Article

POLLEN BIOLOGY AND REPRODUCTIVE ECOLOGY OF SELECTED PALEOTROPICAL DENDROBIUMS AND THEIR COMMERCIAL HYBRIDS

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Abstract—Understanding the reproductive biology is of great importance in the development of novel hybrids in ornamental plants. Pollen fitness-related traits are crucial for the pollination success in any plant species including dendrobiums. The aim of the study was to determine and compare the fitness traits of ten commercial *Dendrobium* hybrids and two indigenous *Dendrobium* species, *D. crumenatum* and *D. anosmum* found in Sri Lanka. We measured pollen viability, pollen germinability, and fruit production after controlled pollination. The effect of storage temperature on *D. crumenatum* pollen viability was evaluated to establish a suitable pollen storage method to improve future breeding programmes, as the flowering of dendrobiums is seasonal. The reproductive ecology of selected dendrobiums was studied by the observations of visits of natural pollinators and by assessing floral morphology to predict their potential pollinators. Six commercial hybrids had non-viable pollen while *D. crumenatum* showed the highest pollen germinability under both *in vivo* and *in vitro* conditions. Ninety percent of the commercial hybrids failed *in vitro* pollen germination whereas under *in vivo* conditions 50% were successful. Self-incompatibility in *D. crumenatum* was observed in both hand-pollination and under natural pollination. Pollen of *D. crumenatum* can be stored for two weeks at 9°C maintaining viability and germinability. Selected dendrobiums have shown adaptations to melittophily, suggesting the pollination by bees. Findings indicated a reduction of male fitness in most of the commercial *Dendrobium* hybrids and a higher fruit set is seen in selfing than cross-pollination. The present study provides information for developing conservation strategies and future hybridization programmes in paleotropical dendrobiums.

Keywords—*Dendrobium*, male fitness, reproductive ecology, artificial pollination, *Dendrobium crumenatum*, commercial *Dendrobium* hybrids

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INTRODUCTION

Orchidaceae is one of the largest angiosperm families with significant ornamental and economic value (Hinsley et al. 2018). With increasing market circulation and rising trade, breeders are compelled to develop new hybrids with unique characteristics (Li et al. 2021). The development of attractive floral morphologies of high commercial value goes back more than 150 years and nearly 100,000 commercial hybrids have been developed to date (Vendrame et al. 2008). This places orchids commerce on the 8% share of the global floriculture trade, with annual sales of more than US $4 billion (Dehgahi & Joniyas 2017; Zhang et al. 2018). Among orchids, *Dendrobium* Swartz. is one of the most popular ornamental orchid genera for its cosmopolitan distribution, a high number of florets per inflorescence, recurrent flowering with a wide range of colours, sizes and shapes, year-around availability, and long flowering life; from several weeks to months (Martin & Madassery 2006; Moudi et al. 2013). It is the third largest genus of the Orchidaceae family, including 1,200–1,500 species that are distributed predominantly throughout paleotropical regions. Sri Lanka and the Western Ghats of India belong to one of the 34 world biodiversity hotspots, with ten indigenous *Dendrobium* species reported in Sri Lanka (Xiang et al. 2016; Priyadarshana et al. 2020). Among them, *D. crumenatum* is an epiphytic pseudobulb with white, fragrant flowers and distributed in wild

throughout Taiwan to tropical Asia (Jayaweera 1981; Govaerts et al. 2019). Fernando and Ormerod (2008) reported *D. crumenatum* as an adventive species, introduced and naturalized in Sri Lanka. *Dendrobium anosmum* is a lithophyte or pseudobulb epiphyte with purple or white flowers and dark purple lip, distributed in Sri Lanka, Indo-China to New Guinea (Govaerts et al. 2019).

In commercial orchid hybrid production, attractive floral traits always play a key role in selecting parental materials. Although some of the wild species are very attractive, widely grown and demanding, the majority of orchids available in the market are hybrids mainly from Taiwan, Thailand and Malaysia. Nevertheless, the development of attractive hybrids using Sri Lankan indigenous *Dendrobium* species is scarce **(**Padmini & Kodagoda 2017; Priyadarshana et al. 2020). The success of pollination and hybridization depends on various environmental, physiological, biochemical or genetic factors such as pre-zygotic barriers and inbreeding depression. In addition to female reproductive success (fruit or seed production), male fitness traits such as pollen viability and pollen germinability also need to be considered for a successful hybridization process (Rao & Ong 1972). According to Niu et al. (2018), pollen grains of commercial dendrobiums may have reduced viability and germinability over time. In fact, previous attempts to generate new hybrids using commercial hybrids with highly promising characters in the market have failed (Pers. com., 2017).

Understanding orchid pollination biology is very important for a successful breeding program. In the family, pollen grains are packed in masses called 'pollinia', which are the pollen dispersal units (Chen & Fang 2016). A pollinium may contain millions of pollen grains and is dispersed by specialized animal pollen vectors while ensuring minimum wastage during pollen transfer (Dressler 1993). Pollinia often are attached to the body of the pollinator and then get detached. A strong association between certain floral traits and functional groups of pollinators that exert similar selective pressures have been observed in other studies (Waser et al. 1996; Zhao & Wang 2015). Pollinator-mediated selection of floral traits relative to the differences in behaviour patterns, sensory abilities and dietary preferences of pollinators are often described (Ollerton et al. 2015; Dellinger 2020). Once pollinia are deposited into the receptive stigmatic cup that is located on the underside of the column, pollen grains are dissolved in the stigmatic fluid (Fonseca et al. 2015). Hydrated pollen grains in the periphery initiate the formation of pollen tubes and upon contacting the stigmatic cup wall and pollen tubes are directed towards the ovary. Fertilization of dendrobiums occurs about 55 to 60 days after pollination (Sagawa & Israel 1964).

Due to the seasonal flowering patterns, pollinia of some *Dendrobium* species or hybrids are not available throughout the year for hybridization and this can hinder the success of breeding programmes. *Dendrobium crumenatum* is one of the seasonally flowering *Dendrobium* species and their flowers are short-lived (one or two days). In order to incorporate pollen of such species in hybrid production, identification of appropriate pollen storage conditions is of utmost importance. Storing at low temperatures is the common pollen storage method available currently. However, no records are available for suitable storage temperatures for *D. cruminatum* and *D. anosmum* (Vendrame et al. 2008). The present study focuses on these aspects of the reproductive biology of dendrobiums, ultimately providing information on potential parents, for the production of new hybirds. We assessed the fitness traits of two indigenous *Dendrobium* species, *D. crumenatum* (pigeon orchid) and *D. anosmum,* and ten promising commercial *Dendrobium* hybrids in terms of pollen viability, pollen germinability (male fitness traits) and fruit set (female fitness traits). Pollination ecology in terms of pollination syndromes and floral visitors was also studied.

