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Cytotaxonomy of the endemic Karst and Danxia ferns in *Adiantum* (Pteridaceae)

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Abstract

Chromosome traits may differ between closely related ferns growing in different geological landscapes. *Adiantum* series *Gravesiana* (Pteridaceae) includes six species, of which, five species are exclusively distributed in Karst landforms, and one, *Adiantum juxtapositum*, grows on Danxia landform. Additionally, there are some populations or individuals whose morphology are between different species, which may be the results of polyploidization or hybridization. Here we studied chromosome numbers of species in series *Gravesiana* primarily using crosiers. Results are: *A. longzhouensis* and *A. juxtapositum* were diploids with 2n = 60 and 2n = 64, respectively; *A. dentatum* was deduced to be diploids with 2n = ca. 60; *A. mariesii* had two cytotypes, 2n = 60 (diploid) and 2n = ca. 120 (tetraploid), respectively; *A. gravesii* had four cytotypes including 2n = ca. 60 (diploid), ca. 120 (tetraploid), ca. 150 (pentaploid), and ca. 180 (hexaploid). All these different cytotypes suggest polyploidization and hybridization have occurred in *A. gravesii* and *A. mariesii*, so we treated these two species as two "complex group". All chromosome numbers in this study are the first record.

Keywords: cytotaxonomy, chromosome numbers, polyploidy, cytotypes, *Adiantum*

Introduction

Analyses of chromosome numbers are basic and essential for botanists researching taxonomy, systematic and evolution of plants (Guerra 2008). The genetic information carried by chromosomes of each species or subspecies can help us understand the phylogeny of plants at each classification level such as family, genus, etc. (Windham & Yatskievych 2003, Glick & Mayrose 2014).

Data on chromosome numbers of pteridophytes is still insufficient. Chromosome numbers have been reported for more than 2,000 ferns, accounting for ca. 20% of all ferns throughout the world (Walker 1973). According to Wu (1984), sampling is somewhat improved at the genus level: chromosome data are available for almost 60% genera of ferns (ca. 207 genera). In China, chromosome numbers have been counted for ca. 21 lycophytes (0.81%) and 550 ferns (accounting for 21.2%) of the total 2600 pteridophytes, of which, 85.5% are leptosporangiates and most belong to Pteridaceae, Dryopteridaceae, Thelypteridaceae, Polypodiaceae, or Athyriaceae (Hsieh *et al.* 2008, Cheng & Zhang 2010, Dong 2011, Qi & Zhang 2011, Wang *et al.* 2011, Wang *et al.* 2013, Wang *et al.* 2018). Among the 550 ferns, data for about 112 species (20.4%) were obtained after 2000 year, while since the year 2010, only ca. 33 species were reported with clear chromosomes pictures.

