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RESEARCH ARTICLE

Asexual Bulbil Development and Diversification of Reproductive Strategy between *Remusatia vivipara* and *Remusatia pumila* (Araceae)

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ABSTRACT: *Remusatia vivipara* and *Remusatia pumila* can produce both sexual flowers and asexual bulbils. However, *R. vivipara* seldom set seeds, whereas *R. pumila* have regular seed set. Our aim was to understand the asexual mode of bulbil development and the reasons for sexual failure in *R. vivipara*. Asexual bulbil development was observed by scanning electron and light microscopy. Pollen viability and germination rate were counted for at least 200 pollen grains in triplicate for each inflorescence. Chromosome counting was performed to confirm *R. vivipara* as triploid species. The bulbiliferous shoots of both *R. vivipara* and *R. pumila* initiate from the upper portions of tubers. As they elongate upward, bulbil clusters generates in each node of the shoot. Bulbils develop several hooked scales on top simultaneously, which facilitate dispersal because they can easily attach to the animal's fur. Although *R. vivipara* produces showy flowers in spring, their pollen grains were inviable and unable to germinate in vitro in sucrose solution, when compared with *R. pumila*. These two bulbiliferous species have diversified their reproductive strategies, with respect to *R. vivipara* being able to reproduce completely asexually and *R. pumila* being able to reproduce both sexually and asexually.

KEY WORDS: Araceae, hooked scale, pollen viability, pollen germination, *Remusatia vivipara*, *Remusatia pumila*.

INTRODUCTION

Bulbiliferous plants can generate asexual bulbils on the stems or in the flowers (Ceplitis and Bengtsson, 2004). Bulbil production may be beneficial because it allows both sexual reproduction via seeds and asexual reproduction via bulbils. Bulbiliferous species have evolved independently in several angiosperm families; thus, although these species may share a common developmental mechanism in forming bulbils, they have different evolutionary origins. In most bulbiliferous species, a single floral meristem is replaced by a single bulbil (e.g., *Ranunculus reptans* L., *Polygonum viviparum* L., *Festuca vivipara* (L.) Sm., *Allium vineale* L., *Butomus umbellatus* L.; Engell, 1973; Diggle, 1997; Prati and Schmidt, 2000; Ronsheim and James, 2000; Brown and Eckert, 2005). Other bulbiliferous species, for examples of *Mimulus gemmiparus* W.A. Weber, *Dicentra cucullaria* (L.) Bernh., *Diossorea alata* L., produce bulbils from modified axillary buds on the stems, leaves, or roots (e.g., *Mimulus gemmiparus* W.A. Weber, *Dicentra cucullaria* (L.) Bernh., *Dioscorea alata* L.; Wickham *et al*., 1982; Walton and Hufford, 1994; Moody *et al*., 1999).

Most bulbiliferous species are annual herbs and grow in the temperate zones, frigid zones, Arctic Circle, and Alpine areas. Generation of bulbils may be an adaptive trait for the species growing in harsh environments (Callaghan *et al*., 1992). Considering the high altitude and cold temperature, the optimal growing season in these regions is short and the flowering individuals may not receive sufficient pollinator visits (Wickham *et al*., 1982; Walton and Hufford, 1994; Moody *et al*., 1999). In addition, flowers may not be able to develop completely in these harsh environments; thus, reproduction by bulbils provides an alternative (Billings and Mooney, 1968; Dormann *et al.*, 2002).

However, the distributions of bulbiliferous species are not limited to the alpine and temperate regions, and several tropical bulbiliferous species such as *Dioscorea bulbifera* L., *Titanotrichum oldhamii* Soler, and *Globba cernua* Baker have been documented (Wickham *et al*., 1982; Wang and Cronk, 2003; Box and Rudall, 2006). Bulbil initiation may have different pathways in the species that grow in the temperate and tropical regions. Bulbiliferous plants grow generally as epiphytes on limestone and cliffs in tropical and subtropical regions and in seasonally drying habitats, where growth conditions are poor (Silvertown, 1983; Stuntz *et al*., 2002; Kingston and Waldren, 2003; Wang and Cronk, 2003). In these environments, bulbil production may also act as a reproductive alternative. For example, *T. oldhamii* can only grow on limestone cliffs with seeping water under dense forest canopies. Although its flowers are blooming in summer, the lack of pollinator visits and the seasonally drying habitat are believed to trigger bulbil development in autumn. Similar to

several other tropical bulbiliferous plants, sexual reproduction is blocked by bulbil development in *Titanotrichum* (Wang and Cronk, 2003; Wang *et al*., 2004).

The genus *Remusatia* (Araceae) consists of 4 bulbiliferous species in tropical Africa and Asia. *Remusatia vivipara* (Roxb.) Schott is widely distributed in East and West Africa, Himalayan area, East Asia and Tropical Asia. The karyotypes of *R. vivipara* are polymorphic: The populations of *R. vivipara* from Nepal, South India are diploides, 2n = 28, those from Yunnan Dali are triploides (Long *et al*., 1989). The flowers or fruits of *R. vivipara* are seldom observed in Taiwan or Yunnan (China), whereas those of *Remusatia pumila* (D. Don) H. Li & A. Hay are commonly observed in the field. Although *R. vivipara* has been observed to flower, the factors underlying its failure to set fruit are unknown in Nantou County, Taiwan. In our previous observation, both *R. vivipara* and *R. pumila* produce bulbiliferous shoots from tubers, and their asexual bulbils occur in the bulbiliferous shoots rather than in the inflorescences or leaves. The development of their bulbils differs markedly from that of the other bulbiliferous plants.

