743590	0.9743590	0.9500000	0.9743590	0.9743590	1.0000000	0.9375000	********																					
26	0.9375000	0.9375000	0.9375000	0.8658537	0.9625000	0.6666667	0.6595745	0.9500000	0.9750000	0.9250000	0.9250000	0.8375000	0.6956522	0.9135802	0.9012346	0.6956522	0.9375000	0.9500000	0.9500000	0.9750000	0.9500000	0.9500000	0.9750000	0.9146341	0.9750000	********																				
27	0.9615385	0.9615385	0.9615385	0.8875000	0.9375000	0.6813187	0.6739130	0.9250000	0.9500000	0.9487179	0.9487179	0.8589744	0.7111111	0.9367089	0.9240506	0.7111111	0.9615385	0.9743590	0.9743590	0.9500000	0.9743590	0.9743590	1.0000000	0.9375000	1.0000000	0.9750000	********																			
28	0.9615385	0.9615385	0.9615385	0.8875000	0.9375000	0.6813187	0.6739130	0.9250000	0.9500000	0.9487179	0.9487179	0.8589744	0.7111111	0.9367089	0.9240506	0.7111111	0.9615385	0.9743590	0.9743590	0.9500000	0.9743590	0.9743590	1.0000000	0.9375000	1.0000000	0.9750000	1.0000000	********																		
29	0.9493671	0.9493671	0.9493671	0.8765432	0.9259259	0.6739130	0.6666667	0.9135802	0.9382716	0.9367089	0.9367089	0.8481013	0.7032967	0.9493671	0.9367089	0.7032967	0.9493671	0.9620253	0.9620253	0.9382716	0.9620253	0.9620253	0.9873418	0.9259259	0.9873418	0.9629630	0.9873418	********																		
30	0.9102564	0.9102564	0.9102564	0.8607595	0.8875000	0.7126437	0.7045455	0.8750000	0.9000000	0.9220779	0.8974359	0.8000000	0.7441860	0.9102564	0.8974359	0.7441860	0.9350649	0.9230769	0.9230769	0.9000000	0.9230769	0.9230769	0.9487179	0.8875000	0.9487179	0.9250000	0.9487179	0.9487179	0.9367089	********																		
31	0.9259259	0.9259259	0.9259259	0.8554217	0.9506173	0.6595745	0.6526316	0.9382716	0.9629630	0.9135802	0.9135802	0.8271605	0.6881720	0.9259259	0.9135802	0.6881720	0.9259259	0.9382716	0.9382716	0.9629630	0.9382716	0.9382716	0.9629630	0.9036145	0.9629630	0.9876543	0.9629630	0.9629630	0.9753086	0.9135802	********																		
32	0.9259259	0.9259259	0.9259259	0.8554217	0.9506173	0.6595745	0.6526316	0.9382716	0.9629630	0.9135802	0.9135802	0.8271605	0.6881720	0.9259259	0.9135802	0.6881720	0.9259259	0.9382716	0.9382716	0.9629630	0.9382716	0.9382716	0.9629630	0.9036145	0.9629630	0.9876543	0.9629630	0.9629630	0.9753086	0.9135802	1.0000000	********																		
33	0.9375000	0.9375000	0.9375000	0.8658537	0.9625000	0.6666667	0.6595745	0.9500000	0.9750000	0.9250000	0.9250000	0.8375000	0.6956522	0.9135802	0.9012346	0.6956522	0.9375000	0.9500000	0.9500000	0.9750000	0.9500000	0.9500000	0.9750000	0.9146341	0.9750000	1.0000000	0.9750000	0.9750000	0.9629630	0.9250000	0.9876543	0.9876543	********																		
34	0.9375000	0.9375000	0.9375000	0.8658537	0.9625000	0.6666667	0.6595745	0.9500000	0.9750000	0.9250000	0.9250000	0.8375000	0.6956522	0.9135802	0.9012346	0.6956522	0.9375000	0.9500000	0.9500000	0.9750000	0.9500000	0.9500000	0.9750000	0.9146341	0.9750000	1.0000000	0.9750000	0.9750000	0.9629630	0.9250000	0.9876543	0.9876543	1.0000000	********																		
35	0.9493671	0.9493671	0.9493671	0.8765432	0.9259259	0.6739130	0.6666667	0.9135802	0.9382716	0.9367089	0.9367089	0.8481013	0.7032967	0.9493671	0.9367089	0.7032967	0.9493671	0.9620253	0.9620253	0.9382716	0.9620253	0.9620253	0.9873418	0.9259259	0.9873418	0.9629630	0.9873418	0.9873418	1.0000000	0.9367089	0.9753086	0.9753086	0.9629630	0.9629630	********																		
