# Optimization of Chromosome Preparation and Study on Chromosome Number and Ploidy in Hemiboea Plants: Postprint

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## Abstract

Chromosome number and ploidy are essential fundamental information in research on systematics, evolutionary biology, and genetics. Plants of the genus Hemiboea (Gesneriaceae) possess significant medicinal and ornamental value, comprising approximately 44 species primarily distributed in southern China. Owing to the characteristics of delicate, intertwined root systems and small chromosome size, obtaining root tip materials for chromosome preparation and cytological studies is often difficult. To date, chromosome numbers have been reported for only three species in this genus, while the chromosome numbers and ploidy levels of the vast majority of species remain unknown. The evolutionary patterns of chromosome numbers and their relationship to species evolution are also unclear. To investigate optimal conditions for chromosome preparation in this genus and the relationship between chromosome number and species evolution, this study leveraged the characteristic of leaf cutting propagation in Hemiboea plants and employed a leaf hydroponic rooting method to obtain root tip materials from six species: Hemiboea subcapitata, H. longgangensis, H. longzhouensis, H. subacaulis var. jiangxiensis, H. follicularis, and H. yongfuensis. The effects of different experimental conditions on chromosome preparation quality were investigated, the experimental conditions for chromosome preparation were optimized, and chromosome counts were conducted. Simultaneously, based on the evolutionary history of chromosome numbers in Hemiboea, the evolutionary patterns of chromosome numbers and their relationship to species evolution were discussed. The results indicated that: (1) sampling between 9:30-10:00 AM, hydrolysis for 10 min, and staining for 15 min constitute suitable conditions for chromosome preparation in Hemiboea; (2) all six aforementioned species of Hemiboea were determined to be diploid, with a chromosome number of 32 (2n=2x=32); (3) aside from variations in chromosome number in a few species, most species in this genus likely have a chromosome number of 2n=2x=32, and chromosome number variation may be attributed to aneuploidy, showing no significant relationship with species evolution. This study provides a reference for chromosome preparation in Hemiboea and taxa with similar leaf regeneration characteristics, and offers insights for research on the classification and systematic evolution of these groups.

## Full Text

# Optimization of Chromosome Preparation and Study of Chromosome Numbers and Ploidy in *Hemiboea*

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**Abstract:** Chromosome number and ploidy are fundamental data of great importance in systematic and evolutionary biology as well as genetics. The genus *Hemiboea* (Gesneriaceae) comprises approximately 44 species with significant medicinal and ornamental value, distributed primarily in southern China. Due to their small, intertwined roots and diminutive chromosomes, obtaining suitable root tip materials for chromosome preparation has proven challenging. To date, chromosome numbers have been reported for only three species in this genus, leaving the chromosome numbers and ploidy levels of most species unknown. Consequently, the evolutionary patterns of chromosome numbers and their relationship to species evolution remain unclear. To address these gaps, this study exploited the characteristic leaf-cutting propagation ability of *Hemiboea* species to obtain root tip materials through a hydroponic leaf-rooting method for six species: *Hemiboea subcapitata*, *H. longgangensis*, *H. longzhouensis*, *H. subacaulis* var. *jiangxiensis*, *H. follicularis*, and *H. yongfuensis.* We systematically explored the effects of different experimental conditions on chromosome preparation quality, optimized the preparation protocol, and performed chromosome counting. Additionally, we reconstructed the evolutionary history of chromosome numbers in *Hemiboea* to investigate their evolutionary patterns and relationship to species evolution. The results demonstrated that: (1) sampling between 9:30–10:00 AM, dissociation for 10 min, and staining for 15 min represent optimal conditions for chromosome preparation in *Hemiboea*; (2) all six studied species are diploid with a chromosome number of $2n = 2x = 32$; and (3) except for a few species with variable chromosome numbers, most species in the genus likely possess $2n = 2x = 32$, with chromosome number variation likely resulting from aneuploidy rather than being directly related to species evolution. This study provides a valuable reference for chromosome preparation in *Hemiboea* and other taxa with similar leaf-regeneration characteristics, offering insights for future research on classification and phylogeny of this group.