MATERIALS AND METHODS

SAMPLE COLLECTION

Two indigenous *Dendrobium* species and ten commercial *Dendrobium* hybrids (Fig. 1) were selected for the study using three individuals per species/hybrid (N = 36). Experiments were conducted during the period of August 2018 to January 2020. Indigenous species, tentatively identified as *D. crumenatum* and *D. anosmum* (Fig. 1) were collected from Gampaha (6.97′ 79.91′) and Naula, Matale (7.70′ 80.64′) districts in Sri Lanka and grown *ex situ* (7.31′ 79.85′) to study the floral

Figure 1. Indigenous *Dendrobium* species (A) *D. crumenatum* and (B) *D. anosmum* and commercial *Dendrobium* hybrids (C) *D.* 'Big Jumbo', (D) *D.* 'Emma White', (E) *D.* 'Pink', (F) *D.* 'Lemon Yellow', (G) *D.* 'Arading Green', (H) *D.* 'Nana Red', (I) *D.* 'Mickey Pinky Splash', (J) *D.* 'Visa Peach', (K) *D.* 'Sonia red' and (L) *D.* 'Happy Star'. (scale bar- 1 cm)

traits and pollen biology. Ten six-month old commercial *Dendrobium* hybrids were purchased from plant nurseries in Gampaha, Sri Lanka and all plants were maintained in the plant house of the University of Kelaniya, Sri Lanka. For the experiments, pollinia were collected from second to sixth fully opened fresh flowers (using four flowers) from the base of each inflorescence at the anthesis around 7.00 – 7.30 am and were immediately brought to the laboratory of the Department of Plant and Molecular Biology, University of Kelaniya, Sri Lanka.

Identification of collected indigenous *Dendrobium* species

Identification of indigenous *Dendrobium* spp. was performed by direct observation of the floral morphology, combined with ITS makers. Genomic DNA of the two indigenous *Dendrobium* species was isolated from young leaves by modified cetyltrimethylammonium bromide (CTAB) method (Farook et al. 2016). Leaf samples frozen at -80ºC were washed with sorbitol washing buffer [Tris HCl (0.1 M), EDTA (0.005 M) β-mercaptoethanol (1 mL/L) and polyvinylpyrrolidone (PVP) (0.1 g/L) (pH 8.0)] to remove the mucilaginous polysaccharides (Tel-Zur et al. 1999). PCR amplification of DNA was performed using an ITS primer pair 17 SE and 26 SE (5′ ACG AAT TCA TGG TCC GGT GAA GTG TTC G 3′ and 5′ TAG AAT TCC CCG GTT CGC TCG CCG TTA C 3′) (Xu et al. 2015). Components of the amplification reaction included 1X buffer, 2.5 mM $MgCl_2$, 0.4 mM each forward and reverse primer, 0.2 mM dNTPs, 0.625 U GoTaq polymerase (Promega Inc., USA) and 2 µL of genomic DNA. PCR conditions were 95°C for 4 min followed by 30 cycles of 95°C for 1 min, 65°C for 30 s, and 72°C for 30 s and final extension at 72ºC for 7 min. Single clean PCR products were sequenced bi-directionally at Genetech, Sri Lanka. A homology search using Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) was conducted. The identity

of the two indigenous species was confirmed by comparing the query sequences to the previously published or vouchered authentic sequences.

VARIATION IN FLORAL TRAITS

Floral traits of the two selected indigenous species and eight hybrids were observed and recorded to identify the pollination syndromes. Fully-opened second flower of the inflorescence of each species or hybrid was selected for floral measurements. Quantitative floral morphological characters were measured to the nearest 0.01 mm in three replicates using a vernier calliper. The eleven floral traits that were used to describe each species or hybrid were: (1) Floral symmetry (radial or bilateral symmetry), (2) Restrictive or unrestrictive floral morphology to determine the accessibility for the floral visitors. Flowers that have easily accessible floral rewards with nectar or/and pollen exerted beyond the petals are unrestrictive. Flowers with a tube radius of at least 2 mm are considered as unrestrictive and less than 2 mm are considered restrictive (Wang et al. 2019), (3) Flower colour variation in the different parts of the flower, (4) Number of flowers in the inflorescence that comprise the display signals for pollinator attraction, (5) Flower length (distance between the tip of the dorsal sepal to the tip of the labellum), (6) Floral width (distance between lateral sepals), (7) Column colour, (8) Labellum colour, (9) Labellum disk colour, (10) Presence or absence of scent (determined by smell), (11) Presence or absence of nectar at the base of the column (determined by observation of naked eye).

POLLEN HISTOCHEMISTRY

Pollinia samples collected from three individuals of each selected indigenous species and hybrids (N = 36) were immersed in drops of IKI solution or drops of Sudan IV solution and examined under a microscope (x1000). A dark bluish-black colour (when stained with IKI) indicates the presence of starch, and a red colour (when stained with Sudan IV) indicates the presence of lipids (Wang et al. 2004). Pollinia were stained with ninhydrin solution to determine the presence of proteins in pollinia (Ram et al. 1988).

DETERMINATION OF POLLEN VIABILITY

To determine pollen viability, pollinia of the hybrids and indigenous species were immersed in 1% 2,3,5-tiphenyl tetrazolium chloride (TTC) (Sigma-Aldrich, USA) at pH 7.0 and incubated at 40°C in dark inside an incubator for 2 hours (Beyhan & Serdar 2008) since the method has been successful in determining pollen viability of some *Dendrobium* spp. (Vendrame et al. 2008; Sulusoglu & Cavusoglu 2014). After incubation, pollen grains were examined under a microscope and those stained in red colour were counted as viable. The number of stained and unstained pollen grains was counted in aliquots of 100 samples for each replicate and the average pollen viability percentage was calculated in three replicates (N = 36).

Generalised linear model with a quasibinomial distribution procedure was performed to assess the significant difference in the proportion of viable pollen among the selected dendrobium species/hybrid using the glm function in R version 4.2.1 (R Development Core Team, 2021) and post hoc test was carried out using the Tukey contrast for pairwise comparisons at $\alpha = 0.05$ using lsmeans function.

IN VIVO POLLEN GERMINATION

Pollen germination on stigma was performed by autogamic self-pollination of the third or fourth fully opened flower from the base of the inflorescence around 7.00 – 7.30 am by hand pollinating, placing the pollinia on to the stigmatic surface of the same flower (Firmage & Dafni 2001; Souza et al. 2020). After 72 hours, pollen was removed from the stigmatic fluid using a clean toothpick and stained with lactophenol cotton blue. Pollen tubes that were elongated as twice as the diameter of the pollen grain were considered as germinated pollen grains (Bellusci et al. 2010). The number of germinated pollen tubes per hundred pollen grains in each replicate were counted and average pollen germination percentage was calculated in three replicates (N = 36). Pollen tube length of ten pollen samples was measured per replicate and the average pollen tube length was calculated for all the *Dendrobium* samples assessed (Bellusci et al. 2010).

