Embryological Characteristics of Floral Morphology and Anther Development in Yunnan Flame Orchid (Postprint)

Authors: Wang Lecheng, Tao Kaifeng, Zhu Yong, Zhang Jin, Luo Yan, Li Lu

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Abstract

Research on the rare and endangered plant genus Renanthera is limited. This study investigated the floral morphology and embryological characteristics of anther development in Yunnan Renanthera using microscopy and paraffin sectioning techniques. The results showed: (1) The floral morphology possesses the following taxonomic characteristics: a trilobed labellum with an inconspicuous spur; a cylindrical gynostemium; a purple anther cap with yellow stripes; and a pair of pollinia connected by a viscidium and stipe. (2) The anther primordium differentiated into a pair of lateral anther locules, with a centrally located, off-axis sterile septal tissue differentiating within the microsporangium of each locule, developing into a pair of unequally sized secondary microsporangia; at anther maturity, degradation of the sterile septal tissue resulted in the formation of two deeply cleft pollinia. (3) The fully developed anther wall comprises 5–6 layers, representing a "multilayered" anther wall type, including: epidermis, 2–3 layers of endothecium, middle layer, and tapetum; the tapetal cells are uninucleate and glandular. At anther maturity, the endothecium undergoes fibrous thickening, and the anther locules dehisce and release pollen at the abaxial end. (4) Microspore mother cells underwent simultaneous cytokinesis, forming tetrahedral and bilaterally symmetrical microspore tetrads; the microspores remained within the tetrads and developed into 2-celled tetrads through mitosis, arranged compactly to form a robust pollinium. No pollen abortion was observed during anther development. Based on existing literature, this study analyzed the taxonomically significant embryological characteristics in the floral morphology and anther development of Yunnan Renanthera, providing new data for the taxonomy and conservation biology of the genus Renanthera.

Floral Morphology and Embryological Features of Anther Development in *Renanthera imschootiana*

Yuecheng Wang[1], **Kaifeng Tao**[1], **Yong Zhu**[1], **Jin Zhang**[1], **Yan Luo**[2], **Lu Li**[1]*

[1]Department of Biodiversity Conservation, Southwest Forestry University, Kunming 650224, China
[2]Department of Horticulture and Gardening, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666300, Yunnan, China

Abstract

Studies on the rare and endangered orchid genus *Renanthera* are scarce. This paper investigates the floral morphology and embryological features of anther development in *Renanthera imschootiana* using microscopy and paraffin sectioning techniques. The results reveal: (1) Floral morphology exhibits the following taxonomic characteristics: a tri-lobed labellum with an inconspicuous spur, a cylindrical column, a purple anther cap with yellow stripes, and a pair of pollinia connected by a viscidium and stipe. (2) The anther primordium differentiates into a pair of lateral thecae, with each theca developing a sterile septum along the central axis of the microsporangium that divides it into two unequal secondary microsporangia; upon anther maturity, degradation of this sterile septum results in two deeply cleft pollinia. (3) The fully developed anther wall comprises 5–6 layers, classified as the "massive type," including the epidermis, 2–3 layers of endothecium, a middle layer, and the tapetum; tapetal cells are uninucleate and glandular. At anther maturity, the endothecium develops fibrous thickenings, and the anther dehisces abaxially to release pollen. (4) Microspore mother cells undergo simultaneous cytokinesis during meiosis, forming tetrahedral and isobilateral microspore tetrads; microspores remain within the tetrads and develop into 2-celled pollen through mitosis, arranged compactly to form a rigid pollinium. No pollen abortion was observed during anther development. By integrating existing data, this study analyzes the taxonomically significant embryological features of floral morphology and anther development in *R. imschootiana*, providing new information for the taxonomy and conservation biology of the genus *Renanthera*.