**Keywords:** Gesneriaceae, *Hemiboea*, hydroponic leaf rooting, chromosome number, genome ploidy

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Chromosomes serve as carriers of genetic information in organisms and often exhibit differentiation among genera, species, and even within species, providing crucial evidence for exploring intergeneric and interspecific evolutionary relationships as well as intraspecific variation patterns. Consequently, chromosome data have been widely used as a basis for plant taxonomy. Chromosome number and ploidy also represent important foundational information for genetic and genomic research. Numerous cytological studies have been conducted on Gesneriaceae plants. The earliest report came from Oehlkers (1923) on *Monophyllaea horsfieldii*, and large-scale cytological investigations began in the 1960s. Chinese research on Gesneriaceae cytology started relatively late, with the first study by a Taiwanese scholar on *Hemiboea bicornuta* (Hsu, 1968). Since then, significant progress has been made, including extensive cytological work on the broadly defined genus *Primulina* s.l., with chromosome numbers reported for approximately 100 species. These comprehensive studies have revealed that Gesneriaceae chromosomes are typically small and show considerable variation in chromosome numbers. Most experiments have utilized root tips as experimental material, with a few employing flower buds. When selecting root tips, the primary methods for obtaining them are seed germination and tissue culture, both of which have limitations. Seed germination is prone to mold contamination, and even with disinfection, some seeds still mold and germinated seedlings may rot. Tissue culture is operationally complex and demands high laboratory standards. Therefore, identifying a more convenient and effective method for obtaining root tip materials is essential for cytological studies of Gesneriaceae.

The genus *Hemiboea* belongs to the family Gesneriaceae and currently comprises approximately 44 species and six varieties, many of which possess important medicinal and ornamental value. The genus is widely distributed in southern China, with a few species extending to northern Vietnam and southern Japan; the karst regions of southern China represent its distribution and diversification center. Cytological research on *Hemiboea* remains scarce, with only three species —*H. bicornuta*, *H. cavaleriei*, and *H. ovalifolia*—having been studied, and these investigations were limited to reporting chromosome numbers and ploidy levels. The evolutionary patterns of chromosome numbers and their relationship to species evolution in *Hemiboea* remain unclear, largely due to the difficulties in karyotype studies within this genus.

Previous studies have encountered significant challenges. For instance, Hsu (1968) used both flower buds and root tips of *H. bicornuta* without pretreatment, reporting only n = 18 bivalents. Lu et al. (2002) studied *H. cavaleriei* using root tips pretreated with 0.1% colchicine for 2 h and dissociated in a 1:1 mixture of 1 $\mathrm{mol \cdot L^{-1}}$ HCl and 45% glacial acetic acid at 60°C for 30 s, but observed poorly dispersed chromosomes with adhesion, likely due to the inherently small chromosome size and insufficient pretreatment and dissociation times. Cao et al. (2003) examined *H. ovalifolia* using seedling root tips dissociated for 30 s in a 2:1 mixture of 1 $\mathrm{mol \cdot L^{-1}}$ HCl and 45% glacial acetic acid at 60°C, resulting in very poor chromosome dispersion with obvious adhesion. These issues stem from the combination of small chromosome size and inadequate dissociation time, which prevents cell wall breakdown and chromosome separation. Furthermore, we observed that *Hemiboea* plants have extremely fine, intertwined root systems, making it difficult to obtain good root tips with meristematic tissue directly from plants. Therefore, new sampling strategies and optimized preparation methods are needed for chromosome number studies in this genus.

Research has shown that Gesneriaceae plants can reproduce vegetatively, with even small cuttings developing into new individuals. Leaf cutting is the most common propagation method, and most Gesneriaceae species can be propagated from leaves, which can also be rooted hydroponically by simply immersing the petiole in water. This method has been successfully applied in cytological studies of the genus *Petrocodon* and, through our experiments, has proven effective for *Hemiboea* as well, providing a new approach for cytological research in this genus.

This study selected six *Hemiboea* species—*H. subcapitata*, *H. longgangensis*, *H. longzhouensis*, *H. subacaulis* var. *jiangxiensis*, *H. follicularis*, and *H. yongfuensis* —to: (1) explore optimal conditions for chromosome preparation; (2) investigate chromosome variation patterns in the genus based on chromosome counts from these six species combined with previously reported data; and (3) reconstruct the evolutionary history of chromosome numbers using molecular phylogenetics to examine their evolutionary patterns and relationship to species evolution. We hope this study will provide a reference for cytological research on *Hemiboea* and other taxa with similar leaf propagation characteristics, offering insights for future investigations on classification, systematics, and speciation in this group.

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## 1 Materials and Methods

**1.1 Materials** The study materials are illustrated in Figure 1 [Figure 1: see original paper] and their sources are listed in Table 1 . All plants are currently cultivated in the greenhouse of the Systematics and Evolution Laboratory at the Institute of Watershed Ecology, Nanchang University. Voucher specimens are deposited at the Nanchang University Herbarium (JXU).

