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Pattern and Significance of Seedling Development in *Titanotrichum oldhamii* (Gesneriaceae)

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Abstract: The seedling development of *Titanotrichum oldhamii* (Hemsl.) Solereder has been investigated to provide insight into the evolution and systematic position of *Titanotrichum*. In *T. oldhamii*, the size differentiation of the two cotyledons begins while the hypocotyl grows. However, both of the two cotyledons develop normally and locate at the same level. Finally, the two cotyledons are almost equal in size. The aerial shoot (including stem and leaves) is produced from the permanent activity of the apical meristem in the plumular bud. Even though the seedling development in *Titanotrichum* basically conforms to the general growth pattern of the seedling in the Cyrtandroideae, it is remarkably different from that of other Cyrtandroideae. Based on the revealed evidence in seedling development in *Titanotrichum* and other comparative data, the authors have evaluated the possible evolutionary pathway of *Titanotrichum* and further discussed the familial placement of this genus.

Key words: seedling development; cotyledon; functional evolution; familial placement; *Titanotrichum*; Gesneriaceae

The species *Titanotrichum oldhamii* (Hemsl.) Solereder is distributed from Fujian and Taiwan of China to southern Japan. It was first described by Forbes and Hemsley[1] as a member of the Scrophulariaceae with the name *Rehmannia oldhamii* Hemsl. Meanwhile, Forbes and Hemsley suggested that the unilocular ovary of this species pointed to Gesneriaceae, which was the reason why he noted with a question mark after *Rehmannia*[1,2]. Solereder[3] lifted this species to a generic rank, *Titanotrichum* Solereder, as a distinct and monospecific genus transferred to the subfamily Cyrtandroideae in the Gesneriaceae. But, Sealy[4] suggested that *Titanotrichum* was closer to another subfamily Gesnerioideae than to the Cyrtandroideae. Kvist and Pedersen[5] still considered *Titanotrichum* closer to Scrophulariaceae than to Gesneriaceae. However, Wang *et al*[6] raised *Titanotrichum* to the status of a monogeneric tribe Titanotricheae in the Cyrtandroideae (Gesneriaceae), also recognized by Burtt and Wiehler[7]. The position of *Titanotrichum* as the sister to the remainder of the Cyrtandroideae was weakly supported by the *ndh*F sequence data[8,9]. Nevertheless, the cladistic analysis of *ndh*F sequence data also showed that placing *Titanotrichum* in the Scrophulariaceae and the subfamily Gesnerioideae (Gesneriaceae) only required 39 – 50 and 24 additional steps, respectively[9]. The molecular systematic studies indicated that the exact position of *Titanotrichum* was still somewhat in doubt[9].

One morphogenetic pattern was discovered in the subfamily Cyrtandroideae, which was the unequal growth of the cotyledons during seedling development[10 – 12]. Almost all species in the Cyrtandroideae that have been cultivated have had cotyledons which become unequal in size soon after germination[13 – 16]. This seedling developmental pattern serves to differentiate the subfamily Cyrtandroideae in the Gesneriaceae. Therefore, Burtt[13] proposed a system of the Gesneriaceae, in which two subfamilies Cyrtandroideae and Gesnerioideae were delimited mainly by the situation of the cotyledons. He treated *Titanotrichum* as an anomalous group in his system due to no knowledge of the seedling structure of this plant[13]. This system has been widely accepted by other researchers[6,16,17]. However, because its distribution is very limited and flowers are often replaced by clusters of bulbuls serving as vegetative propagules in the upper part of inflorescences, the seeds of *T. oldhamii* are difficult to collect in the field. Therefore, there has been no information of the seedling development and structure in *Titanotrichum* up to date. Two local populations with harvest of seeds recently found in Fujian Province allow us to conduct a detailed study on the seedling development, which is necessary for the understanding of *Titanotrichum* and its familial placement.

The goal of this study is first to explore the developmental pattern and structure of the seedling in *Titanotrichum*. Secondly, comparing the developmental features of the seedling in *Titanotrichum* with those found in related groups, we also evaluate the possible evolutionary pathway of *Titanotrichum*. Finally, the familial placement of *Titanotrichum* is discussed, based on the revealed evidence in seedling development together with comparative data.

1 Materials and Methods

The seeds of *Titanotrichum oldhamii* (Hemsl.)