In addition, the shapes of bulbils differ between the species. Bulbils of *T. oldhamii* have two bracts, which form a V-shape, and bulbils of *G. cernua* are spherical with a pitted surface (Wang *et al*., 2004; Box and Rudall, 2006). Bulbils of *R. vivipara* are very different from those of other bulbiliferous species. The surface of bulbil covered by hooked scales, which can attach easily to the passing mammals or birds for dispersal (Mayo, 1993; Renner and Zhang, 2004).

Herbarium specimens are useful for distinguishing and observing plants. However, most herbarium specimens (in KUN Herbarium and TAI Herbarium) of these bulbiliferous plants lack flowers or fruits but commonly possess asexual bulbils. In particular, most specimens of *R. vivipara* possess only asexual bulbils. Infrequent seed set may be caused by obstacles to sexual reproduction or damaged inflorescences. Successful sexual reproduction requires several stages of development in male and female gametophytes, gametes, embryo, and endosperm and in pollination and fertilization (e.g., *T. oldhamii*, *P. viviparum*, and *Agave macroacantha*). Bulbiliferous plants appear to have adapted to produce asexual bulbils in the tropical zone in response to environmental changes and injuries to flowering shoots. This strategy appears to replace failed sexual reproduction (Arizaga and Ezcurra, 1995; Diggle *et al*., 2002; Wang *et al*., 2004). The evolution of tropical bulbiliferous plants appears to have favored asexual reproduction because they were unable to compete with other plants in the same habitat, resulting in failed sexual reproduction.

Although both asexual and sexual reproductions occur in certain Aroids species such as *Amorphophallus bulbifera*, *Schismatoglottis bulbilifera*, *Arisaema scortechinii*, *Pinellia ternate*, *Pinellia cordata* and *Remusatia species* (Gusman, 2005; Li and Boyce, 2010; Li et al., 1997; Okada et al., 1999). The hooked scales of asexual bulbils of *R. vivipara* are useful to attach furs for widely dispersed in the Araceae. Here, we compared the reproduction and bulbil development in *R. vivipara* with that in *R. pumila*. Our study focused on pollen viability, pollen germination, and bulbil development in these two species. Moreover, we aimed to answer the following questions: (1) how asexual bulbils develop in *R. vivipara* and *R. pumila*? (2) how the hooked scar structure develops? and (3) why *R. vivipara* rarely set seeds in the field? Meanwhile, the diversification of reproductive strategy between *R. vivipara* and *R. pumila* is discussed.

MATERIALS AND METHODS

Source of plant material and growth conditions

One hundred and eighty-five living accessions of *R. vivipara* and *R. pumila* were collected from Taiwan, China, and Thailand, and were cultivated in greenhouses at the National Taiwan University. They were grown in soil medium mixed with moist peat moss and rocks. The inflorescences of *R. vivipara* emerge directly from the tubers in spring, before any leaves are initiated. In contrast, leaves grow first in *R. pumila* during the spring, and then flowers during the summer. Both species initiate bulbils in the autumn, when temperatures are lower (below 18°C). Both the species become dormant in the winter, with only the tubers remaining, whereas all tissues aboveground fade away.

SEM observations

Bulbiliferous shoots, bulbils, and pollen grains at various stages of development were fixed overnight in FAA (70% ethanol:glacial acetic acid: formalin; 18:1:1) and subjected through a graded ethanol series to 100% acetone dehydration before proceeding to critical point drying (CPD) using a Hitachi Critical Point Dryer, HCP-2. The dried samples were immediately mounted on aluminum stubs using gold palladium for 3 min in an Eiko Engineering Ion Coater IB-2. Specimens were examined using a FEI Quanta 200 SEM at a working distance of between 10–20 mm and an accelerating voltage of 10 kV.

LM observations

Bulbils at different stages were dehydrated, pre-infiltrated, infiltrated, and embedded with paraffin. Serial paraffin sections were cut using a Microm HM 315R. After staining with Safranin O, Fast green, and

I-KI, the sections were treated with Xyline, and slides were embedded in Entellan for microscopic study.

Pollen viability and germination test

R. vivipara seldom flowers in the field; however, we had collected eight flowers of *R. vivipara* in 2009. Pollen grains were collected immediately after pollen release on the constriction of the spadix, when flowering, for testing viability and germination. The fluorocromatic procedure utilized fluorescein diacetate and acetone to stain pollen (Heslop-Harrison and Heslop-Harrison, 1970; Nepi *et al*., 2005). Viable pollen grains were brightly illuminated under the 450–490 nm excitation filter of a fluorescence microscope. To test pollen germination, we compared pollen germination at a series of sucrose concentrations (0%, 5%, 7%, 10%, 15%, 20%, 30%, 40%, and 50%). The sucrose solution additionally contained boric acid and calcium nitrate acid (Dafni, 1993). We determined the optional concentration by calculating germination rates for *R. vivipara* and *R. pumila*. Pollen solutions were kept in the dark at room temperature, and germination rates in the optional sucrose concentration were calculated at 24 and 48 h. Pollen viability and germination rates were counted for at least 200 pollen grains in triplicate for each inflorescence.