Generalised linear models with a quasibinomial distribution was performed to test significant differences in the proportion of *in vivo* germinated pollen among the selected dendrobiums, using the glm function. Post hoc test for pairwise comparisons was carried out by

Tukey contrast with the lsmeans function in R version 4.2.1 (R Development Core Team, 2021).

In vitro pollen germination

Pollinia were removed from fully opened second or third flower from the base of the inflorescence to Eppendorf tubes (0.2 mL) at 7.00 am on the first day of anthesis. Pollen germination of *D. crumenatum* was evaluated using (i) sterilized 0%, 10%, 20% and 30% (w/v) sucrose solutions (pH 5.7) (Bellusci et al. 2010). Based on the performance of pollen germination, the best sucrose concentration for maximum pollen germination was identified (N = 12). The best sucrose concentration identified was further enriched with (ii) 0.01% boric acid medium (Shiau et al. 2002) and (iii) BK medium (100 mg/L H_3BO_3, 100 mg/L $CaCl_2$, 100 mg/L $MgSO_4.7H_2O$, and 100 mg/L KNO_3) (Brewbaker & Kwack 1963; Ajeeshkumar & Decruse 2013). Pollen germination of *D. crumenatum* was assessed in the media mentioned above (N = 9). and the optimum medium was selected for *in vitro* germination of pollen from the other study species and hybrids. If pollen germination was not successful in the selected medium, a modified sucrose medium was used by adding the stigmatic fluid from respective *Dendrobium* spp. or hybrids. Three stigmas were removed, dissolved in 3.0 ml sterilized distilled water and added to sucrose media of four different concentrations [0%, 10%, 20% and 30% (w/v)] (Cheng & Fang 2016). Pollinia were incubated for 24 hours at room temperature (27°C) in triplicates per treatment (N = 36). Percentage mean pollen germination and mean pollen tube lengths of the replicates were calculated. Suitable media for *in vitro* pollen germination was selected by analysing *D. crumenatum* pollen germination percentages using one way ANOVA by aov function. Generalised linear model with a quasibinomial distribution procedure was performed using *in vitro* pollen germination data (proportion of germinated pollen) with each selected dendrobium species/hybrid using glm function and post hoc test were carried out using the Tukey contrast using lsmeans function. Pearson correlation analysis was performed to determine the correlation between pollen viability, and *in vivo* germination using cor.test function. All statistical analysis were performed using R version 4.2.1 (R Development Core Team, 2021).

Controlled pollination and seed production success

Self- and cross-pollinations were performed artificially for indigenous *Dendrobium* species and commercial dendrobiums at 7.00 am on the first day of anthesis (Borba et al. 1999). In self-pollination experiments, pollinia were removed and placed on the stigma of the same flower whereas, in the cross-pollination experiments, the stigma of different individuals of the same species/hybrid were pollinated. A total of 72 crosses consisting of 36 self-pollinations and 36 out-crosses were performed with three individuals (a total of six crosses including three self- and three cross-pollinations per each species/hybrid) per each indigenous *Dendrobium* species or hybrid (N = 36). Indigenous *D. crumenatum* was cross-pollinated with commercial *D.* 'Nana Red' due to the availability of pollen at the time of flowering of *D. crumenatum*. The plants were carefully maintained until hybrid seeds were produced in 3-4 months. The viability of seed samples removed from all fruits was determined by incubating them in 1% TTC, at pH 7.0 for 24 hours in dark at 40 ºC. Embryos stained in red colour were considered as viable and others were counted as non-viable.

Effect of storage temperature on the pollen viability and germinability

Dendrobium crumenatum pollen were collected into microfuge tubes (0.2 mL) from fully opened fresh flowers at the onset of the anthesis around 7.00 – 7.30 am. A set of pollen was dried in silica for 24 hours before storage and the other set was directly stored at -80°C, -20°C, -1°C, 9°C and 28°C temperatures. Cryopreservation of pollen at -80°C and -20°C was also conducted using a modified Murashige and Skoog (MS) medium (pH 5.7). Glycerol [30% (w/v)], ethylene glycol [15% (w/v)] and dimethyl sulfoxide (DMSO) [15% (w/v)] were added to the MS medium to prevent ice crystallization during cryopreservation (Vendrame et al. 2008). Treatments were performed in triplicates (N = 250). Samples of stored pollen were removed after 1, 3, 7, 14, 30 and 60 days, *in vitro* pollen germinability, and pollen viability were tested in the 10% sucrose medium and in 1% TTC respectively, to confirm pollen viability and germinability, pollen of *D. crumenatum* stored at 9°C for 7-day period was used in controlled cross-pollination process with the hybrids; *Dendrobium* 'Pink Stripe,' *D.* 'Sonia

Red' and *D.* 'Pink New Splash' as these hybrids were available at the time. Performance in pollen storage was analysed by the Kruskal-Wallis rank sum test using the Kruskal.test function in FSA package in R version 4.2.1.

Natural pollination and observation of floral visitors

Natural pollination and insects visiting the inflorescences of all selected indigenous species and hybrids were observed at the plant house of the University of Kelaniya, Sri Lanka and in a home garden in Gampaha district, Sri Lanka. Observations were carried out in the morning and afternoon several times of the day from anthesis to wilting of flowers from August 2018 to January 2020. Insects that collected pollen and contacted stigmas were recorded as pollinators. Fruit set production by natural pollination was observed for all selected indigenous species and hybrids.

RESULTS

IDENTIFICATION OF THE TWO INDIGENOUS *DENDROBIUM* SPECIES

Sample PO (GenBank Accession No. MZ540353) showed 99.38% sequence similarity to the ITS of *D. crumenatum* deposited by Takamiya et al. (2014) under the accession number AB593537.1. Sample DA (GenBank Accession No. MZ540354) had the highest sequence similarity of 99.88% of *D. anosmum* deposited by the same research group under the accession number AB593499.1. Samples PO and DA were therefore identified as *D. crumenatum* and *D. anosmum*, respectively, after consideration of morphological traits as well.