Keywords: *Renanthera*, morphology, pollinium development, massive type anther wall, microsporogenesis and male gametophyte development

Introduction

The genus *Renanthera* belongs to the tribe Vandeae, subfamily Epidendroideae of Orchidaceae. This relatively small genus comprises only 20 species distributed primarily in Southeast Asia and the tropical Himalayan region (Ji et al., 1999). China hosts three species with narrow geographic distributions (Jin et al., 2019), yet research data remain limited. *Renanthera imschootiana* has a broader distribution, occurring in southern Yunnan, Guangxi, and Hainan in China, as well as in Myanmar, Thailand, Laos, and Vietnam (Chen et al., 1999; Wu, 2003; Jin et al., 2019). *Renanthera imschootiana* from Yunnan is endemic to China, found only at the type specimen locality (Vietnam) and in southern Yunnan (Yuanjiang) (Ji et al., 1999). A recently described new species, *R. sinica*, is also endemic to China, restricted to Wenshan, Yunnan (Liu et al., 2003). *Renanthera* species are large epiphytic orchids with climbing stems, leathery leaves, panicles or racemes bearing multiple flowers, and large, flame-red, orange, or spotted flowers of high ornamental value (Chen et al., 1999). Several artificial hybrid cultivars have been developed (Luo et al., 2012; Cao et al., 2014).

Due to their narrow geographic distribution, sparse wild populations, and limited herbarium collections, *Renanthera* species are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), included as critically endangered in China's Red List of Biodiversity—Higher Plants (Yang, 2013), incorporated into the List of National Key Protected Wild Plants (Ministry of Agriculture and Rural Affairs of State Forestry and Grassland Administration, 2021), and recorded in the List of Plant Species with Extremely Small Populations in Yunnan (2021 Edition) (Sun, 2021). Currently, information on geographic distribution, population structure, biology, and reproductive ecology of *Renanthera* remains scarce (Li et al., 2018), necessitating further research. Field surveys conducted between 2014 and 2018 identified two population sites of *R. imschootiana* in Yuanjiang, Yunnan, at elevations of 1,100–1,200 m in mixed evergreen and deciduous broad-leaved forests, where plants grew epiphytically on *Quercus variabilis* and *Cyclobalanopsis helferiana* trunks. Only 12 individuals were found, including just one seedling, indicating a declining population. Low fruit set despite flowering was identified as a direct cause of its endangered status (Li et al., 2018). Therefore, studies on reproductive biology and pollination ecology are urgently needed.

Floral morphology and anther development constitute major aspects of pollination biology, breeding systems, and embryonic development in Orchidaceae, encompassing pollination syndromes (Pansarin & Maciel, 2017; Huang & Guo, 2000; Zhang et al., 2022), differentiation of pollen dispersal units at anther maturity (Singer et al., 2008), and embryological features of microsporogenesis and male gametophyte development during anther development (Swamy, 1949; Rao, 1967; Sood, 1989; Kant & Hossain, 2010; Zhang et al., 2019; Tan et al., 2020; Li et al., 2020). These features play crucial roles in species classification and systematic evolution at subfamily, tribal, subtribal, generic, and specific levels within Orchidaceae (Dressler, 1986, 1993; Freudenstein et al., 2002; Pridgeon et al., 2005; Freudenstein & Chase, 2015; Mosquera-Mosquera et al., 2019). The three Chinese *Renanthera* species exhibit remarkable similarity in plant morphology and floral color, often causing confusion during field surveys (Chen et al., 1999; Wu, 2003; Jin et al., 2019). When Liu et al. (2003) first described *R. sinica* as a new species, they emphasized comparative analysis of floral morphological features, identifying inflorescence type, flower color, and labellum lobes as key characteristics for species identification within the genus. A study on *R. bella* from Malaysia under artificial cultivation conditions documented its growth and development process, providing new information for artificial breeding of *Renanthera* (Mohamad & Rusdi, 2020). Considering the importance of floral morphology and embryology and the scarcity of research data on *Renanthera*, this study examines *R. imschootiana* using dissection microscopy and paraffin sectioning to observe its floral morphology and embryological features of anther development. Combined with existing data, we conduct a comparative analysis of their taxonomic significance to provide new information for species identification and conservation biology of this genus.