Chromosome counting

For pretreatment, collected root tips of both species (approximately 0.5 cm long) were treated with 8-hydroxyquinline for 5 h at 18°C. Then root tips were fixed in freshly prepared aceto–alcohol fixative (absolute ethyl alcohol:glacial acetic acid; 3:1). The root tips were placed in fixative and stored in the fridge for 24 h. Subsequently, these root tips were washed two times with distilled water. Root tips were then treated with enzyme complex (2% cellulose:3% pectinase; 1:1) and were macerated in 1 M HCl for 5 min at 60°C. Once macerated root tips had been washed with distilled water three times, the apex of the root tip (approximately 1 mm) was cut with a sharp dissecting blade on a glass slide and stained with aceto–orcein for examination. Photographs were acquired using a camera mounted on a light microscope under bright illumination. For study of meiosis, anthers of *R. vivipara* were first fixed in Farmer's 3:1 solution (absolute alcohol:acetic acid), then transferred to 70% alcohol, and stored at 4°C until use.

RESULTS

The development of bulbiliferous shoots and bulbil clusters

All the bulbils observed develop on bulbiliferous shoots and none on inflorescences (Fig. 1 and Fig. 2). Bulbiliferous shoots of both *R. vivipara* and *R. pumila* initiate from the upper part of the tuber (Figs. 1C and 1D; Figs. 2 A2 and B2). In both the species, 3–5 bulbiliferous shoots are generated by each individual. This shoot-like structure is a novel structure, as most of the Araceae species lack true shoots. The bulbiliferous shoots grow differently in *R. vivipara* and *R. pumila*. *R. vivipara* has an erect and thick bulbiliferous shoot, whereas *R. pumila* has a creeping and branching shoot. Bulbil clusters of both the species develop in the node of the bulbiliferous shoots. Each cluster contains at least 6–10 bulbils. Therefore, each bulbiliferous shoot produces approximately 70–110 bulbils. The mature bulbils have hooked or ciliated scales on top. *R. vivipara* has a short, hooked scaly structure, whereas *R. pumila* produces a long, ciliated scaly structure (Figs. 1E and 1F; Figs. 2 A3 and B3). These hooked scales enable attachment to animal for dispersion.

Detailed bulbil development under SEM and light microscopy

When bulbil development initiates at the node of the bulbiliferous shoot in *R. vivipara*, a primordium will develop by extruding from the epidermis at the node (Figs. 3A and 3B). This primordium then elongates and finally bends in one direction (Figs. 3C and 3D). Next, the second scale primordium initiates from the inside of the first scale primordium. This second scale primordium will also elongate and bend in almost the opposite direction to the first scale primordium (Figs. 3E and 3F). Subsequently, a third scale primordium (Figs. 3G and 3H) will initiate from the inner portion of the second scale primordium (Figs. 3G and 3H). This third scale primordium then elongates and bends in a direction approximately 120° apart from the second scale primordium. Thereafter, the fourth, fifth, sixth, or even seventh scale primordia initiate in a similar sequence (Figs. 3I and 3J). This finally forms a mature bulbil surrounded by 5–8 scale primordia (Figs. 3K and 3L). Scale primordia of *R. pumila* initiate following the same developmental process as *R. vivipara*. However, the scale primordium becomes much longer than in *R. pumila* (Figs. 3M and 3N). The scale primordium of *R. pumila* can elongate as much as five times as long as *R. vivipara*. It is worthwhile to notice that the tips of these scale primordia will eventually curve down, becoming hook-like (Figs. 3I and 3J).

To summarize the development of hooked scales in *Remusatia spp*., the new hooked scale develops at the base of bulbil, before the scale primordia, and the hooked scale primordia developed on the top of bulbil opposite sides of an 120°–150° angle (Fig. 4). We did not observe bulbil dispersal by animals in the field, but this hooked scale structure may greatly aid in the dispersal of bulbils as it easily attached to feathers of