VARIATION IN FLORAL TRAITS

All studied commercial hybrids and indigenous species have inflorescences with bilateral symmetrical restrictive flowers where the pollinia are hidden or partially hidden within the corolla. Column of *D. anosmum* and *Dendrobium* 'Visa Peach' is completely covered by labellum and not visible to outside while other commercial hybrids and *D. crumenatum* columns are partially covered by the folded tri-lobbed labellum. All hybrids and indigenous species have white coloured anther cap covering the yellow colour four pollinia attached to the tip of the column. The stigma is ovoid-shaped on the ventral face of the column containing a mucilaginous secretion. Nectar droplets or wetness were not observed at the base of the columns in the selected indigenous species or in the hybrids. *Dendrobium crumenatum* is the only scent-emitting species among the selected dendrobiums. Flowers of *D. crumenatum* are pure white and small with a bright yellow disc on the labellum that emits a strong sweet fragrance, which is present from early morning at the onset of the anthesis. *Dendrobium anosmum* does not emit fragrance but produces attractive purple-coloured flowers with tubular-shaped single-lobed labellum with a large dark-purple spot to attract pollinators and guide them towards the column where pollinia are located. Thus, hairs are present on the margin of the labellum. All the selected *Dendrobium* hybrids except white colour *D.* 'Big Jumbo' and *D.* 'Emma White' and red-coloured *D.* 'Nana Red' have colour variations in the labellum, disk and column in order to guide pollinators to the direction of pollinia.

Flower morphologies of studied dendrobiums showed adaptations to melittophily, suggesting bees as the pollinators. Flowers of selected indigenous species and hybrids are commonly red, yellow, or purple in colour and zygomorphic flowers and those features are typically associated with bee pollination (Faegri & van der Pigl 1979; Nikkeshi et al. 2015). The labellum acts as landing surface, however, forming a semi-closed chamber restrict movement of floral larcenists and provide opportunity to enter small insects to collect hidden nectar. Also, colour variations at the end of the labellum highlights the column, potentially acting as nectar guides to the nectaries that are located at the base of the column. *Dendrobium crumenatum* is fresh and sweet scented, which is a common trait in bee-pollinated flowers to act as additional chemical sensory for bees to locate them (Table 1).

POLLEN HISTOCHEMISTRY

Dendrobium crumenatum and *D. anosmum* pollen stained in dark brown with IKI while the pollen of commercial dendrobiums stained in yellow indicating the presence of starchy pollen. All *Dendrobium* pollinia stained pink with Sudan IV indicating the presence lipids. Pollinia failed to stain with ninhydrin solution indicating the absence of proteins in tested *Dendrobium* pollinia.

Table 1: Floral morphological traits of *Dendrobium* species and hybrids (*N* = 36).

Dendrobium species/hybrid*	Floral color	Number of flowers per inflorescence (mean)	Flower length (cm) (mean)	Flower width (cm) (mean)	Labellum color	Disk color	Column color	Scent
D. crumentatum	White	10.6	3.33	4.06	White	Yellow	White	Present
D. anosmum	Purple	9.7	4.64	5.76	Purple	Dark Purple	Purple	Absent
Dendrobium 'Big Jumbo'	White	5.6	6.95	8.17	White	White	White	Absent
Dendrobium 'Emma White	White	6.3	4.94	5.46	White	White	White	Absent
Dendrobium 'Pink'	Purple	7	5.33	6.33	Purple	Purple	Purple	Absent
Dendrobium 'Lemon Yellow'	Green	10.7	5.09	6.00	Purple and green	Purple	Green	Absent
Dendrobium 'Arading green'	Green	7.3	4.46	5.91	Purple and green	Purple	Green	Absent
Dendrobium 'Nana Red'	Red	8.3	5.45	7.12	Red	Red	Red	Absent
Dendrobium 'Mickey Pinky Splash'	Pink and yellow	9.33	4.60	4.96	Pink	Yellow	Purple	Absent
Dendrobium 'Variety Peach'	Light orange and white	8	6.62	8.06	Light orange and white	White	White	Absent
Dendrobium 'Sonia red'	Purple and white	7.3	6.99	8.51	Purple	White	White	Absent
Dendrobium 'Happy Star'	White yellow and purple	5.7	6.83	8.42	Purple	Pink	Purple	Absent

*All results are based on three replicates using three plants of each species or hybrid

POLLEN VIABILITY

Pollen of commercial hybrids and indigenous species were stained with 1% TTC. Highest percentage of pollen viability was showed in *Dendrobium* 'Big Jumbo' (89.33 ± 3.48%) followed by indigenous species, *D. crumenatum* (89.0 ± 3.60%), the hybrids, and *D.* 'Pink' (88.0 ± 4.35%). Tukey post hoc comparisons revealed that *Dendrobium* 'Big Jumbo' and *D.* 'Pink' have significantly higher pollen viability than the other two viable hybrids; *D.* 'Mickey Pinky Splash' and *D.* 'Visa Peach' (N = 36; $P < 0.05$). However, significant difference was not observed between two indigenous species, *D. crumenatum* and *D. anosmum* stained with 1% TTC (N = 36; $P < 0.05$). Pollen of *D.* 'Emma White', *D.* 'Lemon Yellow', *D.* 'Arading Green', *D.* 'Nana Red', *D.* 'Sonia red' and *D.* 'Happy Star' were not stained with 1% TTC (Fig. 2).

IN VIVO AND *IN VITRO* POLLEN GERMINATION

Pollen incubated on the stigmatic fluid of *Dendrobium* species and hybrids showed pollen tube elongation after 72 hours incubation period. The highest percent mean pollen germination was found in *D. crumenatum* (99.33 ± 0.67%) followed by *D. anosmum* (76.00 ± 5.57%) forming the longest pollen tubes of 31.31 ± 2.47 μm and 47.78 ± 16.60 μm respectively. According to the Tukey post hoc comparisons, *in vivo* pollen germinability of indigenous *D. crumenatum* were significantly different from imported commercial hybrids while no significant difference was observed between the two indigenous species (N = 36; $P < 0.05$). *D.*

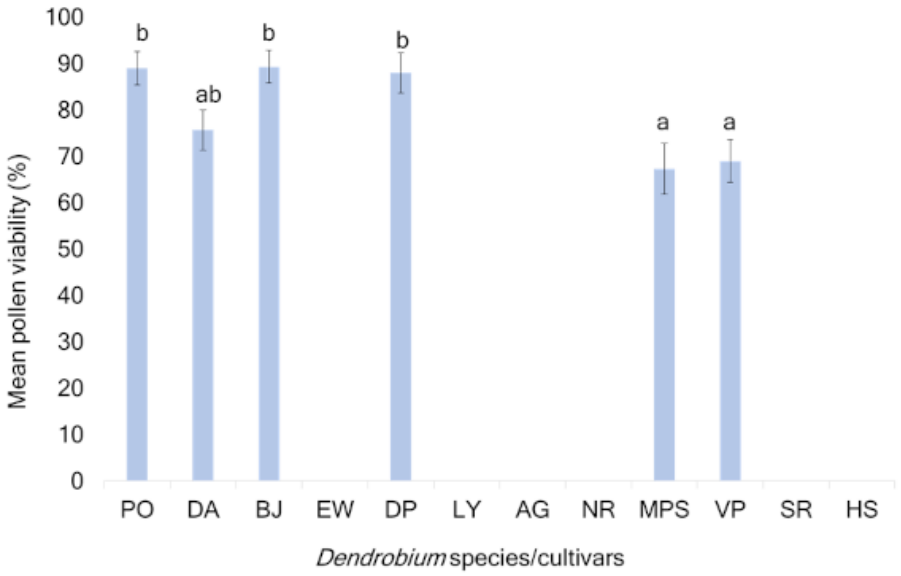

Figure 2. Pollen viability of *Dendrobium* species and hybrids stained with 1% TTC after 2-hour incubation at 40°C in the dark. Error bars indicate S.E. values (Mean ± S.E.). (*N* = 36)

The letters indicate significant differences among the *Dendrobium* species and hybrids at $P < 0.05$.