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Adiantum Lineanus (1753: 1094) is a genus of subfamily Vittarioideae of Pteridaceae. It has more than 200 species from cold temperate zone to tropics, mostly in South America, and 34 species (16 endemic) in China (Zhang *et al.* 2013). So far, chromosome numbers are known for ca. 46 species of *Adiantum* (Mehra & Verma 1960; Roy & Sinha 1961; Abraham *et al.* 1962; Manton *et al.* 1970; Irudayaraj & Manickam 1987; Manickam & Irudayaraj 1988; Paris & Windham 1988; Kato *et al.* 1992; Paris 1993; Wagner & Boydsten 1978; Perwati & dan Purnomo 2002; Tindale & Roy 2002; Nakato & Kato 2005; Pedro *et al.* 2006; Bir & Verma 2010; Saggoo & Kaur 2016; Vijayakanth *et al.* 2018). Many more cytotaxonomic studies on this genus are urgently needed to help clarify classification among closely related species, since polyploidization and hybridization are common in the *A. caudatum* Linnaeus (1771: 308) complex and *A. pedatum* Linnaeus (1753: 1095) complex (Manton *et al.* 1970, Nakato & Kato 2005). *Gravesiana* Ching ex Y.X. Lin (1980: 101), a series of *Adiantum*, consists of six species *A. gravesii* Hance (1875: 197), *A. mariesii* Barker (1880: 494), *A. longzhouensis* A.H. Wang, F.G. Wang & F.W. Xing (2017: e0172729), *A. obovatum* A.H. Wang, F.G. Wang & F.W. Xing (2017: e0172729), *A. dentatum* A.H. Wang, F.G. Wang & F.W. Xing (2017: e0172729) and *A. juxtapositum* Ching (1957: 312). These species exclusively grow in chalk soil near dripping entrances of karst caves or wet cliffs of the Karst landform, or grow in Danxia soils of wet cliffs and crevices of the Danxia landform (Shing & Wu 1990; Yan *et al.* 2012; Wang *et al.* 2017). Karst landforms are formed by dissolution, water erosion, potential erosion and collapse of soluble carbonate rocks (limestones). Karst caves, sinkholes, karst hills, isolated peaks, stalagmites, and stalactites are typical Karst landforms (Zheng & Kuang 2021). Accounting for 12% of the earth's land surface area, Karst landforms are widely distributed throughout the world, but the southwestern China is the largest karst region (Zhou *et al.* 2020). Named by Chinese geologists, Danxia landforms refer to an erosional landscape developed on red-stratum mudstone, characterized by scarp slopes (Huang *et al.* 2015). They are mainly distributed in China (concentrated in SE, SW, NW), the western United States, Central Europe and Australia (Zheng & Kuang 2021). Both Karst and Danxia habitat are characterized by high exposure rate of bedrock, low process of soil formation, shallow and discontinuous soil, and poor water retention (Wang & Zhang 1999; Cao *et al.* 2003; Li *et al.* 2004; Zhang & Wang 2009; Liu *et al.* 2011; Zhang *et al.* 2011; He *et al.* 2012; Liu *et al.* 2013; Liu *et al.* 2020). Due to their special habitat, fibrous roots of *Adiantum* series *Gravesiana* are well developed in the wild for embedding into thin topsoil (2-9 cm depth in Zhang & Wang 2009, Liu *et al.* 2013), and the plants are very small, which makes it hard to obtain clear chromosome numbers through squashing their root tips. During our field surveys, we sometimes observed morphology of populations or individuals intermediate between two or three species, which may be the results of polyploidization or hybridization. To test this, we conducted their cytotaxonomic study here.

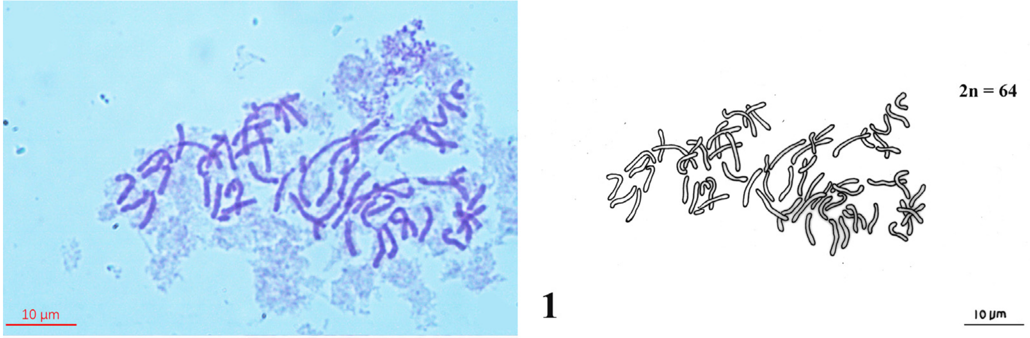
Materials and methods

Materials for mitotic studies were collected from wild plants, including 16 populations of series *Gravesiana* in Karst and Danxia regions of China (Table 1). We used apical meristem (ca. 1mm) of the crosier (leaf roll) for mitotic chromosome observation, rather than root tips. All voucher specimens were deposited in the herbarium of South China Botanical Garden, Chinese Academy of Science (IBSC).