36	0.9740260	0.9740260	0.9740260	0.8987342	0.9493671	0.6888889	0.6813187	0.9367089	0.9375000	0.9358974	0.9358974	0.8461538	0.7191011	0.9240506	0.9113924	0.7191011	0.9487179	0.9615385	0.9615385	0.9620253	0.9870130	0.9870130	0.9871795	0.9250000	0.9871795	0.9625000	0.9871795	0.9871795	0.9746835	0.9358974	0.9506173	0.9506173	0.9625000	0.9625000	0.9746835	********																		
37	0.9615385	0.9615385	0.9615385	0.8875000	0.9375000	0.6813187	0.6739130	0.9250000	0.9259259	0.9240506	0.9240506	0.8354430	0.7111111	0.9367089	0.9240506	0.7111111	0.9367089	0.9493671	0.9493671	0.9500000	0.9743590	0.9743590	0.9746835	0.9135802	0.9746835	0.9506173	0.9746835	0.9746835	0.9873418	0.9240506	0.9629630	0.9629630	0.9506173	0.9506173	0.9873418	0.9871795	********																	
38	0.9615385	0.9615385	0.9615385	0.8875000	0.9375000	0.6813187	0.6739130	0.9250000	0.9259259	0.9240506	0.9240506	0.8354430	0.7111111	0.9367089	0.9240506	0.7111111	0.9367089	0.9493671	0.9493671	0.9500000	0.9743590	0.9743590	0.9746835	0.9135802	0.9746835	0.9506173	0.9746835	0.9746835	0.9873418	0.9240506	0.9629630	0.9629630	0.9506173	0.9506173	0.9873418	0.9871795	1.0000000	********																
39	0.9740260	0.9740260	0.9740260	0.8987342	0.9493671	0.6888889	0.6813187	0.9367089	0.9375000	0.9358974	0.9358974	0.8461538	0.7191011	0.9240506	0.9113924	0.7191011	0.9487179	0.9615385	0.9615385	0.9620253	0.9870130	0.9870130	0.9871795	0.9250000	0.9871795	0.9625000	0.9871795	0.9871795	0.9746835	0.9358974	0.9506173	0.9506173	0.9625000	0.9625000	0.9746835	0.9871795	0.9871795	********																
40	0.9740260	0.9740260	0.9740260	0.8987342	0.9493671	0.6888889	0.6813187	0.9367089	0.9375000	0.9358974	0.9358974	0.8461538	0.7191011	0.9240506	0.9113924	0.7191011	0.9487179	0.9615385	0.9615385	0.9620253	0.9870130	0.9870130	0.9871795	0.9250000	0.9871795	0.9625000	0.9871795	0.9871795	0.9746835	0.9358974	0.9506173	0.9506173	0.9625000	0.9625000	0.9746835	1.0000000	0.9871795	0.9871795	1.0000000	********

Table 2 Similarity matrix based on simple matching coefficients for 40 individuals of *S. monophylla*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	********																																								
2	1.0000000	********																																							
3	1.0000000	1.0000000	********																																						
4	0.9545455	0.9545455	0.9545455	********																																						
5	0.9166667	0.9166667	0.9166667	0.8750000	********																																					
6	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	********																																				
7	0.8750000	0.8750000	0.8750000	0.9130435	0.9583333	0.8750000	********																																			
8	0.9347826	0.9347826	0.9347826	0.8913043	0.8600000	0.9347826	0.8200000	********																																		
9	0.9347826	0.9347826	0.9347826	0.8913043	0.8600000	0.9347826	0.8200000	0.9565217	********																																	
10	0.5576923	0.5576923	0.5576923	0.5192308	0.5178571	0.5576923	0.4821429	0.5769231	0.5471698	********																																