PO- *D. crumenatum*; DA- *D. anosmum*; BJ- *D.* 'Big Jumbo'; EW- *D.* 'Emma White'; DP- *D.* 'Pink'; LY- *D.* 'Lemon Yellow'; AG- *D.* 'Arading Green'; NR *D.* 'Nana Red'; MPS- *D.* 'Mickey Pinky Splash'; VP- *D.* 'Visa Peach'; SR- *D.* 'Sonia red'; HS- *D.* 'Happy Star'.

'Lemon Yellow', *D.* 'Arading Green', *D.* 'Emma White', *D.* 'Sonia Red' and *D.* 'Happy Star' were not successful in germination on the stigma under *in vivo* conditions (Table 2).

Dendrobium crumenatum pollen germinated in all the tested media and the highest *in vitro* germination resulted in 10% sucrose (97.67 ± 1.45%) with the mean pollen tube length of 24.32 ± 1.36 µm ($N = 12$; $P < 0.05$) (Fig. 3A). A medium with 10% sucrose showed the highest mean pollen germination compared to the 10% sucrose with boric acid (26.33 ± 6.67%) and BK (23.33 ± 4.37%) germination media ($N = 9$; $P < 0.05$). Hence, 10% sucrose was selected for further germination experiments of other species/hybrids (Table 2). Percent pollen germination of *D. anosmum* was 9.33 ± 1.8% and *D.* 'Pink' was 37.33 ± 7.9% on 10% sucrose. However, the other commercial hybrids failed to germinate in 10% sucrose or modified sucrose media with the stigmatic fluid (Table 2).

Table 2. Pollen germination percentage and mean pollen tube length of *Dendrobium* species/hybrids (A) *in vivo* and (B) *in vitro* in 10% sucrose solution (*N* = 36).

	(A) In vivo pollen germination*		(B) In vitro pollen germination*	
Dendrobium species/hybrids	Mean pollen germination (%)	Mean pollen tube length (µm)	Mean pollen germination (%)	Mean pollen tube length (µm)
D. crumentatum	99.33 ± 0.67c	31.31 ± 2.47	97.67 ± 1.45 c	24.32 ± 1.36
D. anosmum	76.00 ± 5.57bc	47.78 ± 16.60	9.33 ± 1.88a	20.01 ± 5.05
D. 'Big Jumbo'	58.67 ± 9.93b	66.53 ± 18.64	0.00	-
D. 'Emma White'	0.00	-	0.00	-
D. 'Pink'	64.67 ± 4.91b	50.83 ± 19.08	37.33 ± 7.99 b	14.15 ± 4.22
D. 'Lemon Yellow'	0.00	-	0.00	-
D. 'Arading Green'	0.00	-	0.00	-
D. 'Nana Red'	56.33 ± 7.63b	44.38 ± 14.66	0.00	-
D. 'Mickey Pinky Splash'	67.00 ± 2.52b	14.56 ± 0.55	0.00	-
D. 'Visa Peach'	29.33 ± 6.35a	37.83 ± 13.14	0.00	-
Dendrobium 'Sonia red'	0.00	-	0.00	-
Dendrobium 'Happy Star'	0.00	-	0.00	-

***** Different letters indicate significant differences between the *Dendrobium* species and hybrids at $P < 0.05$.

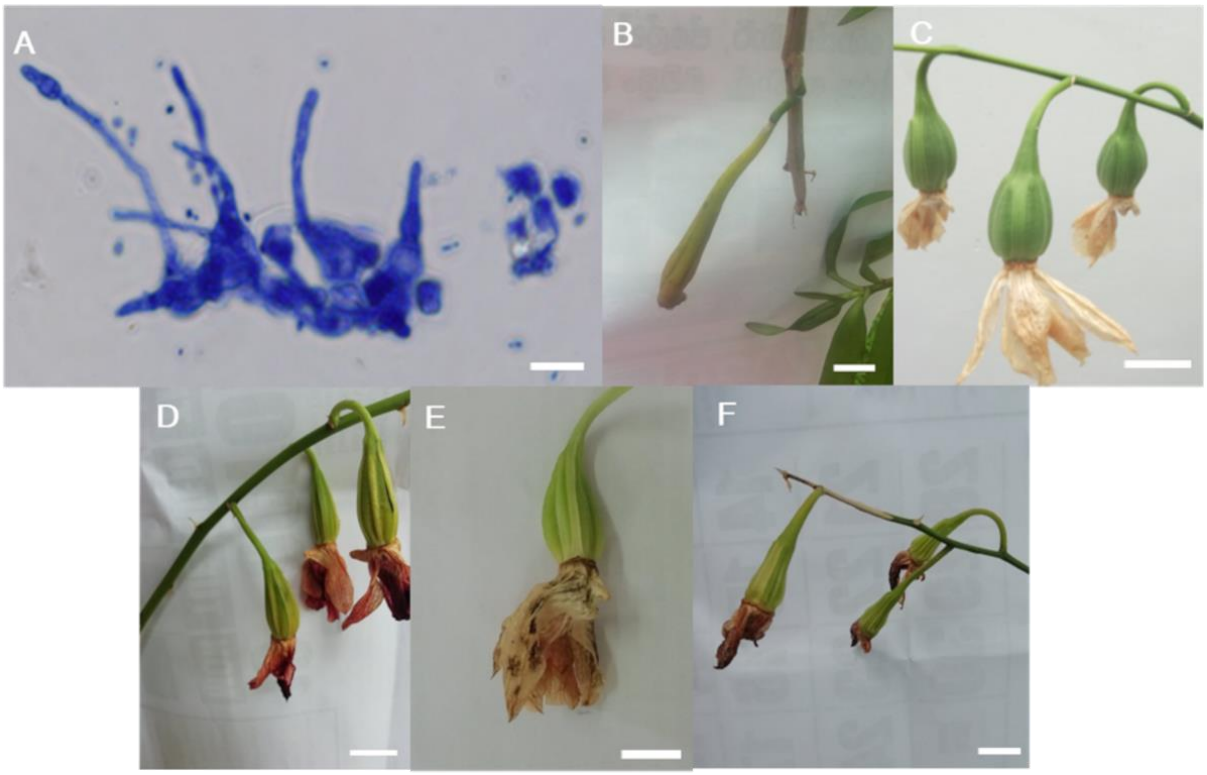

Figure 3. (A) *Dendrobium crumenatum* pollen germinating *in vitro* after 24 hours on 10% sucrose (scale bar: 10 µm) and *Dendrobium* fruit set resulted from controlled self- and cross-pollination (B) *D. anosmum*, (C) *D.* 'Big Jumbo', (D) *D.* 'Nana Red', (E) *D.* 'Visa Peach' and (F) *D.* 'Nana Red' x *D. crumenatum* (bar represents 1 cm length).