Materials and Methods

Renanthera imschootiana plants cultivated at the Southwest Forestry University Research and Teaching Practice Base (Yunnan Fengchunfang Biotechnology Co., Ltd. Orchid Germplasm Resource Nursery) were growing and flowering normally, with a flowering period from April to May each year. From 2017 to 2020, flower buds and open flowers at different developmental stages were collected for four consecutive years and fixed in 50% ethanol for storage at room temperature. Fresh open flowers were dissected and observed under a stereomicroscope (Leica M165 FC) to document and photograph the morphology, color, and size of floral structures including the labellum, column, anther cap, pollinia, viscidium, and stipe. Fixed bud materials were divided into five developmental stages based on morphology and size, with 5–10 buds sampled per stage. Samples were dehydrated through an ethanol series, cleared in xylene, infiltrated with paraffin, and embedded using conventional paraffin sectioning techniques (Leica RM 2235) at a thickness of approximately 6–8 m. Sections were stained with iron alum-hematoxylin and mounted in neutral resin (Li, 2006). After drying, sections were observed and photographed under a microscope, with images arranged using Adobe Photoshop 2020.

Results

2.1 Floral Morphological Structure

Renanthera imschootiana is a large epiphytic herb bearing typical axillary racemes when flowering, with slender, multi-branched inflorescence axes (Plate I: A) bearing 5–30 flowers. Flowers are bilaterally symmetrical, with dorsal sepals and lateral petals oblanceolate and pale yellow; the two lateral sepals are narrowly spatulate and orange-red (Plate I: B). The fleshy labellum is small and tri-lobed, with lateral lobes deep purple, triangular, sharply acute, erect, and extending beyond the column, with two irregularly margined yellow lamellae at the base (Plate I: C–E). The mid-lobe is ovate, recurved, with a dark red apex and white tuberculate ridges at the base (Plate I: F, G). The spur is inconspicuous, a sac-like protrusion approximately 2 mm long. The column is short, 4–5 mm long, cylindrical with a white base and purple-red patches, featuring a deeply sunken stigmatic cavity with a semicircular lower portion and a deeply fissured opening at the upper portion forming inward-closing column wings (Plate I: D, G). The anther cap is helmet-shaped, semicircular, light purple externally, with a clearly divided two-chambered inner wall, leaving residual anther wall tissue after dehiscence and pollinia release (Plate I: H, I). Each theca contains one pollinium, unequal in size and deeply cleft, with the abaxial side larger and the adaxial side smaller, connected by a white, slender cylindrical stipe and yellow viscidium to form a pollinarium with accessory structures (Plate I: J, K).

2.2 Developmental Pattern of a Pair of Deeply Cleft Pollinia

Early anther primordia differentiated into a pair of lateral, juxtaposed thecae that curved toward the labellum. Each theca differentiated into a microsporangium composed of archesporial cells with large nuclei and uniform morphology (Plate II: A, B), which subsequently differentiated into a deeply stained microsporangium and a lightly stained sterile septum (Plate II: A, C, D). The sterile septum consisted of lightly stained parenchyma cells that clearly differed from the actively dividing, deeply stained microspore mother cells (Plate II: E). It originated from the middle of the microsporangium near the connective tissue, extended along the longitudinal axis of the anther chamber to the central region of the microsporangium, then bent transversely toward the inner side of the anther, terminating at the 5–6 layers of microspore mother cells near the anther wall, differentiating into a transversely curved, incompletely penetrating tissue (Plate II: C) that formed a pair of unequal, deeply cleft secondary microsporangia (Plate II: C). During meiosis of microspore mother cells, cells of the sterile septum near the microspore mother cells differentiated into 1–2 layers of small, nearly spherical, uninucleate inner tapetal cells (Plate II: E, F). From the microspore tetrad to male gametophyte developmental stages, the inner tapetum and sterile septum cells began to degrade, creating spaces that divided the pollen sac derived from the microsporangium into an unequal, deeply cleft pollinium (Plate II: G, H). Consequently, mature anthers of *R. imschootiana* produce a pair of deeply cleft pollinia, consistent with floral morphological observations (Plate II: J, K).

2.3 Massive Type Anther Wall Development

Early anther primordia formed from a mass of deeply stained archesporial cells beneath the epidermis (Plate III: A). Subepidermal archesporial cells differentiated into primary parietal cells (Plate III: B). Through 2–3 periclinal divisions, primary parietal cells successively developed into inner and outer secondary parietal cells (Plate III: C). By the microspore mother cell stage, a complete anther wall had developed, consisting from outside to inside of: epidermis, 2–3 layers of endothecium, middle layer, and tapetum (Plate III: D–F). Thus, the anther wall development type is classified as "massive type" (Sood, 1989). At this stage, epidermal and endothecial cells enlarged and became rectangular; the middle layer was slender; and the tapetum was spherical, uninucleate, and glandular (Plate III: E, F). During meiosis of microspore mother cells, the middle layer and tapetum showed degradation, while epidermal cells degenerated and became irregularly serrated (Plate III: G–I). At the 2-celled pollen stage of male gametophyte development, the epidermis gradually degraded with obvious remnants, the middle layer and tapetum degraded completely creating clear spaces, and two layers of endothecial cells showed prominent annular thickenings (Plate III: H, I), providing the basis for anther dehiscence and pollen release.