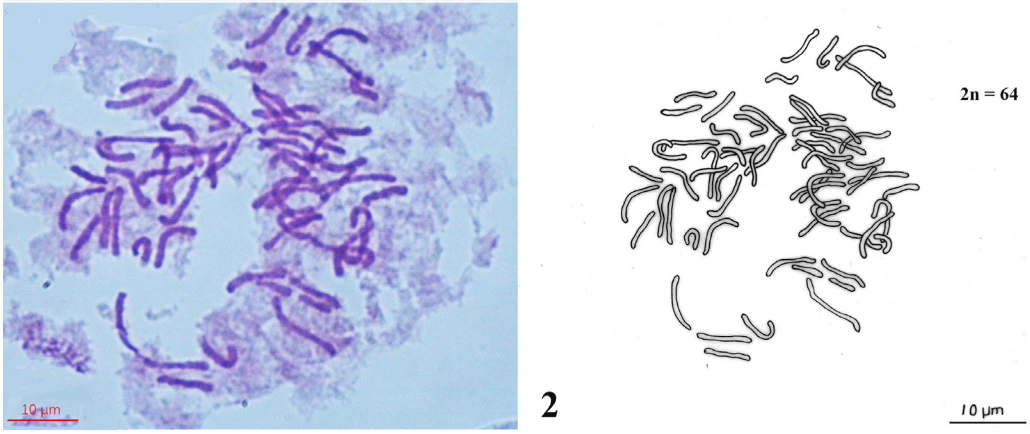
Young crosiers were pretreated with a 0.05% solution of colchicine for 3–4 hr, fixed in Carnoy's fixative (6:3:1) alcohol–chloroform–glacial acetic acid v/v for 24 h at 4 ℃ temperature, macerated in a mixed solution of cellulase and pectinase for 4–6 hr at 4 ℃ refrigeration, stained with 1% acetocarmine for 8–10 min, and then squashed into slides. If the case that squashes could not prepared immediately after immersion Carnoy's fixative, they were transferred to 70% alcohol and stored under refrigeration until use. Finally, we choose the well-prepared fresh slides to observe and photograph with a digital camera OLYMPUS DP26 connected to microscope OLYMPUS BX43. Some fresh slides were made into permanent ones.

Results

Chromosome counts of series *Gravesiana* obtained in this study are listed in Table 1. We observed two plants from two populations of *A. juxtapositum* were diploids with 2n = 64 (Figs 1-2). Three plants from two localities of *A. longzhouensis* were diploids with 2n = 60 (Figs 3-7). The chromosome number of *A. dentatum* (one plant from one population) was not clear but can be deduced to be diploid with 2n = ca. 60 (Fig 8).

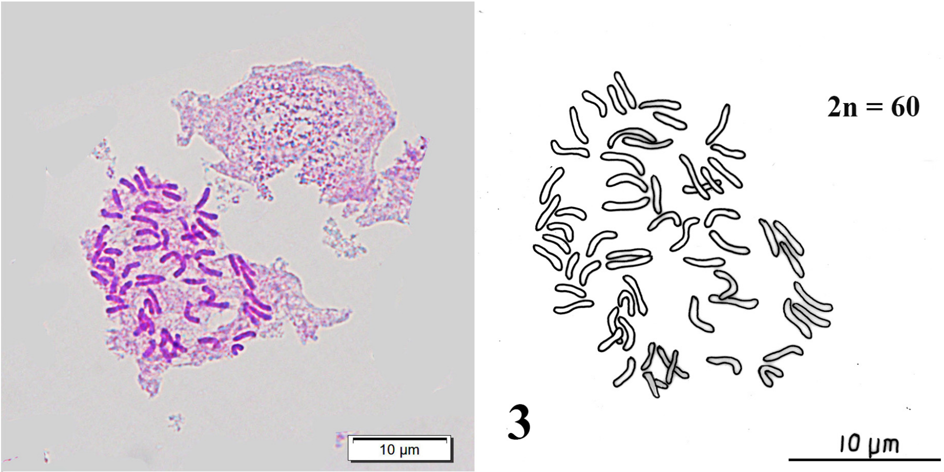


1



2

FIGURES 1–2. Photomicrographs (left) and explanatory diagrams (right) of chromosomes of *A. juxtapositum* (2n = 64). 1: individuals collected in GSD (*WAH029*); 2: individuals collected in HCZ (*wah20191115001*). All short names were listed in Table 1.