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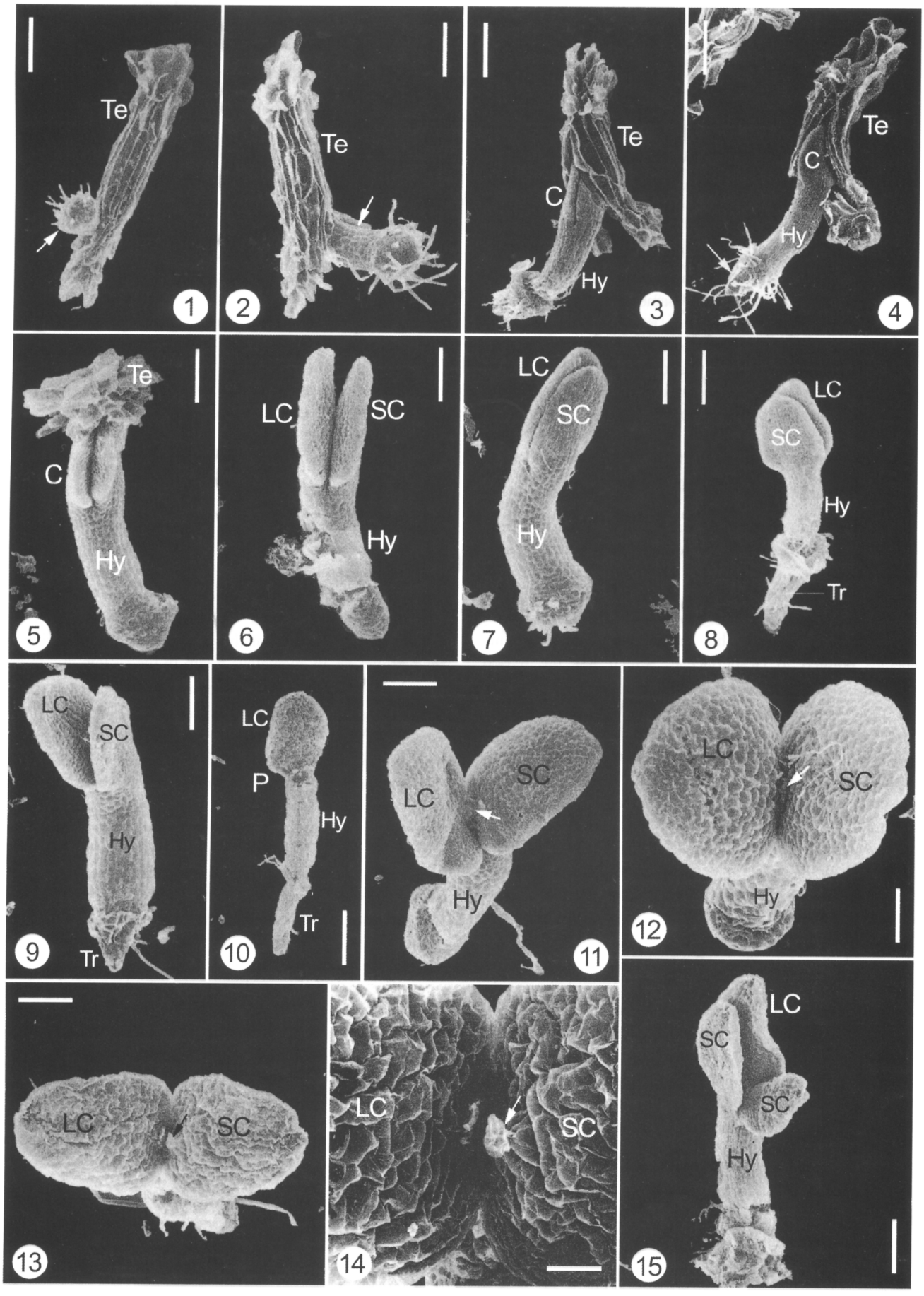
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Solereder were collected from two natural populations in Fujian Province. Voucher specimens (LI Zhen-Yu and ZOU Xiu-Hong, 11327, 11328) are deposited in the Herbarium (PE), Institute of Botany, the Chinese Academy of Sciences. Seeds for germination were spread on the surface of the filter paper saturated with sterile water in the petri dish. Seedlings at different developmental stages were fixed in formalin-acetic acid-alcohol (FAA) and then dehydrated in a series of ethanol (75%, 85% and 95%). Materials were checked in 95% ethanol and further dehydrated in three changes of 100% ethanol, 100% ethanol-amylacetate (1:1) and amylacetate, and then critical point dried with a Hitachi HCP-2 Critical Point Dryer and Au/Pd sputter coated with a SPI-MODULE Sputter Coater. The prepared samples were examined with a Hitachi S-800 scanning electron microscope (SEM) and recorded on Shanghai Panchromatic film.