11	0.9565217	0.9565217	0.9565217	0.9130435	0.8800000	0.9565217	0.8400000	0.9782609	0.9782609	0.5660377	********																															
12	0.9777778	0.9777778	0.9777778	0.9333333	0.8979592	0.9777778	0.8571429	0.9565217	0.9565217	0.5471698	0.9782609	********																														
13	0.6800000	0.6800000	0.6800000	0.7083333	0.6603774	0.6800000	0.6862745	0.6346154	0.6346154	0.5098039	0.6538462	0.6666667	********																												
14	0.8936170	0.8936170	0.8936170	0.8510638	0.8235294	0.8936170	0.7843137	0.9148936	0.9565217	0.5185185	0.9361702	0.9148936	0.6346154	********																											
15	0.9555556	0.9555556	0.9555556	0.9111111	0.8775510	0.9555556	0.8367347	0.8936170	0.9347826	0.5283019	0.9148936	0.9347826	0.6800000	0.9347826	********																											
16	0.8723404	0.8723404	0.8723404	0.9111111	0.8400000	0.8723404	0.8750000	0.8541667	0.8936170	0.5000000	0.8750000	0.8541667	0.7142857	0.8936170	0.9130435	********																										
17	0.9347826	0.9347826	0.9347826	0.8913043	0.8600000	0.9347826	0.8200000	0.9148936	0.9565217	0.5471698	0.9361702	0.9148936	0.6666667	0.9565217	0.9777778	0.9347826	********																										
18	0.9347826	0.9347826	0.9347826	0.8913043	0.8600000	0.9347826	0.8200000	0.9148936	0.9565217	0.5471698	0.9361702	0.9148936	0.6666667	0.9565217	0.9777778	0.9347826	1.0000000	********																										
19	0.9347826	0.9347826	0.9347826	0.8913043	0.8600000	0.9347826	0.8200000	0.9148936	0.9565217	0.5471698	0.9361702	0.9148936	0.6666667	0.9565217	0.9777778	0.9347826	1.0000000	1.0000000	********																									
20	0.9772727	0.9772727	0.9772727	0.9318182	0.8958333	0.9772727	0.8541667	0.9130435	0.9555556	0.5384615	0.9347826	0.9555556	0.6600000	0.9130435	0.9772727	0.8913043	0.9555556	0.9555556	0.9555556	********																								
21	0.9333333	0.9333333	0.9333333	0.8888889	0.8571429	0.9333333	0.8163265	0.8723404	0.9130435	0.5384615	0.8936170	0.9130435	0.6938776	0.9130435	0.9772727	0.8913043	0.9555556	0.9555556	0.9555556	0.9545455	********																								
22	0.9777778	0.9777778	0.9777778	0.9333333	0.8979592	0.9777778	0.8571429	0.9148936	0.9148936	0.5471698	0.9361702	0.9565217	0.7000000	0.9148936	0.9777778	0.8936170	0.9565217	0.9565217	0.9565217	0.9555556	0.9555556	********																								
23	0.9777778	0.9777778	0.9777778	0.9333333	0.8979592	0.9777778	0.8571429	0.9148936	0.9148936	0.5471698	0.9361702	0.9565217	0.7000000	0.9148936	0.9777778	0.8936170	0.9565217	0.9565217	0.9565217	0.9555556	0.9555556	1.0000000	********																							
24	0.9555556	0.9555556	0.9555556	0.9111111	0.8775510	0.9555556	0.8367347	0.8936170	0.9347826	0.5283019	0.9148936	0.9347826	0.6800000	0.9347826	1.0000000	0.9130435	0.9777778	0.9777778	0.9777778	0.9772727	0.9772727	0.9777778	0.9777778	********																						
25	0.9555556	0.9555556	0.9555556	0.9111111	0.8775510	0.9555556	0.8367347	0.8936170	0.9347826	0.5283019	0.9148936	0.9347826	0.6800000	0.9347826	1.0000000	0.9130435	0.9777778	0.9777778	0.9777778	0.9772727	0.9772727	0.9777778	0.9777778	1.0000000	********																						