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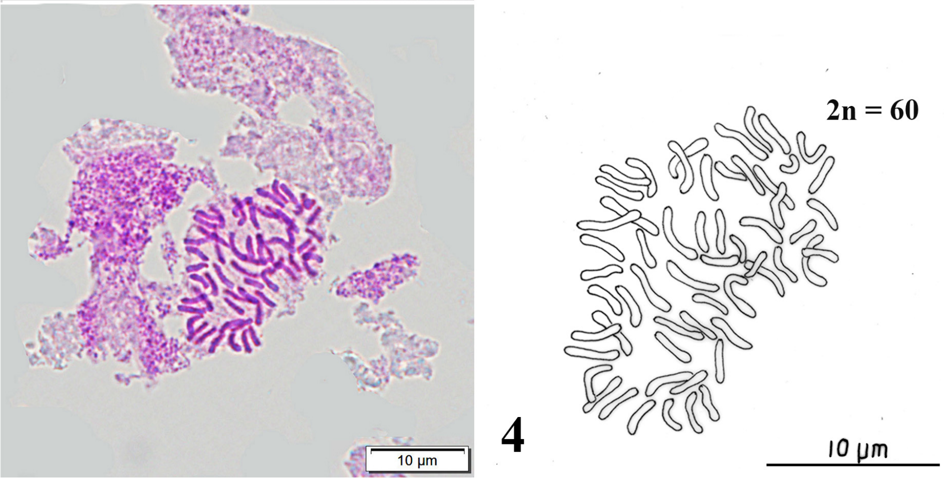