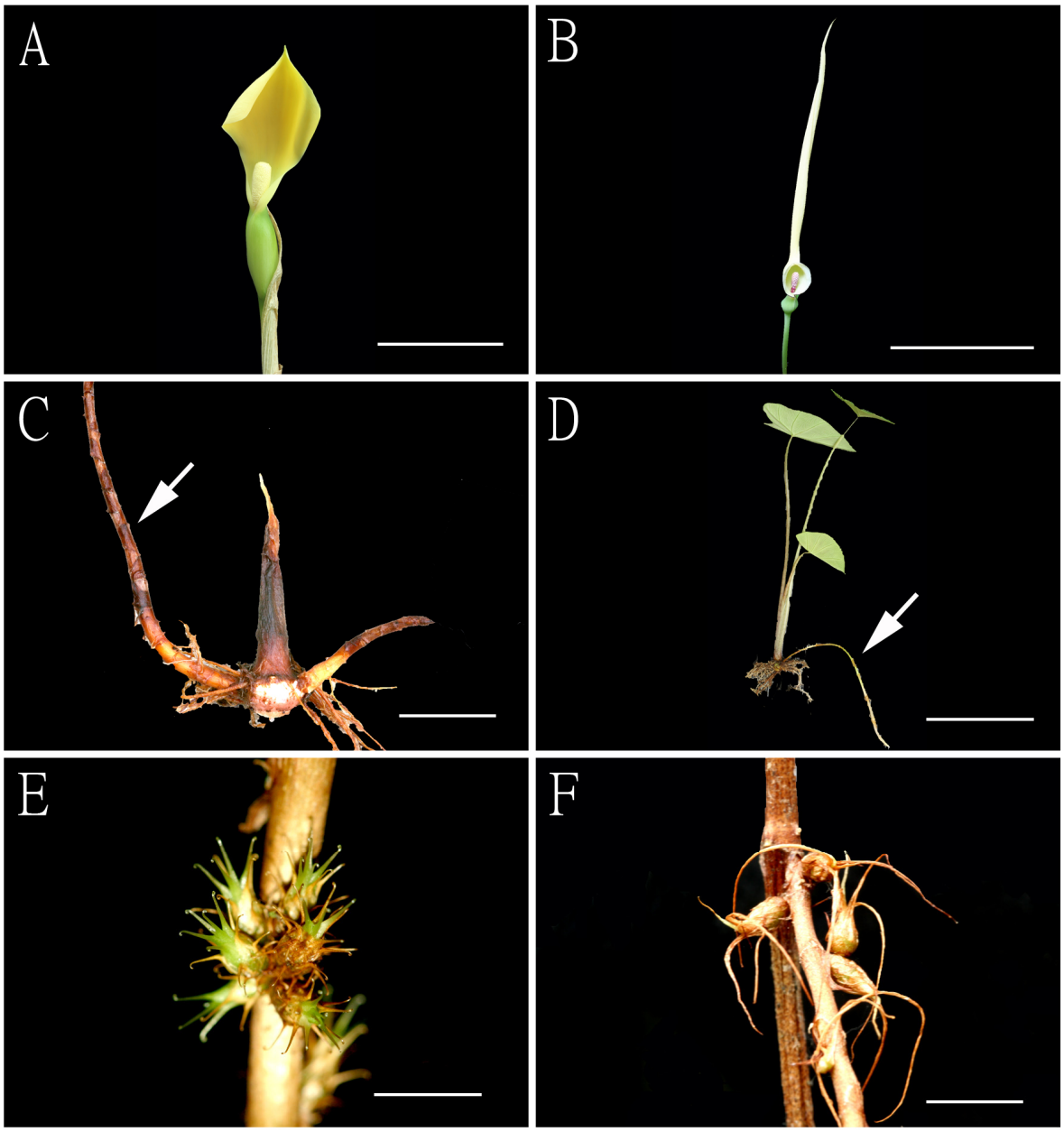

Fig. 1. Growth form of *R. vivipara* (A, C, and E) and *R. pumila* (B, D, and F). A & B: Inflorescences, Bars = 1 cm. C & D: bulbiliferous shoots, Bars = 2 cm. E & F: Bulbils, Bars = 1 cm.

Spilornis cheela in our test (Fig. 5).

Comparison of bulbiliferous shoots and stoloniferous shoots

Starch granules were seldom observed in the cells of the leafstalk and woody stem (Figs. 6A, 6B and 6C). Both the bulbiliferous shoots and stoloniferous shoots grow from the tuber, and we observed starch granules in cells of both types of shoots (Figs. 6D, 6E and 6F). The nodes of the shoots are possibly associated with bulbils. Once the bulbiliferous shoot and stoloniferous shoot are cut off at the ground, the node of the shoot will grow roots and become a new propagule.

Pollen viability and pollen germination rates

In pollen viability tests, no *R. vivipara* pollen could be detected when stained by fluorescein diacetate, indicating that its pollen was inviable. In contrast, a high pollen viability rate (80.8 ± 6.18% of total pollen counted) was observed in *R. pumila* (Fig. 7).

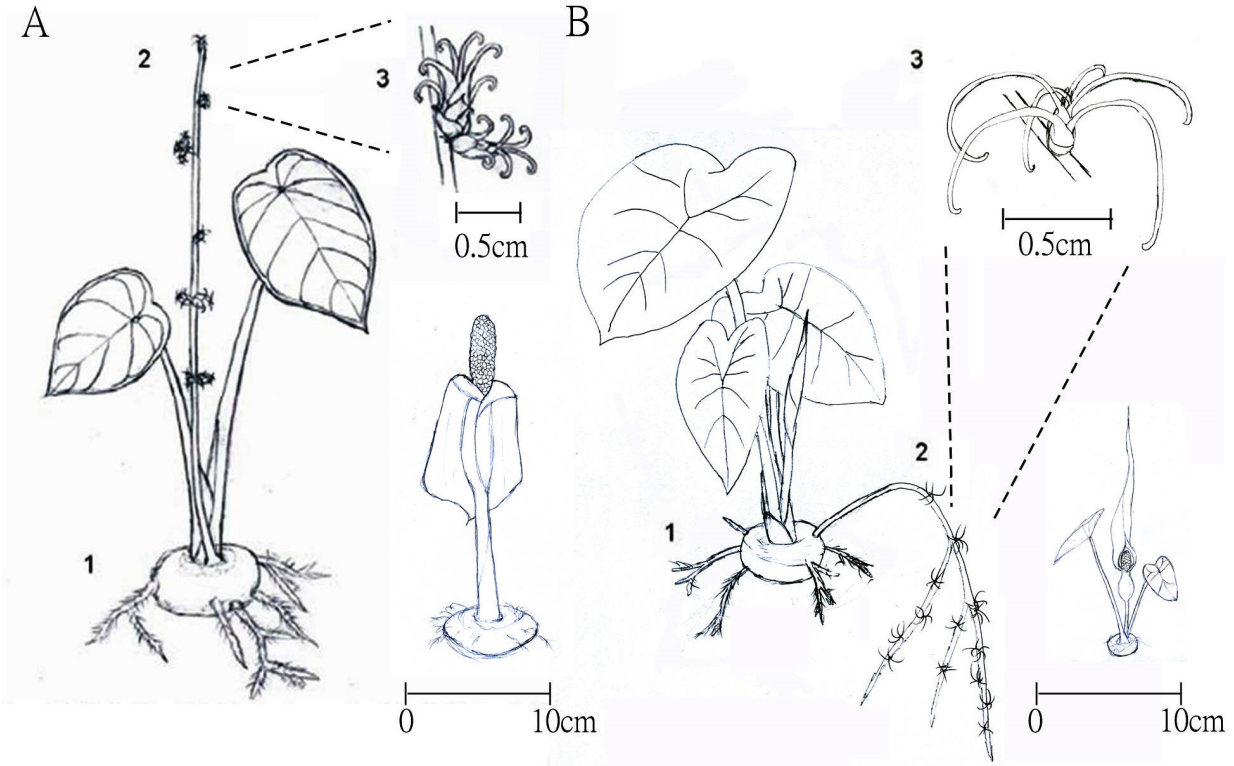

Fig. 2. Plant architecture in natural populations of *R. vivipara* (A) and *R. pumila* (B). The growth form of bulbiliferous shoots are erect in *R. vivipara* (A2) and branching stolons in *R. pumila* (B2). The scaly structure on bulbils is longer in *R. vivipara* (A3) than in *R. pumila* (B3).