**Table 1** Sources of materials and chromosome numbers

| Species | Locality | Voucher | Chromosome No. (2n = 2x) |
|---|---|---|---|
| *Hemiboea subcapitata* | Wuyuan, Jiangxi | XXG et al., 2021482 | 32 |

| Species | Locality | Voucher | Chromosome No. (2n = 2x) |
|---|---|---|---|
| *H. longgangensis* | Daxin, Guangxi | ZLG, YH, LYJ, 2021449 | 32 |
| *H. longzhouensis* | Daxin, Guangxi | XXG et al., 2021594 | 32 |
| *H. subacaulis* var. *jiangxiensis* | Jingangshan, Jiangxi | ZQ, 2021920 | 32 |
| *H. follicularis* | Hezhou, Guangxi | Guangxi Botany Institute | 32 |
| *H. yongfuensis* | Guilin, Guangxi | ZLG, YH, LYJ, 2021594 | 32 |

## 1.2 Methods

### 1.2.1 Optimization of Chromosome Preparation in *Hemiboea* 1.2.1.1 Sampling

Healthy leaves were selected from the six *Hemiboea* species and cultured in water at room temperature for 10-20 days, with water changes every 2-3 days. Once roots developed, healthy root tips were collected at three time intervals: 9:30-10:00 AM, 10:00-10:30 AM, and 10:30-11:00 AM.

#### 1.2.1.2 Pretreatment

Root tips were immersed in 0.002 mol · $L^{-1}$ 8-hydroxyquinoline solution (sufficient to cover the root tips) and pretreated at room temperature for 4-5 h.

#### 1.2.1.3 Fixation

After pretreatment, root tips were rinsed twice with pure water, then transferred to Carnoy' s fixative (anhydrous ethanol:glacial acetic acid = 3:1) and fixed at 4°C for 30 min.

#### 1.2.1.4 Dissociation

Fixed root tips were washed twice with anhydrous ethanol, rinsed with pure water, then transferred to 1 mol · $L^{-1}$ HCl and dissociated at 60°C in a constant-temperature metal bath for 8, 10, or 12 min.

#### 1.2.1.5 Staining

Dissociated root tips were washed twice with pure water (5 min each). The root tip was placed on a slide, and a thin section was cut from the front 乳白色 meristematic region. A small amount of improved phenol fuchsin stain was added, and the material was stained for either 10 min or 15 min.

#### 1.2.1.6 Slide Preparation

Conventional squashing was performed on stained material using a pencil with a rubber head for vertical tapping, taking care not to shift the cover slip to achieve better chromosome dispersion.

#### 1.2.1.7 Microscopy and Chromosome Counting

Prepared slides were examined under a Leica DM2500 microscope. Well-dispersed metaphase cells were selected, observed, and photographed at 100× oil immersion. Chromosome counting followed the plant karyotype analysis standards of Li and Chen (1985).

**1.2.2 Ancestral State Reconstruction of Chromosome Numbers in *Hemiboea*** To investigate the evolutionary history of chromosome numbers in *Hemiboea*, we used 28 *Hemiboea* species, 13 *Lysionotus* species, and 3 *Anna* species as ingroups, with two *Loxostigma* species as outgroups. Nuclear ribosomal ITS and three chloroplast genes (rbcL, matK, and trnL-F) were downloaded from NCBI (Table 2 ). Sequences were aligned using MAFFT v7.407, concatenated with FASconCAT-G v1.04, and a maximum likelihood (ML) tree was constructed using RAxML v8.2.12 with the GTRGAMMA model and 1,000 bootstrap replicates.

Chromosome number data for *Hemiboea* and related genera were collected from the WebCyte database and CCDB (Chromosome Counts Database). For *H. bicornuta*, only n = 18 was reported; its diploid chromosome number remains uncertain and was tentatively analyzed as 2n = 36. Species without reported chromosome numbers were coded as missing. Combining our chromosome data for six *Hemiboea* species with these data, we performed ancestral state reconstruction using Bayesian Binary MCMC (BBM) in RASP v4.2 to infer the evolutionary history of chromosome numbers.

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## 2 Results and Analysis

### 2.1 Optimization of Chromosome Preparation in *Hemiboea*

**2.1.1 Sampling Method Optimization** Experiments demonstrated that all six *Hemiboea* species—*H. subcapitata*, *H. longgangensis*, *H. longzhouensis*, *H. subacaulis* var. *jiangxiensis*, *H. follicularis*, and *H. yongfuensis*—produced healthy roots through the hydroponic leaf-rooting method. Roots obtained via this method were more robust, easier to sample, and yielded better chromosome dispersion than soil-grown roots (Figure 2, Figure 3).