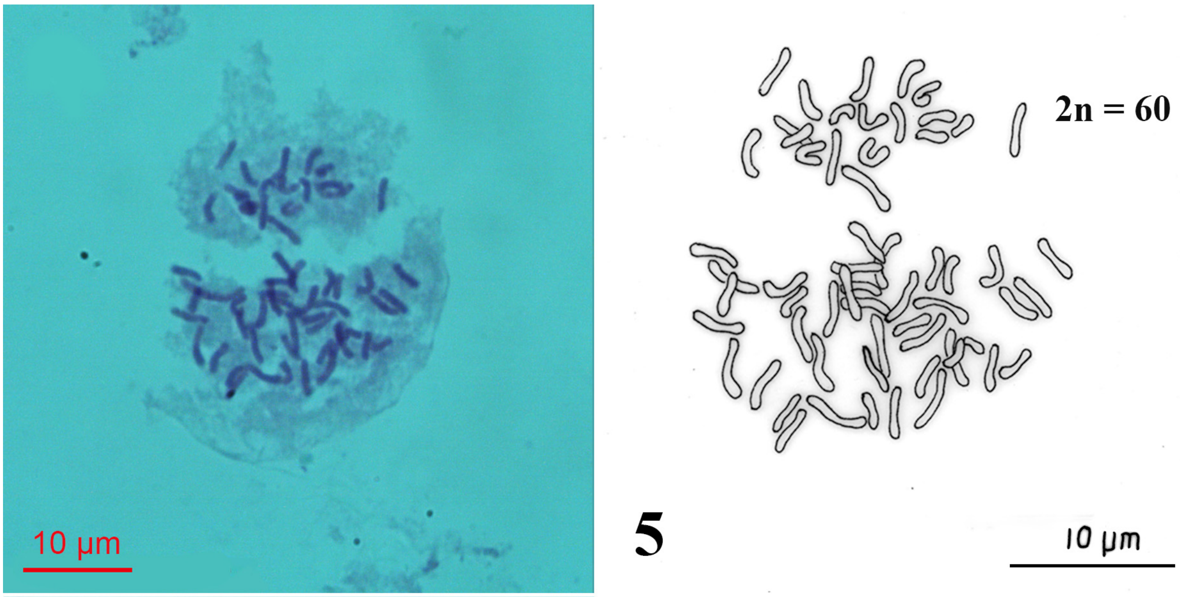
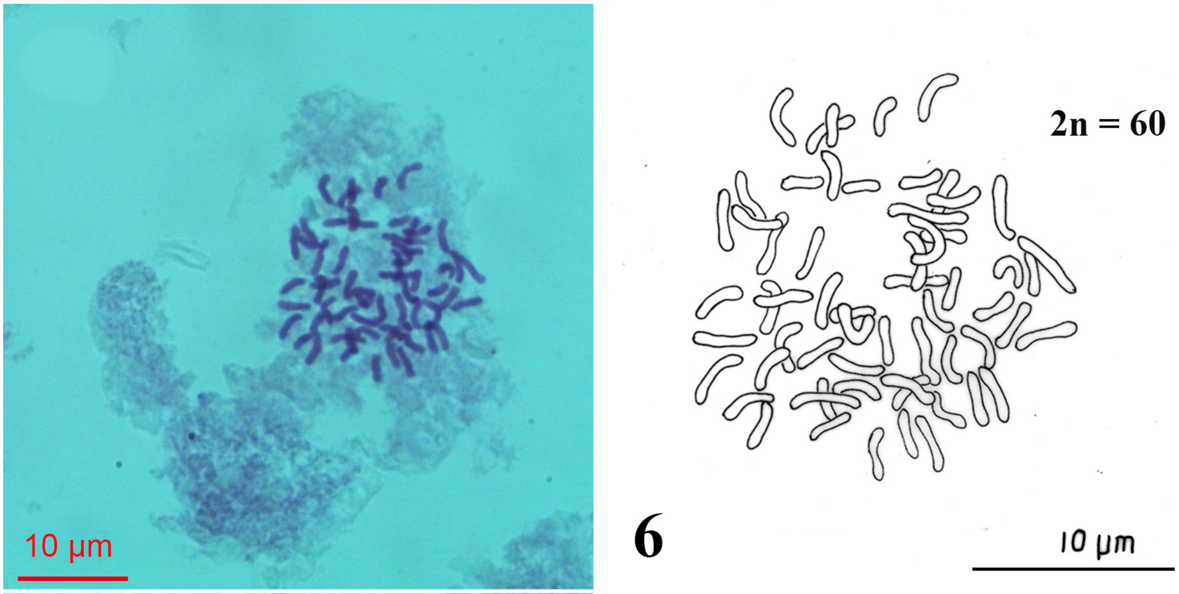
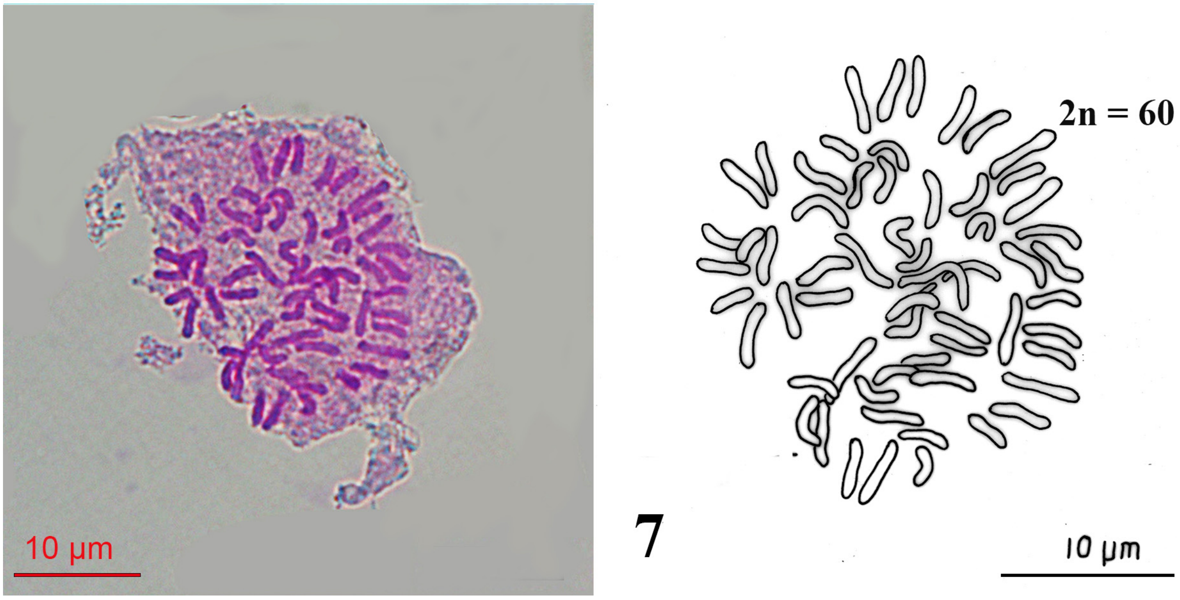