2 Results

In a seed before germination, the two cotyledons are equal in size. Seeds germinated about one week after sowing. At first, the growing hypocotyl pushed the radicle to burst the seed coat, and several fibrous roots were produced from the surface of the radicle (Fig. 1). Afterward, the elongating hypocotyl got out of the seed coat and only the two cotyledons remained in the coat (Fig. 2). As the two cotyledons enlarged, the young seedling gradually escaped from the seed coat (Figs. 3 – 5). Meanwhile, the main taproot gradually developed and continued to grow downward (Figs. 3,4). However, the initiation of both fibrous roots and taproot was delayed sometimes during seedling development (Fig. 5). The seed coat was usually carried up attached to the tip of the two cotyledons or to one of the cotyledons which were greenish in color (Fig. 5). While the growing hypocotyl got out of the seed coat, the primary growth of the two cotyledons showed an initial differentiation in size (Fig. 6). One cotyledon was slightly larger than the other, in which the larger one was 250 – 260 μm long and the small one 245 – 255 μm long (Fig. 6). While the young seedling escaped from the seed coat, two cotyledons became more rapid in wide growth (Figs. 7,8). Meanwhile, The different sizes of the two cotyledons became slightly more manifested, in which the larger one was about 320 μm long and the small one about 260 μm long (Fig. 8). Accompanying the enlargement both in width and length, two cotyledons opened up and gradually spread out flat (Figs. 9,11,12). When the two cotyledons became explanate, the large cotyledon was about 350 μm while the small one was about 290 μm in length (Fig. 13). During the development of the two cotyledons, a plumular bud became visible between two cotyledons (Figs. 10 – 13). The true leaves were initiated in pair (Fig. 14). The first opposite pair of the true leaves was alternate to the two cotyledons (Fig. 16). The second pair of the true leaves arose at the position alternate to the first pair of the true leaves (Fig. 17). At this stage, the two cotyledons were still greenish in color and almost equal in size (Figs. 16, 17). While sequentially detaching the true leaves, the apical meristem was continuously active for apical growth, which formed a simple stem (Figs. 16,17). In an exceptional case, there were three cotyledons developed in the seedling in which one was remarkably larger than the other two (Fig. 15).

3 Discussion

3.1 Comparison between *Titanotrichum* and others in the Cyrtandroideae

Since Crocker[10] first described the unequal growth of the cotyledons in *Streptocarpus*, more and more species have been recognized with more or less unequal development of the cotyledons in the subfamily Cyrtandroideae[11 – 16]. In an extreme case, even though the initial seedling possesses two equal cotyledons, one of the cotyledons increases considerably in size after a few days, and continues to grow until the plant has reached the mature size, while the other remains stationary and eventually dies off[10,11,14,15]. No other leaf is produced in the life of the plant. This growth pattern of seedling has been