The germination of pollen grains at room temperature in different concentrations of sucrose solutions were compared 24 and 48 h after treatment. We found that the optional sucrose concentration for pollen germination was 5–10%. We used sucrose solutions (5%, 7%, 10%) to grow both *R. vivipara* and *R. pumila* to compare their germination rates. Measured at 24 and 48 h after treatment, *R. vivipara* pollens are unable to germinate at all. However, 68.92 ± 12.97% of *R. pumila* pollen germinated at 24 h and 56.17 ± 21.71% had germinated at 48 h (Fig. 7).

Chromosome numbers

Chromosome numbers counted from all flowering samples were triploid (2n = 3X = 42. Fig. 8A), and the chromosome bridge associated with triploidy might cause abnormal meiotic divisions in pollen mother cell in *R. vivipara* (Fig. 8B), resulting in its inviability.

DISCUSSION

Function and structure of the hooked scales

Bulbils can grow more rapidly than seeds, and survive from one growing season to the next in *Polygonum viviparum*, *Allium vineale*, and *Globba cernua* (Engell, 1973; Ronsheim and James, 2000; Box and Rudall, 2006). Gene flow among plants probably depends on sexual reproduction (Walser, 2004; Walser *et al*., 2004). Individuals from clonal propagules are almost genetically identical in these species (Grimsby *et al*., 2007). This is because bulbils are dispersed around the parent plant. To increase dispersal efficiency, some plants have evolved special bulbils. The V-shaped bulbils of *T. oldhamii* may be more easily carried by animals than seeds (Wang and Cronk, 2003). Long-distance dispersal by appendage is considered to be of a particular importance in a plant's life (Honnay *et al*., 2006; Will *et al*., 2007; Vittoz and Engler, 2007). In fact, bulbil of *Remusatia* is ovoid and is covered with scales. The scales are hooked, which can easily attach to human clothes and animal's furs. This may be the reason that we found the populations of *R. vivipara* in dispersed over a long distance in Taiwan.

The ontogeny of *Remusatia bulbils*

Based on the developmental similarities and the location of stored nutrients, brood bulbils have nutrient storage in leaves or leaf bases (e.g., *Mimulus gemmiparus*, Moody *et al*., 1999). These bulbiliferous shoots and new tubers originate from the same site as