There was a significant difference among dendrobiums in, *in vivo* pollen germination ($N = 36$; $P < 0.05$) and *in vitro* pollen germination ($N = 36$; $P < 0.05$). Pearson correlation revealed a significant high correlation between pollen viability (TTC staining) and *in vivo* pollen germination ($N = 36$; $R = 0.81$; $P < 0.05$), moderately significant correlation between pollen viability and *in vitro* pollen germination ($N = 36$; $R = 0.50$; $P < 0.05$), and *in vivo* pollen germination and *in vitro* pollen germination ($N = 36$; $R = 0.61$; $P < 0.05$).

Controlled pollination and seed production

Seventy-two crosses in total were performed using the two *Dendrobium* species and ten commercial hybrids, 36 as self-pollinated crosses while the other 36 as cross-pollinations. From the 36 self-pollination attempts, 14 crosses were successful (38.8% success rate) whereas out of 36 cross-pollinated flowers only 9 crosses (22.2%) were successful *D.* 'Big Jumbo', *D.* 'Nana Red', *D.* 'Pink'. *D.* 'Visa Peach' succeeded in producing 22 fruits in total by both self- and cross-pollination. *D. anosmum* was also able to produce fruits by both self and cross-pollination (Fig. 3B-E).

Dendrobium 'Nana Red' had successful fruit set in all the performed crosses while *D. anosmum*, *D.* 'Big Jumbo', *D.* 'Pink' and *D.* 'Visa Peach' produced higher number of fruits by self-pollination than cross-pollination (Table 3). Seeds obtained from fruits set by both self- and cross-pollinated of all hybrids and *D. anosmum* were mostly viable. Self- pollinated fruit sets resulted 90.38 ± 1.52% mean seed viability while cross-pollinated fruit sets resulted 87.50 ± 1.83% mean seed viability. There was no significant difference in seed viability between self- and cross-pollinated fruit production ($P < 0.05$).

Despite showing the highest *in vivo* germination, *D. crumenatum* failed to produce fruits by either selfing within the same individual or by cross-pollination. Hence, to determine the crossability of *D. crumenatum,* the hybrid *D.* 'Nana Red' was selected due to the availability of flowers at the time of *D. crumenatum* flowering. The crossing produced fruits with viable seeds (91.67 ± 2.03%; Fig. 3F) indicating cross-compatibility.

Table 3. Fruit set percentage of controlled self- or cross-polliation in all studied species and hybrids

Dendrobium species/hybrids	Self-pollination fruit set success (%)	Cross-pollination fruit set success (%)
D. crumentatum	0	0
D. anosmum	100	33.3
D. 'Big Jumbo'	100	66.7
D. 'Emma White	0	0
D. 'Pink'	66.7	33.3
D. 'Lemon Yellow'	0	0
D. 'Arading Green'	0	0
D. 'Nana Red'	100	100
D. 'Mickey Pinky Splash'	0	0
D. 'Visa Peach'	100	33.3
D. 'Sonia red'	0	0
D. 'Happy Star'	0	0

0 = No successful fruit set

EFFECT OF TEMPERATURE ON POLLEN VIABILITY AND GERMINATION

Dendrobium crumenatum pollen had 54.97 ± 12.22 % germinability and 76.0 ± 4.58% viability prior to storage. All the pollen lost viability and germinability after storage at sub-zero temperatures or subjected to cryopreservation. The set of pollen dried in silica for 24 hours prior to storage at all temperatures failed to germinate or to stain with TTC. Pollen stored at 9°C without drying, were viable for 14 days and produced pollen tubes showing 8.63 ± 0.71% germination (Fig. 4).

Although after 7-day storage of pollen *in vitro* pollen germination of *D. crumenatum* was reduced to below 20% (19.57 ± 6.55%) at 9°C, pollen of *D.* 'Pink Stripe,' and *D.* 'Pink New Splash' successfully set fruits (Fig. 5).

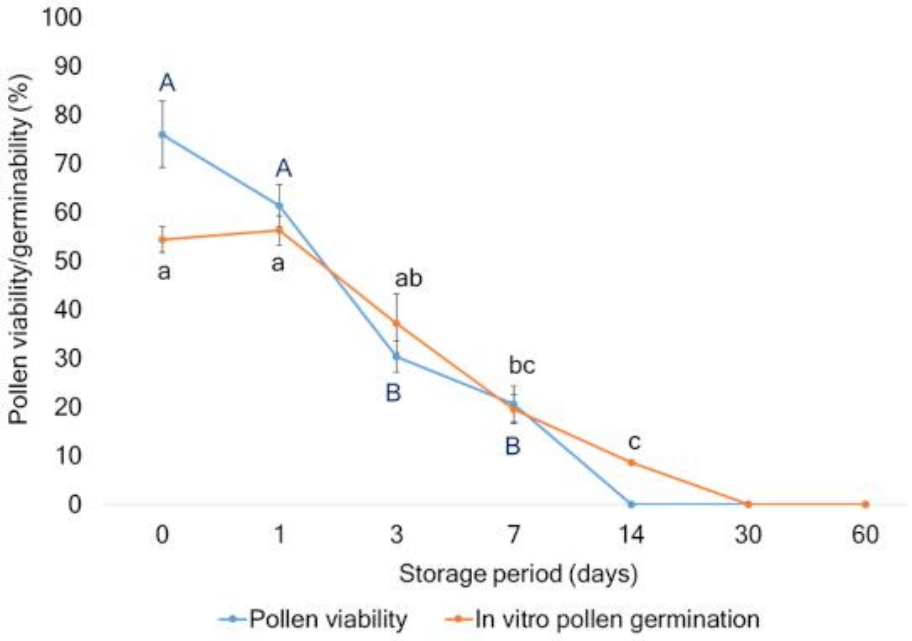

Figure 4. Pollen viability and *in vitro* germinability of *D. crumenatum* pollen stored at 9 °C for 60-day period.