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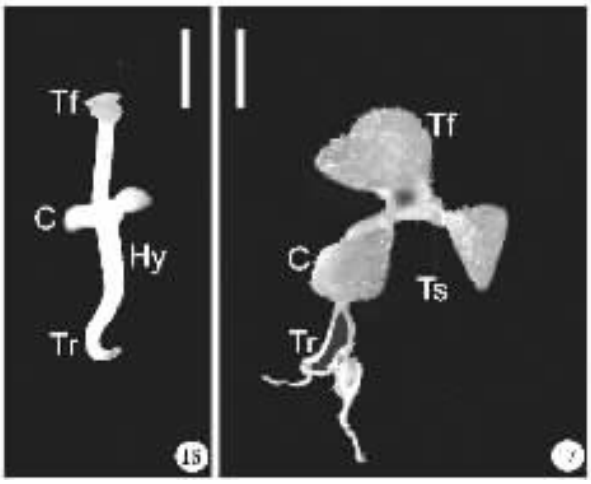
Figs. 1 – 15. SEM micrographs of the seedling development. **1 – 5**. Lateral view, showing the process of the seedling bursting the seed coat. **1**. Radicle (arrow) just bursting the seed coat with several fibrous roots. Scale bar = 209 μm. **2**. Showing that the elongating hypocotyl (arrow) has got out of the seed coat and only two cotyledons remain in the coat. Scale bar = 165 μm. **3,4**. Showing that two cotyledons gradually escape from the seed coat as enlarged in size, meanwhile the main taproot (arrow) gradually develops and continues to grow downward. Scale bar = 209 μm. **5**. Showing the seed coat being carried up attached to the tip of the two cotyledons and the case that both fibrous roots and taproot are delayed in growth during seedling development. Scale bar = 149 μm. **6**. Similar stage to Fig. 2 with the seed coat removed, showing that the primary growth of the two cotyledons demonstrates an initial differentiation in size in which one cotyledon is barely larger than the other. Scale bar = 95 μm. **7,8**. Similar stages to Fig. 3 and Fig. 4 respectively with the seed coat removed, showing that the different sizes of the two cotyledons become slightly more manifested while they become more rapid in wide growth. Scale bar = 120 μm in Fig. 7 and 195 μm in Fig. 8. **9**. Oblique-lateral view, showing two cotyledons being opening up accompanying their enlargement both in width and length. Scale bar = 127 μm. **10**. Similar stage to Fig. 9, showing the initial plumular bud. Scale bar = 209 μm. **11,12**. Oblique-polar view of young seedlings, showing that two cotyledons gradually spread out flat with primary development of the plumular bud (arrow). Scale bar = 127 μm in Fig. 11 and 83 μm in Fig. 12. **13**. Polar view of two explanated cotyledons with a plumular bud (arrow) between them, note almost equal size of two cotyledons. Scale bar = 110 μm. **14**. A close-up of the micrograph of Fig. 13, showing the initial true leaves (arrow). Scale bar = 41 μm. **15**. Oblique-lateral view, showing three cotyledons as an exceptional case developed in the seedling in which one is remarkably larger than the other two. Scale bar = 138 μm.

Abbreviations: C, cotyledon; Hy, hypocotyl; LC, large cotyledon; P, plumular bud; SC, small cotyledon; Te, seed coat; Tr, main taproot.



Figs.16,17. Photograph of young seedlings, note that the two cotyledons are still greenish in color and almost equal in size. **16**. A young seedling with the first opposite pair of the true leaves alternate to the two cotyledons. Scale bar = 1 612 μm. **17**. A young seedling with the second pair of the true leaves becoming visible at the position alternate to the first pair of the true leaves. Scale bar = 3 891 μm.
C, cotyledon; Hy, hypocotyl; Tf, first pair of the true leaves; Tr, main taproot; Ts, second pair of the true leaves.

found in several species with unifoliate habit, in which there is only one enlarged and foliaceous cotyledon in the plant life. These species respectively belong to two tribes and several genera, such as *Streptocarpus* and *Monophyllaea*[15,18]. In these species, a typical plumule or apical meristem is lacking[15,18,19]. However, it is more frequent that the two cotyledons differentiate remarkably in size after germination due to accrescence of one cotyledon in the pair as a result of continued activity of basal intercalary anisocotyly meristems[15,19]. In these species, the apical meristem is still maintained even though delayed in activity, which develops a rosette or caulescent plants[19]. This unequal growth pattern of seedling is widely distributed in the Cyrtandroideae.

In *T. oldhamii*, the size differentiation of the two cotyledons begins while the hypocotyl grows. The size difference in the two cotyledons is manifested during the seedling development. However, both of the two cotyledons develop normally and locate at the same level. The two cotyledons are almost equal finally in size. The aerial shoot (including stem and leaves) is produced from the permanent activity of the apical meristem. Apparently, even though the seedling development in *Titanotrichum* basically conforms to the unequal growth pattern of the seedling in the Cyrtandroideae, it is remarkably different from that of other Cyrtandroideae.