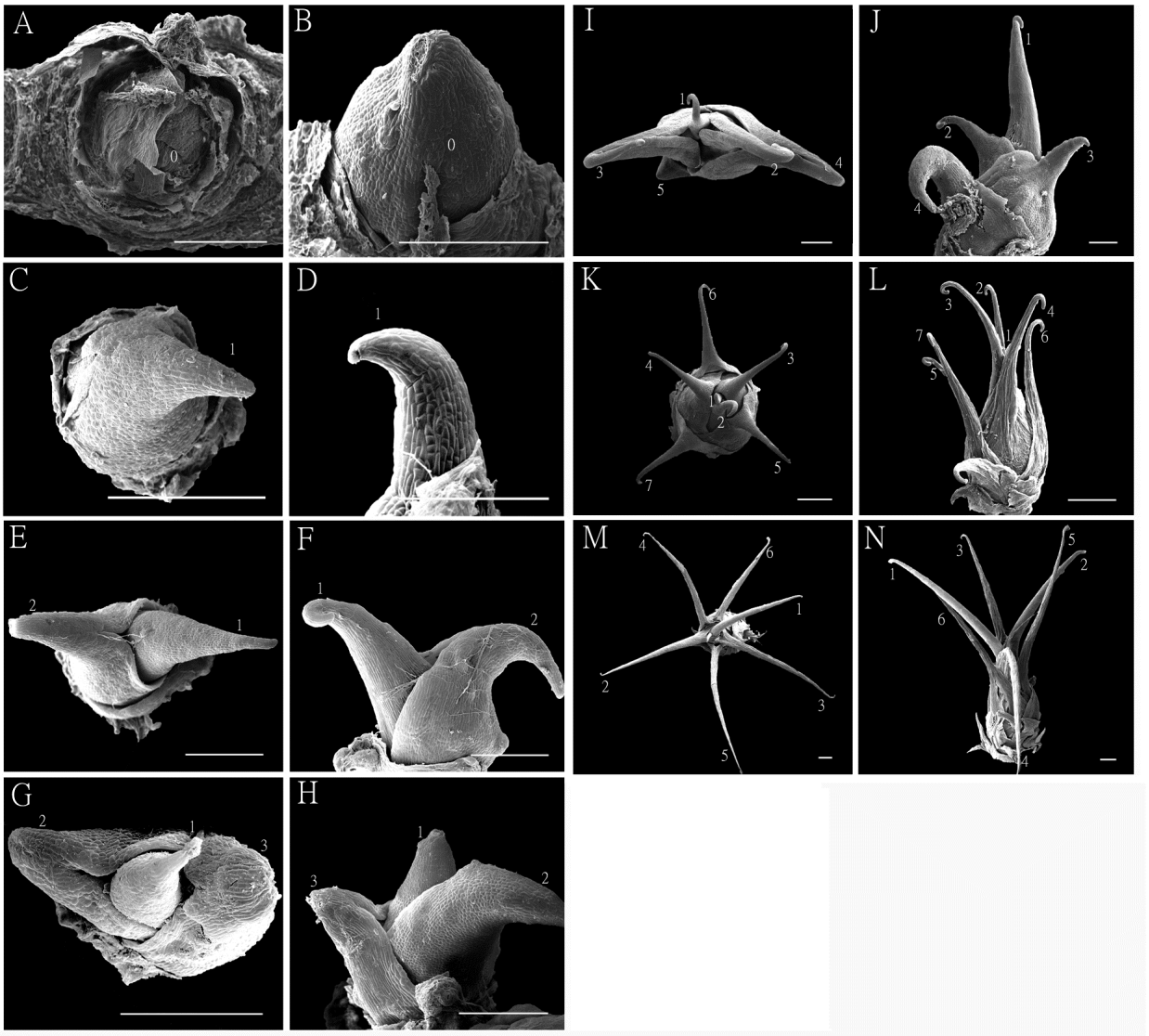

Fig. 3. SEM of bulbil development. A–L: bulbil of *R. vivipara*; M, N: bulbil of *R. pumila*; left A–M: Top view of bulbil. right B–N: side view of bulbil. 0 = meristem, 1–7: sequentially hooked scales. Cells of bulbil grow coarsely and extend to the hooked scale initially, and then the mature hooked scales of bulbil become increasingly thinner. Figure L, N indicates hooked scale of bulbils are different in the two species. Bulbils of *R. vivipara* have hooked scales, but bulbils of *R. pumila* have ciliated scales. Bars = 500 μm.

the tubers. Asexual bulbils are brood tubers in the stem component. Nutrients in the form of starches facilitate germination of the new plant. When detached from parent plant the bulbiliferous shoots or bulbils fall to the ground, and the node between the shoot and bulbils produces new individuals.

Triploid population of R. vivipara seldom set seeds in Taiwan

Bulbiliferous plants display both sexual and asexual reproduction. Asexual reproduction in some species appears to offset a barrier to sexual reproduction that reduces set seeds. The barrier in sexual reproduction of *P. viviparum* appears to be largely due to a low rate of fertilization and embryo/fruit abortion (Diggle *et al*., 2002). In *T. oldhamii*, pollen tubes are apparently unable to locate the position of the micropyle when a bulbil developed at the top of the inflorescence (Wang *et al*., 2004). In *Agave palmeri*, the pollinators are migratory and may fail to arrive in synchrony with the brief mast-flowering period of the flowering stalks (Howell, 1981). Fruit abortion is caused by self-incompatibility in *Opuntia microdasys* (Palleiro *et al*., 2006). These factors lead to low rates of seed set in

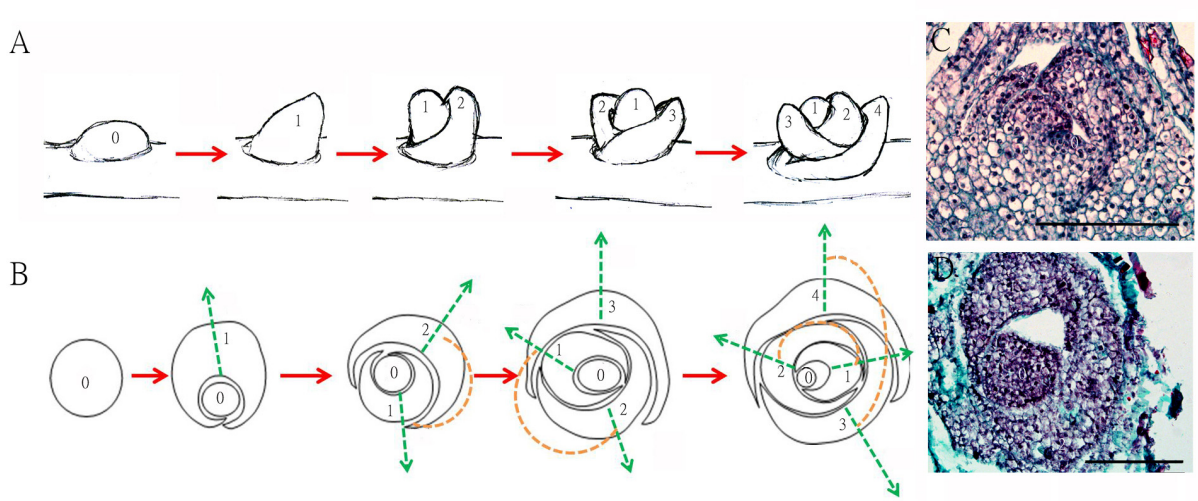

Fig. 4. A, B: Architecture of developing hooked scale side and top view of bulbil; C, D: section of bulbil. 0: meristem, 1–7: sequentially hooked scales. The new scale develops between the base of the previous scale primordial and the scale primordia developed on the top of bulbil, and each scale primordial is arranged at an angle between 120°–150°. Bars = 200 μm.