**2.1.2 Sampling Time Optimization** Comparison of chromosome preparation effects at different sampling times revealed that root tip cells were most actively dividing and chromosomes were best dispersed when sampled between 9:30–10:00 AM. Sampling between 10:00–10:30 AM showed inferior chromosome dispersion, while sampling between 10:30–11:00 AM yielded fewer dividing cells and more chromosome aggregation (Table 3, Figure 4).

**2.1.3 Dissociation Time Optimization** Evaluation of different dissociation times showed that 8 min of dissociation produced insufficiently dispersed chromosomes with adhesion, making counting difficult. A 10-min dissociation time yielded well-dispersed chromosomes suitable for counting, while 12 min caused chromosome breakage, affecting counting accuracy (Table 3, Figure 4).

**2.1.4 Staining Time Optimization** Staining time also significantly affected preparation quality. A 10-min staining period produced faint chromosome staining that was difficult to observe, while 15 min provided excellent staining clarity. Extending staining to 20 min did not improve results and caused dye precipitation that interfered with observation and counting (Table 3, Figure 4).

**Table 3** Effects of different treatments on chromosome preparation

| Treatment | Chromosome Preparation Effect |
|---|---|
| **Sampling time** | |
| 9:30–10:00 am | Many mitotic phases, well-dispersed chromosomes |
| 10:00–10:30 am | Many mitotic phases, partial chromosome aggregation |
| 10:30–11:00 am | Few mitotic phases, chromosome aggregation |
| **Dissociation time** | |
| 8 min | Incomplete cell dispersion, chromosome aggregation |
| 10 min | Good cell and chromosome dispersion |
| 12 min | Easy cell dispersion but chromosome breakage |
| **Staining time** | |
| 10 min | Faint chromosome staining |
| 15 min | Deep chromosome staining |
| 20 min | Particle sedimentation in staining solution |

**2.2 Chromosome Numbers in *Hemiboea* Species** All six *Hemiboea* species exhibited a chromosome number of $2n = 2x = 32$ (Figure 5 [Figure 5: see original paper]).

### 2.2.1 *Hemiboea subcapitata*

Distributed in central and southern China and northern Vietnam, this species grows on slopes, rocky mountains, or under forest shade at 500–1,500 m elevation. Somatic metaphase chromosome number: $2n = 32$ (Figure 5: A, B).

### 2.2.2 *H. longgangensis*

Found in Longzhou, Daxin, Guilin, and Long' an in Guangxi, growing in shaded limestone valley forests at 130–400 m elevation. Somatic metaphase chromosome number: $2n = 32$ (Figure 5: C, D).

#### 2.2.3 *H. longzhouensis*

Distributed in Longzhou, Guilin, Ningming, and Long'an in Guangxi, inhabiting dense limestone mountain forests at 170-800 m elevation. Somatic metaphase chromosome number: 2n = 32 (Figure 5: E, F).

#### 2.2.4 *H. subacaulis* var. *jiangxiensis*

Occurs in Suichuan, Nankang, Jinggangshan, Shangyou, and Ganzhou in Jiangxi, growing on damp rock surfaces in valleys at 750-900 m elevation. Somatic metaphase chromosome number: 2n = 32 (Figure 5: G, H).

#### 2.2.5 *H. follicularis*

Distributed in northern Guangdong, Guangxi, and Guizhou, growing on damp limestone rocks or in rock crevices in forests at 240-1,500 m elevation. Somatic metaphase chromosome number: 2n = 32 (Figure 5: I, J).

#### 2.2.6 *H. yongfuensis*

Endemic to Jinzhong Mountain, Yongfu County, Guilin, Guangxi, growing on karst limestone substrates. Somatic metaphase chromosome number: 2n = 32 (Figure 5: K, L).

**2.3 Evolution of Chromosome Numbers and Ploidy in *Hemiboea*** Ancestral state reconstruction for *Hemiboea* and related genera (*Lysionotus* and *Anna*) suggests that the common ancestor likely had a chromosome number of 2n = 34. *Anna* retains this ancestral number (2n = 34), while *Lysionotus* may have evolved from 2n = 34 to 2n = 32 and then to 2n = 30 through reduction. In *Hemiboea*, chromosome numbers may have evolved from the ancestral 2n = 34 to 2n = 24, 32, and 36, with 2n = 32 appearing multiple times. All three genera are diploid, with no evidence of polyploidization (Yang et al., 2019).

## 3 Discussion and Conclusion

**3.1 Optimization of Chromosome Preparation in *Hemiboea*** *Hemiboea* plants present unique challenges for cytological study due to their extremely fine, intertwined root systems and small chromosomes that resist dispersion and clear observation. This study systematically optimized sampling methods, timing, dissociation duration, and staining time to establish a reliable protocol.