2.4 Microsporogenesis and Male Gametophyte Development

Early anther primordia were surrounded by a mass of deeply stained archesporial cells beneath the epidermis, which after several mitotic divisions differentiated into closely arranged microspore mother cells with large nuclei (Plate IV: A). Subsequently, microspore mother cells increased their nuclear-cytoplasmic ratio and became distinctly polygonal, entering meiosis I (Plate IV: B), producing two daughter nuclei without cell walls (Plate IV: C). As development proceeded, the two daughter nuclei simultaneously underwent the next division, forming four daughter nuclei arranged in tetrahedral or isobilateral configurations within the same cell wall (Plate IV: D). Constrictions then appeared between the four nuclei, each developing a daughter cell wall to form microspore tetrads, predominantly in tetrahedral arrangement (Plate IV: E), occasionally isobilateral (Plate IV: F), with no other arrangement types observed. During male gametophyte development, microspore tetrads were not released from the callose and continued to undergo mitotic division, forming a large central vegetative cell and a small peripheral generative cell (Plate IV: G). Finally, the generative cell was absorbed into the vegetative cell, constituting 2-celled pollen (Plate IV: H). Four mature pollen grains remained within the same callose mass, forming pollen tetrads that were tightly arranged to create a hard, mature pollinium. Notably, approximately 50 flower buds were examined as experimental material, and 100 anther chambers were observed, with no pollen abortion detected.

Discussion and Conclusion

This study reveals the floral morphological characteristics and embryological features of anther development in *Renanthera imschootiana* through dissection microscopy and paraffin sectioning at both structural and cellular-tissue levels. We confirm that the formation of a pair of deeply cleft pollinia is associated with the differentiation and degradation of an incompletely penetrating sterile septum in the center of each theca's microsporangium. Combined with existing data, we discuss their taxonomic and conservation biological significance as follows.

3.1 Floral Morphological Features for Species Identification and Classification within *Renanthera*

First, this study supports previous viewpoints that the position of raceme insertion, flower color, and labellum lobe characteristics serve as diagnostic features for the three Chinese *Renanthera* species (Ji et al., 1999; Liu et al., 2003; Chen et al., 2009; Jin et al., 2019). Axillary racemes occur in *R. imschootiana* and *R. sinica*, while opposite racemes are found in *R. coccinea*. Orange-red flower coloration appears in *R. imschootiana* and *R. coccinea*, whereas pale yellow with purple-red spots characterizes *R. sinica*. All three species have tri-lobed labella, but mid-lobe and lateral lobe features differ significantly among species. A saccate-globose upper mid-lobe occurs only in *R. sinica*, while the other two species lack saccate structures. Erect triangular lateral lobes with a pair of parallel high lamellae at the base appear in *R. imschootiana*; square or suborbicular lateral lobes occur in *R. coccinea*; and ovate or lanceolate lateral lobes characterize *R. sinica* (Liu et al., 2003). This study observed three white fleshy ridge-like protrusions at the base of the mid-lobe and lamellate calli at the base of the lateral lobes in *R. imschootiana*, features not previously described in detail for the other two species and warranting further investigation.