26	0.9333333	0.9333333	0.9333333	0.8888889	0.8571429	0.9333333	0.8163265	0.8723404	0.8723404	0.5686275	0.8936170	0.9130435	0.6600000	0.8333333	0.8913043	0.8125000	0.8723404	0.8723404	0.8723404	0.9111111	0.9111111	0.9130435	0.9130435	0.8913043	0.8913043	********																					
27	0.7755102	0.7755102	0.7755102	0.7346939	0.7169811	0.7755102	0.6792453	0.7600000	0.7600000	0.5384615	0.7800000	0.7959184	0.7291667	0.7600000	0.7755102	0.7058824	0.7600000	0.7600000	0.7600000	0.7551020	0.7916667	0.7959184	0.7959184	0.7755102	0.7755102	0.8297872	********																				
28	0.8750000	0.8750000	0.8750000	0.8333333	0.8076923	0.8750000	0.7692308	0.8571429	0.8571429	0.5090909	0.8775510	0.8958333	0.7551020	0.8571429	0.8750000	0.8000000	0.8571429	0.8571429	0.8571429	0.8541667	0.8541667	0.8958333	0.8958333	0.8750000	0.8750000	0.8163265	0.8936170	********																			
29	0.9333333	0.9333333	0.9333333	0.8888889	0.8571429	0.9333333	0.8163265	0.8723404	0.8723404	0.5686275	0.8936170	0.9130435	0.6600000	0.8333333	0.8913043	0.8125000	0.8723404	0.8723404	0.8723404	0.9111111	0.9111111	0.9130435	0.9130435	0.8913043	0.8913043	1.0000000	0.8297872	0.8163265	********																		
30	0.8163265	0.8163265	0.8163265	0.7755102	0.7547170	0.8163265	0.7169811	0.8000000	0.8367347	0.5471698	0.8200000	0.8367347	0.6666667	0.8367347	0.8541667	0.7800000	0.8367347	0.8367347	0.8367347	0.8333333	0.8333333	0.8367347	0.8367347	0.8541667	0.8541667	0.7600000	0.7600000	0.8571429	0.7600000	********																		
31	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.9361702	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	********																	
32	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	********																
33	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	1.0000000	********															
34	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	1.0000000	1.0000000	********													
35	0.9772727	0.9772727	0.9772727	0.9318182	0.8958333	0.9772727	0.8541667	0.9130435	0.9130435	0.5686275	0.9347826	0.9555556	0.6938776	0.8723404	0.9333333	0.8510638	0.9130435	0.9130435	0.9130435	0.9545455	0.9111111	0.9555556	0.9555556	0.9333333	0.9333333	0.9111111	0.7551020	0.8541667	0.9111111	0.7959184	0.9772727	0.9772727	0.9772727	0.9772727	********											
36	0.8000000	0.8000000	0.8000000	0.7555556	0.7346939	0.8000000	0.6938776	0.8222222	0.8222222	0.5744681	0.8043478	0.7826087	0.6382979	0.8222222	0.8000000	0.7608696	0.8222222	0.8222222	0.8222222	0.8181818	0.7777778	0.7826087	0.7826087	0.8000000	0.8000000	0.7391304	0.7021277	0.7291667	0.7391304	0.6734694	0.8000000	0.8000000	0.8000000	0.8000000	0.8181818	********									
37	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	1.0000000	1.0000000	1.0000000	0.9772727	0.8000000	********							
38	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	1.0000000	1.0000000	1.0000000	0.9772727	0.8000000	1.0000000	********					
39	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	1.0000000	1.0000000	1.0000000	0.9772727	0.8000000	1.0000000	1.0000000	********				