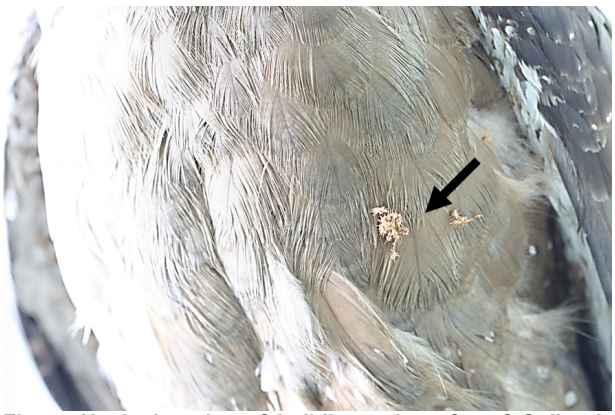

Fig. 5. Hooked scales of bulbil attach to fur of *Spilornis cheela*.

these species. In the Araceae, pollen viability decreases over time in *Arum italicum*, and it must be dispersed immediately between mature and receptive inflorescences for efficient pollination (Gibernau and Macquart, 2003). Although we could not determine all the factors that reduce set seeds in *R. vivipara*, we identified pollen viability as one barrier to successful sexual reproduction; the pollen germination test revealed that *R. vivipara* pollen is inviable. The results reported here will allow us to compare reproductive strategy between *R. vivipara* and *R. pumila*.

Why *R. vivipara* needs conservation in Taiwan

R. vivipara is listed in the red lists of Taiwan as a Vulnerable (VU) species, and mature individuals are less than 1000 (Wang *et al*., 2012). Although *R. vivipara* has barriers in sexual reproduction, it can produce large numbers of bulbils; however, the reason for its rare occurence in the field is ambiguous. *R. vivipara* grows on moist limestone, with several species of moss on trees. In addition, it requires a bright environment; therefore, *R. vivipara* is never found under the ground cover in Taiwan. It appears unable to compete with the other plants on the ground in the same habitat, and it is becoming increasingly rare. However, *R. vivipara* is intermittently distributed across Taiwan. The genetic variation within and between populations of *R. vivipara* needs to be determined to assess the extent of the asexual colonies and to determine whether genetic variation is lower in marginal and threatened populations.

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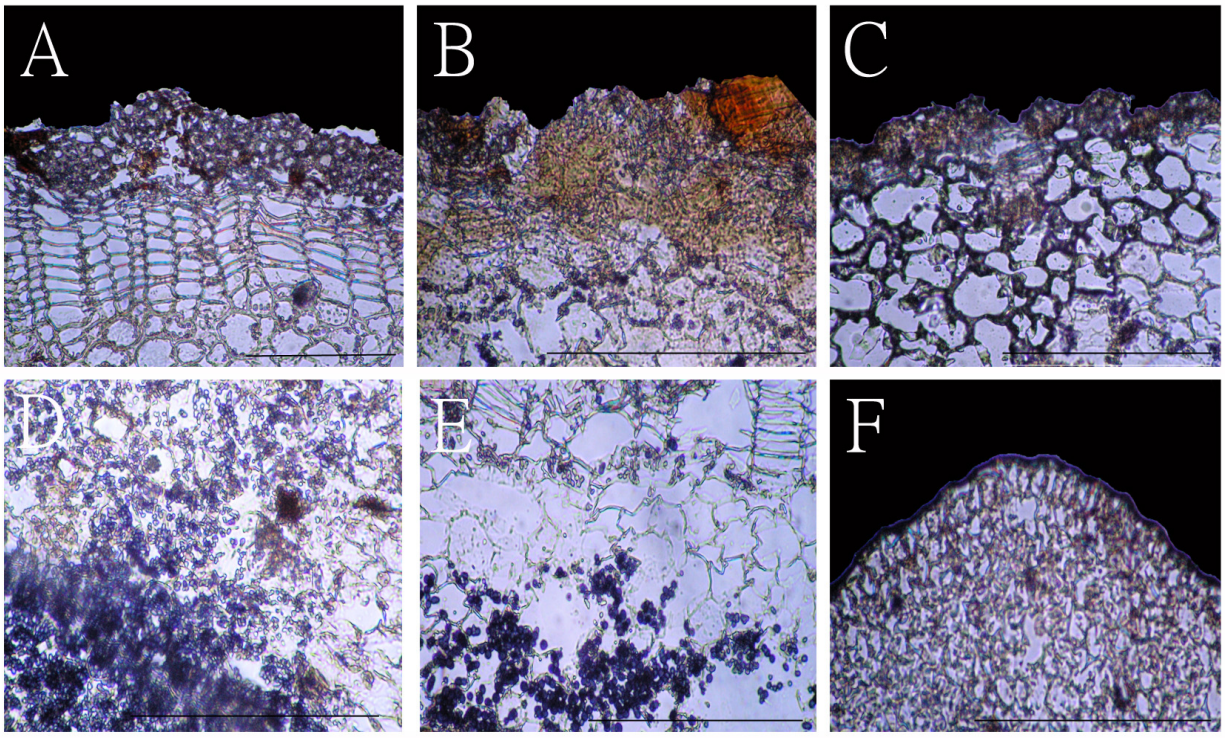

Fig. 6. Distribution of starch granules in the leafstalk (A, B), woody stem (C), bulbiliferous shoot (D, E) and stoloniferous shoot (F). A, D: Leafstalk and bulbiliferous shoot of *R. vivipara*. B, E: Leafstalk and bulbiliferous shoot of *R. pumila*. C: Woody stem of *Epipremnum pinnatum*. F: Stoloniferous shoot of *Colocasia formosanum*. S = Starch granules. Sections were stained with I-KI. Cells of the bulbiliferous and stoloniferous shoots were full of starch granules. Bars = 200 μm.