Second, we consider pollinium number and morphology, along with viscidium and stipe characteristics, to show significant variation among *Renanthera* species and genera, holding important taxonomic value. The subfamily Epidendroideae, to which *Renanthera* belongs, features highly fused pollinia and pollinium accessories, including the anther cap, viscidium, stipe, and caudicle (Dressler, 1993; Singer et al., 2008; Mosquera-Mosquera et al., 2019). However, research on these floral structural features in *Renanthera* is limited (Ji et al., 1999; Liu et al., 2003; Chen et al., 2009; Jin et al., 2019). Using dissection microscopy, this study examined mature anther structures of *R. imschootiana*, revealing that anther dehiscence exposes two unequal, deeply cleft pollinia bonded by a nearly circular, thick viscidium, a feature confirmed through histological sections. This clearly differs from existing descriptions of *Renanthera* as having four nearly equal pollinia with elastic filaments at the base, a slightly long and broad stipe, and a thick, nearly circular viscidium (Ji et al., 1999). When *R. sinica* was described as a new species, although its pollinium number and morphology were not detailed, the line drawing documented a pair of unequal pollinia with a saddle-shaped viscidium (Liu et al., 2003). Pollinium morphology and number in *R. coccinea* remain unclear, but its stipe is described as geniculate at the middle (Ji et al., 1999), while the other two species have straight stipes (Liu et al., 2003). Therefore, detailed features of pollinium morphology, viscidium, and stipe hold significant taxonomic value in *Renanthera* and require accurate description.

3.2 Anther Development Helps Determine Diverse Pollinium Types in the Subtribe Aeridinae

Long-standing controversies exist regarding variation in pollinium morphology and number among different groups in subfamily Epidendroideae, particularly the subtribe Aeridinae, and their taxonomic significance (Dressler, 1993; Freudenstein et al., 2002; Chase et al., 2015). This primarily stems from the concealed position of some species' pollinium features (poricidal, shallowly cleft, or deeply cleft), often hidden at the junction of the two anther chambers and difficult to observe clearly even under dissection microscopy, leading to "misidentification." For example, *Pennilabium yunnanensis* appears to have a pair of entire pollinia under dissection microscopy, but histological sections reveal a pair of finely poricidal pollinia (Li et al., 2020). Freudenstein & Rasmussen (1996) used paraffin sectioning to examine anther development in ten representative genera of Epidendroideae with different pollinium types, revealing that 2, 4, or 8 pollinia result from the early differentiation of sterile septa in varying numbers (2, 4) and arrangements (longitudinal or transverse) within the microsporangium of anther primordia, dividing it into secondary microsporangia that are absorbed and degraded during microsporogenesis and male gametophyte development, creating spaces that separate mature pollen sacs into different numbers of pollinia. Similar studies have confirmed this pattern in other Epidendroideae members. For instance, a pair of poricidal or shallowly cleft pollinia results from an incompletely penetrating sterile septum that differentiates early and degrades at anther maturity, creating spaces, as observed in *Eulophia hormusjii* (Bhanwra & Vij, 2003), *Cymbidium aloifolium* (Kant et al., 2013), and *Vanda coerulea* (Zhang et al., 2019). The eight pollinia of *Arundina graminifolia* develop from a pair of cross-shaped sterile septa in each theca's microsporangium that degrade at anther maturity, dividing the pollen sac into four rod-shaped pollinia (Tan et al., 2020). This study demonstrates at both morphological and histological levels that *R. imschootiana* has two pollinia forming a deeply cleft pair, rather than the four pollinia previously documented for *Renanthera* (Ji et al., 1999). Therefore, histological observation of sterile septum differentiation patterns and their degradation at anther maturity is necessary to understand the relationship with pollinium morphology and number (Li et al., 2020). This approach offers important insights for exploring the taxonomy and systematics of tribe Vandeae–subtribe Aeridinae based on pollinium number and morphology (Freudenstein et al., 2002).

3.3 "Massive Type" Anther Wall Development Pattern in Orchidaceae and Its Correlation with Pollinium Features