Error bars indicate SE values (*N* = 250; *P* < 0.05).

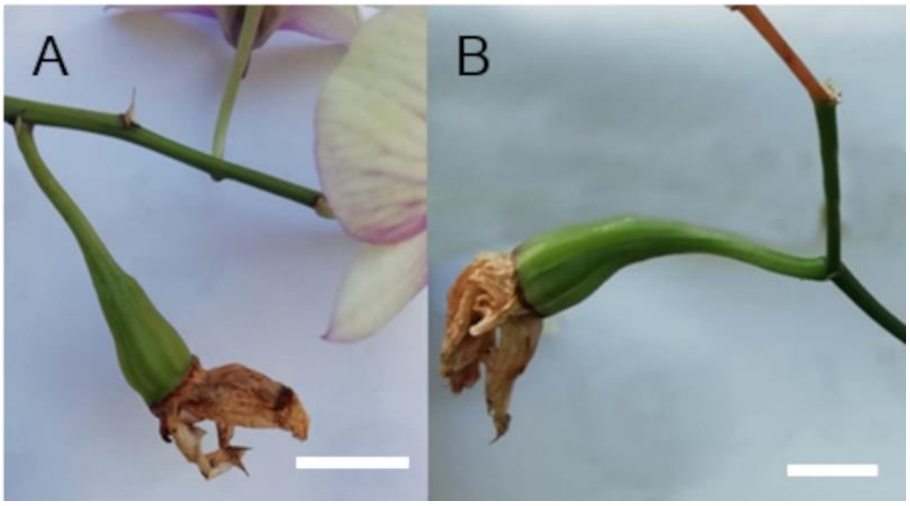

Figure 5. *Dendrobium* fruit set with pollen stored at 9°C for 7-day (A) *D.* 'Pink new splash' x *D. crumenatum* and (B) *D.* 'Pink stripe' x *D. crumenatum* (scale bar 1 cm).

NATURAL POLLINATION AND OBSERVATION OF FLORAL VISITORS

Insect floral visitors or any other pollinator visitations were not observed except for abundant visitations of ants throughout the day in *D. crumenatum*. Ants were able to lift the anther cap of the flower depositing pollinia on the stigma of the same flower, but pollen transfer among flowers by ants was not observed. This self-pollination by ants was observed in most of the flowers. Ants or other potential pollinators were not observed in visiting *D. anosmum* or in any other commercial dendrobiums during the study period. Therefore, ants may be attracted only to *D. crumenatum* due to its sweet fragrance. However, all commercial hybrids and two indigenous species including *D. crumenatum* failed producing fruits by natural pollination (animal-mediated pollen transfer).

DISCUSSION

Dendrobiums are well known for outcrossing and thousands of hybrids with diverse morphs have been successfully introduced into the market. Fitness traits such as pollen viability and pollen germinability, fruit set with viable seeds (Rao & Ong 1972; Cuevas & Polito 2004; Soares et al. 2016) are very important determinants of crossability and hybrid production of any plant species including dendrobiums. Here we compared the fitness traits of selected commercial *Dendrobium* hybrids with two indigenous *Dendrobium* species, *D. crumenatum* and *D. anosmum*, and found that commercial dendrobiums have reduced male fitness over the indigenous *Dendrobium* spp.

Firstly, results of *in vivo* and *in vitro* pollen germinability studies supports this observation. Pollen of both indigenous and five commercial dendrobiums were germinated under *in vivo* conditions forming long pollen tubes while *D. crumenatum* showing a significantly higher pollen germinability than the others. *In vivo* germination studies provide a simulated natural pollination condition thus, more informative than vitality staining or *in vitro* germination tests (Dafni & Firmage 2000). Slater (1991) suggested that the stigmatic mucilage has a pivotal role in *D. speciosum* pollination. Modification of pollen germination media by adding stigmatic extracts and additives, such as calcium, magnesium, potassium, and especially boron (Shiau et al. 2002; Cheng & Fang 2016; Tushabe & Rosbakh 2021) have shown enhanced germinability. However, in the present study, the simplest medium containing pure sucrose (10%) solution resulted the best performance of *in vitro* pollen germination. In this case also, pollen of both indigenous species germinated successfully and except for *Dendrobium* 'Pink', the other tested hybrids failed to germinate. Continuous attempts with various modifications also produced the same results agreeing with the reduced fitness of commercial dendrobiums as inferred by pollen germinability tests as the first line of evidence.

Second line of evidence of reduced fitness of commercial dendrobiums comes from pollen viability tests conducted by TTC staining. For an example, both indigenous species showed ~ 89%-75% viability whereas only four (out of ten)

commercial hybrids showed ~ 89%-67% viability. To determine whether pollen viability is in agreement with pollen germinability, a relationship studies were conducted among these. Pollen viability observed in TTC staining was positively correlated with *in vivo* pollen germination. Therefore, collective results of *in vivo* pollen germination and TTC staining would provide a comprehensive estimation of *Dendrobium* pollen viability.

The third fitness trait studied was fruit set. Disregarding the viability or germinability of pollen, all 12 dendrobiums were subjected to controlled pollination. It was interesting to note that except of *D. crumenatum,* all dendrobiums showing *in vivo* germination led to successful fruit set when subjected to both self- and cross-pollination. Failure of repeated attempts of self-pollination might be dure to the self-incompatibility of *D. crumenatum.* Johansen (1990) also reported similar findings. Further supporting this, hand-pollination of *D. crumenatum* with commercial hybrids, *D.* 'Pink Stripe,' *D.* 'Nana Red', and *D.* 'Pink New Splash' resulted fruit set with viable seeds showing the potential the indigenous species in creating novel hybrids. On the other hand, self- pollination of indigenous *D. anosmum* produced fruits with viable seeds. Out of the commercial hybrids, self-pollination of *D.* 'Big Jumbo', *D.* 'Pink', *D.* 'Nana Red', and *D.* 'Visa Peach' also produced fruits with viable seeds. Niu et al. (2018) reported that 50% of wild *Dendrobium* species are self-incompatible. Failures in fruit set in commercial hybrid *D.* 'Mickey Pinky Splash' and *D. crumenatum* may have been caused by post fertilization barriers or infra-specific cross-incompatibility barriers, presenting a high inbreeding depression thus probably these commercial cultivars might have been originated from the same or closely related parental materials. Further studies on barriers in intra-specific cross-incompatibility and post zygotic stage may resolve these issues with an insight in developing successful crosses (Raimondi et al. 2003).