40	1.0000000	1.0000000	1.0000000	0.9545455	0.9166667	1.0000000	0.8750000	0.9347826	0.9347826	0.5576923	0.9565217	0.9777778	0.6800000	0.8936170	0.9555556	0.8723404	0.9347826	0.9347826	0.9347826	0.9772727	0.9333333	0.9777778	0.9777778	0.9555556	0.9555556	0.9333333	0.7755102	0.8750000	0.9333333	0.8163265	1.0000000	1.0000000	1.0000000	1.0000000	0.9772727	0.8000000	1.0000000	1.0000000	1.0000000	********			

Table 3 Similarity matrix based on simple matching coefficients for 40 individual of *S. pulchella*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	********																																							
2	0.9612403	********																																							
3	0.9248120	0.9172932	********																																						
4	0.8955224	0.8740741	0.9104478	********																																					
5	0.9037037	0.8823529	0.9473684	0.9618321	********																																				
6	0.8656716	0.8582090	0.8529412	0.8518519	0.8467153	********																																			
7	0.9085109	0.8832117	0.9051095	0.9044118	0.9264706	0.8613139	********																																		
8	0.8897059	0.8823529	0.8905109	0.9179104	0.9259259	0.8880597	0.9407407	********																																	
9	0.9097744	0.8880597	0.8686131	0.8814815	0.8759124	0.8796992	0.8633094	0.8897059	********																																
10	0.8750000	0.8955224	0.9037037	0.8888889	0.9253731	0.8321168	0.8978102	0.9253731	0.8613139	********																															
11	0.8686131	0.8888889	0.9111111	0.8823529	0.9185185	0.8394161	0.8913043	0.9044118	0.8823529	0.9323308	********																														
12	0.8888889	0.9097744	0.9037037	0.8888889	0.9111111	0.8455882	0.8978102	0.9111111	0.8888889	0.9248120	0.9769231	********																														
13	0.9185185	0.9253731	0.9333333	0.9185185	0.9407407	0.8750000	0.9270073	0.9264706	0.9044118	0.9402985	0.9477612	0.9548872	********																												
14	0.8714286	0.8776978	0.8992806	0.8714286	0.9064748	0.8428571	0.9071429	0.9064748	0.8714286	0.9197080	0.9411765	0.9338235	0.9489051	********																											
15	0.8970588	0.9037037	0.9402985	0.8970588	0.9333333	0.8540146	0.9197080	0.9191176	0.8832117	0.9473684	0.9402985	0.9473684	0.9774436	0.9416058	********																											
16	0.8897059	0.9104478	0.9044118	0.8897059	0.9117647	0.8602941	0.9264706	0.9402985	0.8759124	0.9253731	0.9473684	0.9545455	0.9552239	0.9343066	0.9477612	********																											
17	0.8666667	0.8731343	0.8814815	0.8666667	0.8750000	0.8507463	0.8759124	0.8888889	0.8394161	0.9022556	0.8955224	0.9022556	0.9179104	0.8978102	0.9303939	0.9029851	********																										
18	0.8823529	0.8750000	0.9253731	0.8962963	0.9185185	0.8394161	0.9191176	0.9185185	0.8823529	0.9179104	0.9111111	0.9179104	0.9477612	0.9130435	0.9696970	0.9328358	0.9097744	********																									
19	0.8814815	0.8880597	0.9104478	0.9097744	0.9037037	0.8656716	0.8905109	0.9037037	0.8814815	0.9172932	0.9248120	0.9029851	0.9473684	0.9124088	0.9398496	0.9179104	0.9234641	0.9104478	********																							
20	0.8888889	0.8814815	0.9179104	0.8888889	0.9253731	0.8592593	0.9117647	0.9253731	0.8888889	0.9248120	0.9469697	0.9303939	0.9548872	0.9481481	0.9473684	0.9398496	0.9022556	0.9469697	0.9172932	********																						