This study observed that the fully developed anther wall of *R. imschootiana* has 5–6 layers, belonging to the "massive type" (Sood, 1989), which holds important systematic and pollination ecological significance. Typically, angiosperm anther walls have fewer than 5 layers and can be categorized into four developmental types: basic, monocotyledonous, dicotyledonous, and reduced types (Johri et al., 1992; Hu, 2005). However, anther wall layer number varies considerably among orchid subfamilies and genera, including both 4-layered monocotyledonous types and 6–7-layered massive types, warranting further data collection for taxonomic analysis. In subfamilies Apostasioideae (Kocyan & Endress, 2001), Cypripedioideae (Swamy, 1949; Ghimire et al., 2020), and Orchidoideae (Sood, 1986, 1988; Kant & Goel, 2013), anther walls typically have 4 layers, representing the monocotyledonous type. In Epidendroideae, however, anther walls include both 4-layered monocotyledonous types and massive types with 6–7 layers. For example, monocotyledonous anther walls occur in *Malaxis* (Sood, 1992), *Dendrobium* (Gurudeva, 2016), and *Pennilabium yunnanensis* (Li et al., 2020). The massive type was first described in *Liparis* (Sood, 1989) and subsequently reported in *Cymbidium* (Kant et al., 2013), *Spathoglottis plicata* (Sriyot et al., 2015), and *Eulophia* (Bhanwra et al., 2003). Notably, *Vanda coerulea* has more than 7 anther wall layers, reaching up to 9 layers (Zhang et al., 2019), which is relatively rare among existing reports. Beyond Orchidaceae, massive anther walls have also been reported in Apocynaceae sensu lato (Johri et al., 1992; Ai et al., 2021), such as *Voacanga*, which typically has 8–9 layers, sometimes reaching 12–16 layers (Maheshwari, 1971). Therefore, we propose that the occurrence of massive anther walls correlates with pollinium formation in the classification systems and pollination ecological adaptations of both Orchidaceae and Apocynaceae (Endress, 2016), deserving further attention.

3.4 Systematic Significance of Cytokinesis Type in Microspore Meiosis and Diversity of Microspore Tetrad Arrangements

This study observed that microspore mother cells in *R. imschootiana* undergo simultaneous cytokinesis to form tetrahedral and isobilateral microspore tetrads, which then develop into 2-celled pollen tetrads through mitosis, forming compact, rigid pollinia. This pattern aligns with existing research on Orchidaceae (Johri et al., 1992). Simultaneous cytokinesis is common during anther development in most orchid groups, including Cypripedioideae (*Cypripedium*; Sood & Rao, 1986) and Epidendroideae (*Gastrodia alata*; Liang, 1983; *Spathoglottis plicata*; Sriyot et al., 2015; *Pennilabium yunnanensis*; Li et al., 2020; *Arundina graminifolia*; Tan et al., 2020). In contrast, successive cytokinesis is less common, occurring in Orchidoideae (*Ophrys*; Aybeke, 2012; *Spiranthes*; Kant et al., 2013) and Epidendroideae (*Vanda coerulea*; Zhang et al., 2019). Regarding systematic evolutionary significance, simultaneous cytokinesis is considered the ancestral condition in angiosperms, while successive cytokinesis represents a derived trait (Furness et al., 2002). Similar perspectives exist for monocotyledons and Asparagales, the order containing Orchidaceae (Rudall et al., 1997; Furness & Rudall, 1999). Therefore, we support the view that simultaneous cytokinesis is common and ancestral in orchid evolution, also occurring in Cypripedioideae (Swamy, 1949; Sood & Rao, 1986; Ghimire et al., 2020).

This study observed two microspore tetrad arrangements in *R. imschootiana*:

tetrahedral and isobilateral, consistent with most orchid species. Current literature reports diverse microspore tetrad arrangements in Orchidaceae, typically 2–3 types, occasionally up to 5 types, including tetrahedral, isobilateral, decussate, T-shaped, and linear arrangements (Hu, 1982; Johri et al., 1992). Microspore tetrad arrangement generally corresponds to the arrangement of pollen tetrads at anther maturity, confirmed in Orchidoideae with sectile pollinia (Kant & Arabaci, 2010; Kant et al., 2013) and Epidendroideae with compact pollinia (Zhang et al., 2019; Tan et al., 2020; Li et al., 2020). Based on existing data, we propose that although each orchid species exhibits multiple microspore tetrad arrangements, tetrahedral and isobilateral types are most common and represent universal features of microsporogenesis in Orchidaceae.

3.5 Conservation Biological Significance of Floral Morphology and Anther Development in *R. imschootiana*

This study found no pollen abortion during microsporogenesis or male gametophyte development in *R. imschootiana*. Therefore, the small wild population size previously reported (Li et al., 2018) is unrelated to pollen development. However, further reproductive biology research is necessary on ovule development, megasporogenesis, female gametophyte formation, and post-fertilization embryonic and seed development to provide new evidence for understanding reproductive biology and endangerment mechanisms in *Renanthera*.

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