High-quality experimental material is paramount for successful chromosome analysis. While hydroponic rooting has been used for other plants such as daylily, garlic, and onion, its application in Gesneriaceae has been limited to *Petrocodon*. Our study demonstrates that *Hemiboea* species readily produce healthy roots through a simple hydroponic method: inserting healthy leaf petioles into water at room temperature. This approach, likely facilitated by the ease of leaf-cutting propagation in Gesneriaceae, is more straightforward than previous methods. Hydroponically grown roots were more robust and yielded better chromosome dispersion than soil-grown roots (Figure 2, Figure 3), providing an excellent foundation for cytological analysis.

To maximize metaphase cells, sampling must coincide with peak mitotic activity. While most plants show optimal division between 8:00–11:00 AM, the ideal window varies among taxa. For the six *Hemiboea* species examined, the optimal sampling period was 9:30-10:00 AM (Figure 3: A), consistent with conventional timing for many plants.

Dissociation time critically affects preparation quality: insufficient time prevents cell wall breakdown, while excessive time causes chromosome breakage. Previous Gesneriaceae studies typically used 30 s dissociation, but our results show that 10 min yields optimal chromosome dispersion in *Hemiboea* (Table 3, Figure 4: E). This longer duration aligns with the 9–10 min optimum reported for marigold, which also has small chromosomes and high chromosome numbers, but contrasts sharply with the 30 s used for *H. cavaleriei* and *H. ovalifolia*, which produced inferior results.

Staining time also significantly impacts visualization. Previous *Hemiboea* studies provided limited description of staining duration, but our results demonstrate that 15 min produces optimal staining intensity (Figure 4: H, Figure 5: A–L), similar to the 8–12 min optimum reported for marigold.

In conclusion, optimal chromosome preparation protocols vary among taxa and must be tailored to specific biological characteristics through systematic experimentation. Our hydroponic leaf-rooting method effectively solved the sampling challenges in *Hemiboea*, and our optimized conditions for sampling, dissociation, and staining enabled reliable chromosome counting. This protocol provides a valuable reference for *Hemiboea*, Gesneriaceae, and other taxa with similar biological characteristics.

**3.2 Chromosome Numbers and Ploidy in *Hemiboea*** Within *Hemiboea*, *H. bicornuta* clusters with *H. longgangensis* and *H. longzhouensis*. The former was initially studied using flower buds, reporting n = 18 bivalents (Hsu, 1968), suggesting a possible chromosome number of 2n = 2x = 36, though this requires confirmation. The latter two species are confirmed as 2n = 2x = 32. Weber et al. (2011) merged the genus *Metabriggsia* (including *H. ovalifolia* and *H. purpureotincta*) into *Hemiboea*, consistent with our phylogenetic results. *H. ovalifolia*, positioned basally in *Hemiboea*, has the lowest known chromosome number in the genus (2n = 2x = 24) (Cao et al., 2003), while the related *H. purpureotincta* lacks chromosome data. *H. cavaleriei* (Lu et al., 2002) and the six species examined in this study all share the same diploid chromosome number (2n = 2x = 32) (Figure 5, Figure 6).

Overall, chromosome number variation in *Hemiboea* ranges from 2n = 2x = 24 to 32, consistent with previous reports (Yang et al., 2019). The n = 18 count for *H. bicornuta* requires verification. Phylogenetic analysis reveals no clear evolutionary trend in chromosome number variation (Figure 6); except for a few variable species, chromosome numbers remain relatively stable, with most species likely possessing 2n = 2x = 32. Further cytological studies on additional species are needed to determine the full range of chromosome variation.

### 3.3 Evolutionary Patterns of Chromosome Numbers in *Hemiboea*

Aneuploidy is common in plant evolution and plays an important role in speciation, often resulting from chromosome fission or fusion that changes chromosome number without altering genetic content (De Storme & Mason, 2014). Our ancestral state reconstruction (Figure 6) indicates that the common ancestor of *Hemiboea* and its relatives likely had 2n = 34. While *Anna* retained this number, *Lysionotus* may have undergone reduction from 2n = 34 to 32 and then to 30. *Hemiboea* appears to have diversified from 2n = 34 to 24, 32, and 36, with 2n = 32 emerging multiple times in parallel. The chromosome number changes in *Hemiboea* and *Lysionotus* likely involve aneuploid evolution, similar to patterns observed in other Gesneriaceae such as *Didymocarpoideae* (Yang et al., 2019). However, limited cytological data and lack of high-resolution interspecific phylogenetic relationships necessitate further integrated cytological and molecular systematic studies to fully elucidate chromosome evolution in this group.

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