21	0.8897059	0.8823529	0.8633094	0.8623188	0.8705036	0.9166667	0.8848921	0.8978102	0.9037037	0.8695652	0.8768116	0.8832117	0.9124088	0.8794326	0.8913043	0.8978102	0.8478261	0.8768116	0.8897059	0.8832117	********																				
22	0.8905109	0.8695652	0.8642857	0.8500000	0.8714286	0.9029851	0.8857143	0.8905109	0.8705036	0.8776978	0.8705036	0.8992806	0.9071429	0.8785714	0.8848921	0.8489209	0.8776978	0.8768116	0.8978102	0.9552239	********																				
23	0.9037037	0.8823529	0.9185185	0.8897059	0.9259259	0.8740741	0.9124088	0.9259259	0.9037037	0.9111111	0.9328358	0.9398496	0.9552239	0.9343066	0.9477612	0.9402985	0.8888889	0.9328358	0.9037037	0.8694656	0.8978102	0.8985507	********																			
24	0.9037037	0.8823529	0.9185185	0.9037037	0.9117647	0.8602941	0.9124088	0.9117647	0.9037037	0.9111111	0.9044118	0.9111111	0.9552239	0.9064748	0.9477612	0.9259259	0.8888889	0.9473684	0.9179104	0.9398496	0.8840580	0.8848921	0.9548872	********																		
25	0.9323308	0.9104478	0.9185185	0.9037037	0.9259259	0.8880597	0.9264706	0.9259259	0.9037037	0.9111111	0.9185185	0.9253731	0.9699248	0.9202899	0.9477612	0.9402985	0.9029851	0.9328358	0.9323308	0.9545455	0.9117647	0.9124088	0.9696970	0.9696970	********																	
26	0.8970588	0.8897059	0.9259259	0.8832117	0.9191176	0.8676471	0.9057971	0.9051095	0.8832117	0.9185185	0.9259259	0.9185185	0.9481481	0.9416058	0.9407407	0.9191176	0.8962963	0.9259259	0.9111111	0.9621212	0.8776978	0.8920863	0.9624060	0.9477612	0.9624060	********																
27	0.9044118	0.8832117	0.9191176	0.8905109	0.9264706	0.8613139	0.9130435	0.9124088	0.8905109	0.9259259	0.9333333	0.9259259	0.9555556	0.9489051	0.9481481	0.9264706	0.9037037	0.9333333	0.9185185	0.9696970	0.8848921	0.8992806	0.9699248	0.9552239	0.9699248	0.9924242	********														
28	0.9248120	0.9029851	0.9191111	0.8823529	0.9185185	0.8666667	0.9191176	0.9185185	0.8962963	0.9037037	0.9111111	0.9179104	0.9477612	0.9130435	0.9402985	0.9328358	0.8955224	0.9253731	0.9104478	0.9469697	0.9044118	0.8913043	0.9621212	0.9473684	0.9770992	0.9548872	0.9624060	********													
29	0.8888889	0.8814815	0.9179104	0.8888889	0.8970588	0.8731343	0.9117647	0.9111111	0.8750000	0.8962963	0.8897059	0.8962963	0.9259259	0.8920863	0.9328358	0.9253731	0.8880597	0.9323308	0.9172932	0.9248120	0.8970588	0.8705036	0.9398496	0.9545455	0.9545455	0.9473684	0.9402985	0.9618321	********												
30	0.9111111	0.8897059	0.9402985	0.8970588	0.9333333	0.8540146	0.9057971	0.9051095	0.8970588	0.9185185	0.9259259	0.9185185	0.9481481	0.9136691	0.9552239	0.9191176	0.8962963	0.9402985	0.9253731	0.9473684	0.8776978	0.8785714	0.9624060	0.9624060	0.9624060	0.9552239	0.9626866	0.9696970	0.9473684	********										
31	0.8920863	0.8714286	0.9343066	0.8920863	0.9275362	0.8500000	0.9142857	0.9000000	0.8785714	0.9130435	0.9202899	0.9130435	0.9420290	0.9496403	0.9489051	0.9136691	0.8913043	0.9343066	0.9197080	0.9270073	0.8732394	0.9007092	0.9416058	0.9416058	0.9416058	0.9347826	0.9420290	0.9343066	0.9270073	0.9632353	********								