3.2 Phylogenetic consideration

The subfamily Cyrtandroideae is characteristic of unequal growth of the cotyledons accompanied with the delayed formation of a plumule. Burtt[20] suggested that small and numerous seeds and an accrescent cotyledon were interlocked during functional evolution in the Cyrtandroideae. As compensating for the lack of food reserves in the seed, the continued growth of one cotyledon may act as an additional photosynthetic tissue before energy is required to mobilize a plumular growing point and new leaves[20]. The delayed development of the shoot apical meristem and the continued growth of one cotyledon require an uncoupling of the strict developmental linkage between meristematic activity and the shoot apex[21]. This uncoupling may result from gain-of-function dominant mutations in plant-meristem-determining genes such as *stm* (shootmeristemless)[21,22]. The unifoliate form, as an extreme case, would be derived from indefinite delay of the activity of the shoot apical meristem and extreme development of the meristematic cotyledon[21]. The interplay of the reproductive strategy and photosynthetic economy gains a more advantage for adapting to different environmental conditions.

In *Titanotrichum*, seeds are averagely 900 μm long and 200 μm wide. The seed size of *Titanotrichum* is larger than the average size of seeds in the Cyrtandroideae. It seems that *Titanotrichum* is not strongly involved in the functional evolution toward remarkable accrescence of one cotyledon and mini-size of seeds. This suggestion is further confirmed by the normal development of the plumule in *Titanotrichum*, which indicates that there exists no uncoupling of the developmental linkage between meristematic activity and the shoot apex in *Titanotrichum*. In addition, a very curious reproductive strategy is developed in *Titanotrichum oldhamii*. In the upper part of the racemose inflorescence, clusters of bulbuls serving as vegetative propagules are often produced in the axils of bract, instead of a flower. The bulbuls are very deciduous, which gives rise to a new plant. The development of the bulbuls shows that each cluster of bulbuls is developed from the same floral primordium as a flower (personal communication with Dr. WANG Cun-Neng). This curious vegetative propagation developed in *Titanotrichum* is parallel to the generative propagation with numerous miniseeds and continued accrescence of one cotyledon as in other Cyrtandroideae in the reproductive strategy. Therefore, it is likely that *Titanotrichum*, representing a lineage, diverges early in the evolution of this subfamily, and does not strongly evolve toward remarkable accrescence of one cotyledon, but toward additional vegetative propagation in the functional evolution. This evolutionary change may be related to lack of convenient pollinators. In addition to the seedling development and vegetative propagation, some morphological features are also exclusive with *Titanotrichum* in the Cyrtandroideae, such as the terminal racemes, dimorphic flowers and scaly stolons. The above facts are coincident with the cladistic analysis of *ndh*F sequence data which show an isolated position of *Titanotrichum* within the Cyrtandroideae[8,9]. Putting the evidence of seedling development together with comparative data into consideration, we suggest that *Titanotrichum* should be considered as the sister to the remainder of the subfamily Cyrtandroideae within the Gesneriaceae. The final judgement should be waited for further information from more detailed sampling of DNA sequences, pollination and reproductive ecology.

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台闽苣苔(苦苣苔科)幼苗的发育式样及其意义

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摘要: 为解决台闽苣苔族(Titanotricheae)这一单种族的科级系统位置,通过扫描电镜观察了台闽苣苔(*Titanotrichum oldhamii* (Hemsl.) Solereder)植物的种子发芽和幼苗发育过程。随着下胚轴的向下伸长,两个子叶开始不明显的异率生长,其中一片子叶略大于另一片子叶。但两片子叶均正常发育并位于同一高度。当真叶发出后,两片子叶几乎等大,并且两个子叶柄等长。在幼苗生长期间,随着子叶的生长,胚芽也正常萌发出茎的顶芽。顶芽持续进行顶端生长产生交互对生的真叶。这一幼苗生长式样和苦苣苔亚科其他类群的仅一片子叶发育与胚芽被抑制的幼苗生长式样有明显区别。考虑到台闽苣苔植物在总状花序的上部大量簇生无性珠芽,并落地迅速生长出新的植株这一在苦苣苔科中独特的无性繁殖方式及相关性状,台闽苣苔族可能较早地从苦苣苔亚科中分化出来,并在繁殖体的功能进化方面和其他类群发生歧化进而获得独特的无性繁殖方式。台闽苣苔族在系统发育上应该被认为是其他苦苣苔亚科类群的姊妹群,应当提升为亚科等级。

关键词: 幼苗发育;子叶;功能进化;科级系统位置;台闽苣苔属;苦苣苔科

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