In the present study, we have observed and recorded floral morphology to predict the pollination syndrome of selected dendrobiums and to facilitate the understanding on diverse pollinators of these commercial hybrids. Based on the floral morphological traits of selected dendrobiums, it can be suggested that the pollinators are mainly bees. They are initially attracted to sweet odour and then to flashy colourful flowers. Bee pollinators generally prefer zygomorphic flowers with depth than the actinomorphic flowers (Faegri & van der Pigl 1979). The labellum acts as a mechanically strong landing platform that gives a good foothold to bees. It consists of markings or patterns, which are useful as nectar guides for pollinators directing them to nectar glands hidden at the base of the column (Faegri & van der Pigl 1979; Wojcik. 2021). In the present study, single indigenous species *D. anosmum,* (dark purple spot on the labellum) and four commercial hybrids *D.* 'Lemon Yellow', *D.* 'Mickey Pinky Splash', *D.* 'Sonia red' and *D.* 'Happy Star' had coloured markings (stripes or dark colouration) on their labellums.

According to previous studies, honeybees, fruit flies and birds commonly pollinate paleotropical *Dendrobium* species (Slater & Calder 1988; Adams & Lawson 1993; Funamoto 2019). Adams (2011) described Australian epiphytic dendrobiums as non-specific with bee pollination syndrome, based on their mass floral displays, and being *Trigona* spp. its generalist pollinator. For some terrestrial dendrobiums no known pollinator specificity was identified (Adams 2011). *Trigona* spp., *Lasioglossum* spp. and *Apis* spp. are commonly suggested pollinator species that are generally found in Sri Lanka and have been reported as pollinators of *Dendrobium* spp. in paleotropical regions (Adams et al. 1992; Adams & Lawson 1993; Karunaratne et al. 2008; Funamoto 2019; Jia & Huang 2021). Leong and Wee (2013) observed *Apis cerana* pollinating *D. crumenatum* in Sian Tuan Avenue, Singapore and reported that the sweet fragrance and the white colour of the flowers attract bees. According to Karunaratne et al. (2008), *A. cerana* is a common bee species found all around in Sri Lanka. However, according to the present results, two indigenous *Dendrobium* species failed fruit set by natural pollination where the only floral visitors recoded were small ants for *D. crumenatum*. Ants are ineffective pollinators because of the small size as they move with the pollinarium from one flower to another and not flying (Funamoto 2019). Even though they can drop the pollinia onto the stigma of the same flower, lack of fruit set may be due to self-incompatibility as previously discussed (Johansen 1990; Carr 1928) in pollinator

observations of European and Paleotropical *D. crumenatum*. In addition, the lack of bees and other pollinators may be the reason for the reduction of effective pollination in the suburban environments in the western province, Sri Lanka where orchid cultivation is popular. In contrast, those pollinators may quite be abundant in natural areas where indigenous species naturally exist. However, it is an accepted fact that orchids, particularly dendrobiums, have a low rate of pollinator visits (Wang et al. 2019). Even though the floral morphology, colourfulness, flashy and comparatively large size tend to attract pollinators, the scarcity of specific pollinators in the native environment may be the cause for the absence of pollinator visitation of the imported hybrids. Thus, hybridization can affect several phenotypic traits (shape, size and colour of petals etc.) of parental traits, which have caused changes in the pollinator niche altering the pollinator visitations (Vereecken et al. 2010).

Dafni (1992) reported that pollen histochemistry is possibly related to pollination mode, pollinator foraging behaviour, and phylogeny. Teck (2011) has observed *Trigona* (stingless bee) collect pollinia of *D. mircogalaphys* while Slater & Calder (1998) also found that pollen of *D. speciosum* is transported on the dorsal thorax of bees (*Trigona* and *Apis melifera*). All the selected dendrobiums had starchy pollen grains with storage lipids, which could act as rewards for pollinators. Many studies have shown that all angiosperm pollen contains some amount of lipids, while starch is not always present. Although no effective pollinator was observed in our investigation, insect-pollinated (entomophilous) species show a greater or lesser replacement of starch by sugar or lipids (Baker & Baker 1979; Wang et al. 2004).

Wang et al. (2019) reported cleistogamous autogamy as the main pollination strategy for *D. wangliangii* while the fruit set in *D. wangliangii* under hand self-pollination was higher than that of the hand cross-pollination. Our observations for *D. anosmum*, *D.* 'Big Jumbo' and *D.* 'Visa Peach' coincided with their findings. Therefore, the prevalence of autogamy in imported hybrids may be due to the high degree of relatedness among them or due to extensive selfing in the breeding process. Although autogamy provides reproductive assurance, especially if it occurs without insect visitation (autonomous autogamy) in areas where potential pollinators are scarce, progeny produced by selfing is usually inferior to outcrossed progeny due to inbreeding depression (Eckert 2000). However, imported hybrids may have reduced inbreeding depression because most deleterious alleles have already been removed from the selection process of the breeding programmes. Thus, in the absence of inbreeding depression, fewer biotic pollinators in the vicinity could favour self-compatibility and self-pollination (Wheelwright et al. 2006).

According to Marks et al. (2014), pollen of *D. fuchsii* stored at – 20°C for 6 years showed viability and had produced fruits after artificial pollination. Since pollen germinability and viability of *D. crumenatum* were significantly reduced even before the storage (Table 2, Fig. 2 and Fig. 4), long-term storage using subzero temperatures (-80°C, -20°C, -1°C) and cryopreservation may not be appropriate for the storage of *D. crumenatum* pollen. However, *D. crumenatum* pollen stored for short periods (7 days) at 9°C maintained their viability and germinability for successful performance in cross-pollination.

In summary, pollen of both indigenous species; *D. crumenatum* and *D. anosmum,* and commercial hybrids; *D.* 'Big Jumbo', *D.* 'Pink', *D.* 'Nana Red', and *D.* 'Visa Peach' are suitable for breeding programmes in developing novel hybrids. Nevertheless, as the majority of the commercial hybrids selected failed in fruit set and pollen germination, plant breeders have to be selective in choosing suitable parents for breeding programmes. Incorporating an additional *in vivo* pollen germination test or quick TTC staining test in the field to select viable pollen before the hybrization might be productive and cost effective for breeders to overcome these constraints. Using the TTC staining method followed by *in vivo* pollen germination test can be recommended for the determination of both pollen viability and germinability of the studied *Dendrobium* pollen to obtain more conclusive results. Due to the lack of pollinator visits, hybridization in examined commercial dendrobiums occurred only by controlled hand-pollination hence, occurrence of natural hybrids is extremely low. In addition, due to the self-incompatibility, indigenous *D.*

crumenatum can only be used as pollen donor in cross-pollination. For the other species, appropriate incompatibility breaking methods should be identified. The findings of the present study provide valuable information on pollination biology with a view of facilitating the selection and conservation of parental materials with promising features to create novel hybrids of paleotropical orchids.

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