32	0.8913043	0.8705036	0.9057971	0.8642857	0.8992806	0.8357143	0.9000000	0.8857143	0.8776978	0.9124088	0.8920863	0.8985507	0.9275362	0.9352518	0.9343066	0.8992806	0.8768116	0.9197080	0.8913043	0.9124088	0.8591549	0.8865248	0.9270073	0.9270073	0.9270073	0.9202899	0.9275362	0.9338235	0.9124088	0.9485294	0.9708029	********							
33	0.9402985	0.9185185	0.9264706	0.9117647	0.9338235	0.9104478	0.9202899	0.9338235	0.9402985	0.9191176	0.9264706	0.9333333	0.9629630	0.9280576	0.9411765	0.9338235	0.8832117	0.9284706	0.9117647	0.9477612	0.9338235	0.9343066	0.9626866	0.9481481	0.9626866	0.9411765	0.9485294	0.9407407	0.9191176	0.9411765	0.9352518	0.9208633	********						
34	0.8955224	0.8880597	0.8823529	0.8676471	0.8759124	0.8656716	0.8905109	0.9037037	0.8814815	0.8750000	0.8823529	0.8888889	0.9185185	0.8848921	0.8970588	0.9191176	0.8529412	0.8962963	0.8814815	0.9029851	0.8897059	0.8768116	0.9179104	0.9179104	0.9323308	0.9111111	0.9044118	0.9393939	0.9338182	0.9111111	0.8920863	0.9191176	0.9259259	********					
35	0.9179104	0.9104478	0.9328358	0.8897059	0.9259259	0.8740741	0.9124088	0.9117647	0.9037037	0.9253731	0.9328358	0.9253731	0.9552239	0.9343066	0.9477612	0.9259259	0.9029851	0.9328358	0.9179104	0.9545455	0.8840580	0.8985507	0.9548872	0.9402985	0.9548872	0.9772727	0.9699248	0.9621212	0.9398496	0.9624060	0.9416058	0.9411765	0.9481481	0.9323308	********				
36	0.9328358	0.9111111	0.9333333	0.8905109	0.9264706	0.8750000	0.9130435	0.9264706	0.9185185	0.9402985	0.9191176	0.9259259	0.9555556	0.9208633	0.9626866	0.9264706	0.9037037	0.9477612	0.9185185	0.9402985	0.8985507	0.8992806	0.9552239	0.9552239	0.9552239	0.9481481	0.9555556	0.9624060	0.9402985	0.9774436	0.9562044	0.9558824	0.9629630	0.9185185	0.9699248	********			
37	0.9402985	0.9185185	0.9264706	0.8978102	0.9338235	0.8823529	0.9202899	0.9338235	0.9259259	0.9333333	0.9264706	0.9333333	0.9629630	0.9280576	0.9555556	0.9338235	0.8970588	0.9407407	0.9117647	0.9477612	0.9057971	0.9064748	0.9626866	0.9481481	0.9626866	0.9555556	0.9629630	0.9699248	0.9333333	0.9701493	0.9492754	0.9489051	0.9703704	0.9259259	0.9774436	0.9924812	********		
38	0.9328358	0.9253731	0.9333333	0.8905109	0.9264706	0.8888889	0.9130435	0.9264706	0.9185185	0.9259259	0.9191176	0.9259259	0.9555556	0.9208633	0.9481481	0.9264706	0.8897059	0.9333333	0.9044118	0.9402985	0.8985507	0.8992806	0.9552239	0.9407407	0.9552239	0.9626866	0.9555556	0.9624060	0.9402985	0.9626866	0.9402290	0.9416058	0.9629630	0.9328358	0.9848485	0.9849624	0.9924812	********	
39	0.9029851	0.8955224	0.8897059	0.8478261	0.8832117	0.9051552	0.8840580	0.8970588	0.8750000	0.8823529	0.8759124	0.8823529	0.9117647	0.8785714	0.9044118	0.8970588	0.8602941	0.8897059	0.8750000	0.8962963	0.8695652	0.8705036	0.9111111	0.8970588	0.9253731	0.9185185	0.9117647	0.9323308	0.9104478	0.9185185	0.8992806	0.8985507	0.9191176	0.9029851	0.9398496	0.9402985	0.9477612	0.9548872	********	
40	0.9044118	0.8970588	0.9191176	0.9044118	0.9124088	0.9029851	0.9130435	0.9552239	0.9044118	0.8978102	0.9191176	0.9117647	0.9270073	0.9071429	0.9197080	0.9407407	0.8897059	0.9191176	0.9185185	0.9259259	0.8985507	0.8992806	0.9407407	0.9264706	0.9407407	0.9338235	0.9270073	0.9333333	0.9402985	0.9338235	0.9208576	0.9000000	0.9485294	0.9328358	0.9407407	0.9411765	0.9485294	0.9555556	0.9259259	********

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