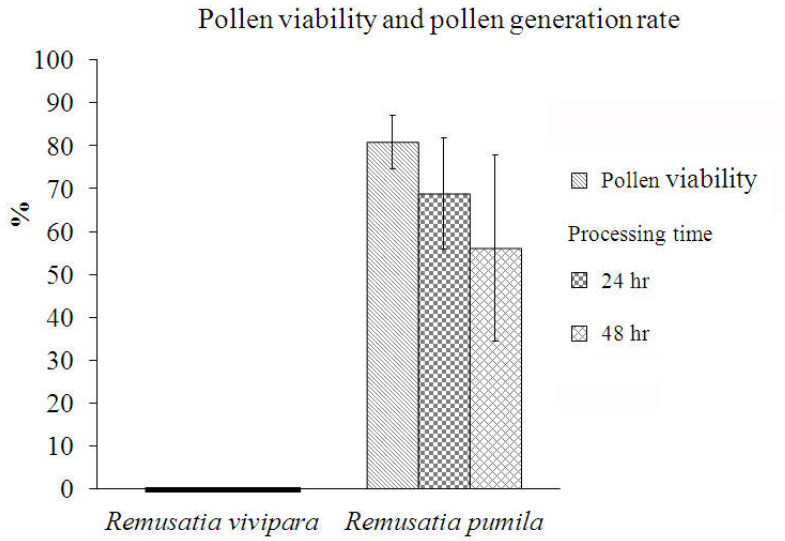

Fig. 7. *In vitro* viability and germination of *R. vivipara* and *R. pumila* pollen grains at different sucrose concentrations.

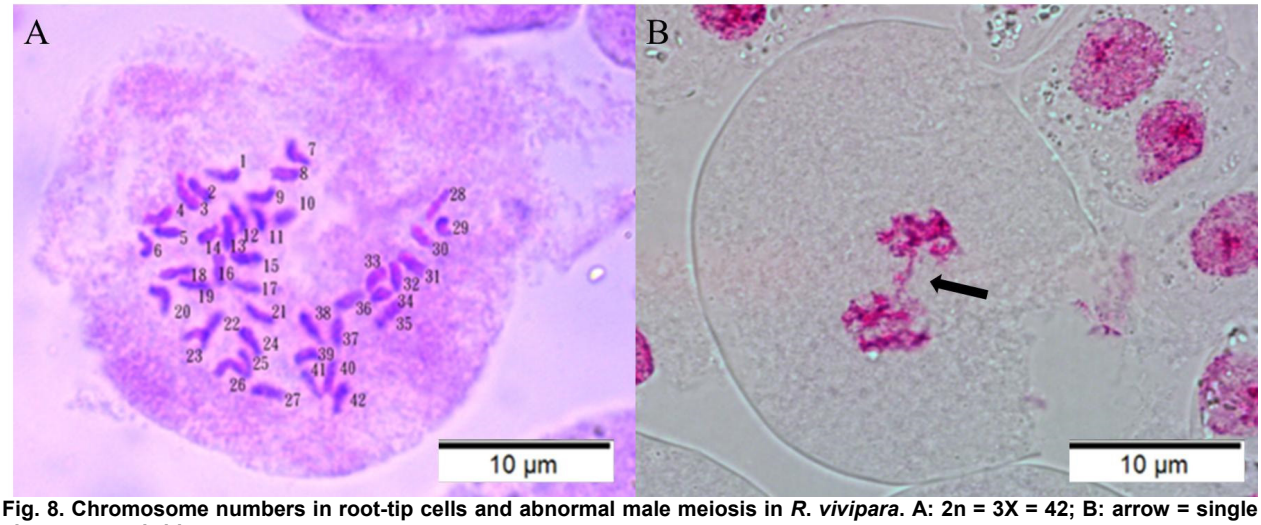

Fig. 8. Chromosome numbers in root-tip cells and abnormal male meiosis in *R. vivipara*. A: 2n = 3X = 42; B: arrow = single chromosome bridge.

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天南星科 *Remusaia vivipara* 岩芋（臺灣目賊芋）和 *Remusaia pumila* 曲苞岩芋的無性珠芽發育和生殖策略的多樣化

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摘要：岩芋（臺灣目賊芋）和曲苞岩芋能產生有性花和無性繁殖珠芽。然而岩芋很少產生種子，而曲苞岩芋卻是常見種子與珠芽。本文的研究目的是要了解無性繁殖的珠芽發育和岩芋有性生殖的問題。岩芋和曲苞岩芋的珠芽枝條均從塊莖的上部發育出來，而珠芽則簇生在珠芽枝條的節上。利用掃描式電子顯微鏡和光學顯微鏡的觀察結果顯示珠芽發育時，珠芽外附倒鉤鱗片，此鱗片的發育與葉子的發育順序及位置相似，各錯開約120°逐漸發出佈滿珠芽外側。鱗片能氈附動物皮毛，有助於珠芽向外傳播。花粉活性及花粉管萌發實驗的結果顯示曲苞岩芋的花粉具有活性，能產生花粉管，然而岩芋當小孢子母細胞分裂時，出現橋鏈的情況，使岩芋產生無活性的花粉，花粉管無法生長。進一步進行染色體的計數，確認岩芋為三倍體植物。就這兩種珠芽植物的生殖策略而言，岩芋完全依賴無性生殖，而曲苞岩芋則兼具有性生殖及無性生殖。然而珠芽枝條的發育是天南星科一個獨特的演化現象，這群植物也就是依據此特徵而歸類成為岩芋屬。

關鍵詞：天南星科、倒鉤鱗片、花粉活力、花粉萌發、岩芋、曲苞岩芋。