4

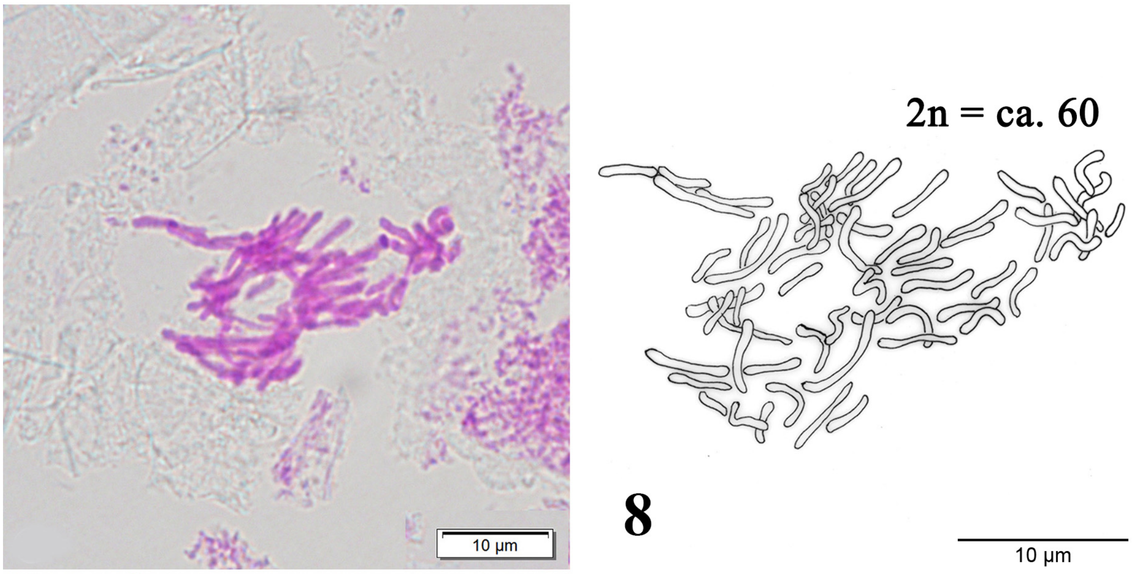
FIGURES 3–7. Photomicrographs (left) and explanatory diagrams (right) of chromosomes of *A. longzhouensis* (2n = 60). 3-6: individuals collected in DX (*wah2020060616*); 7: individuals collected in DB (*wah2020060618*).







FIGURES 3–7. (Continued) Photomicrographs (left) and explanatory diagrams (right) of chromosomes of *A. longzhouensis* (2n = 60). 3-6: individuals collected in DX (*wah2020060616*); 7: individuals collected in DB (*wah2020060618*).



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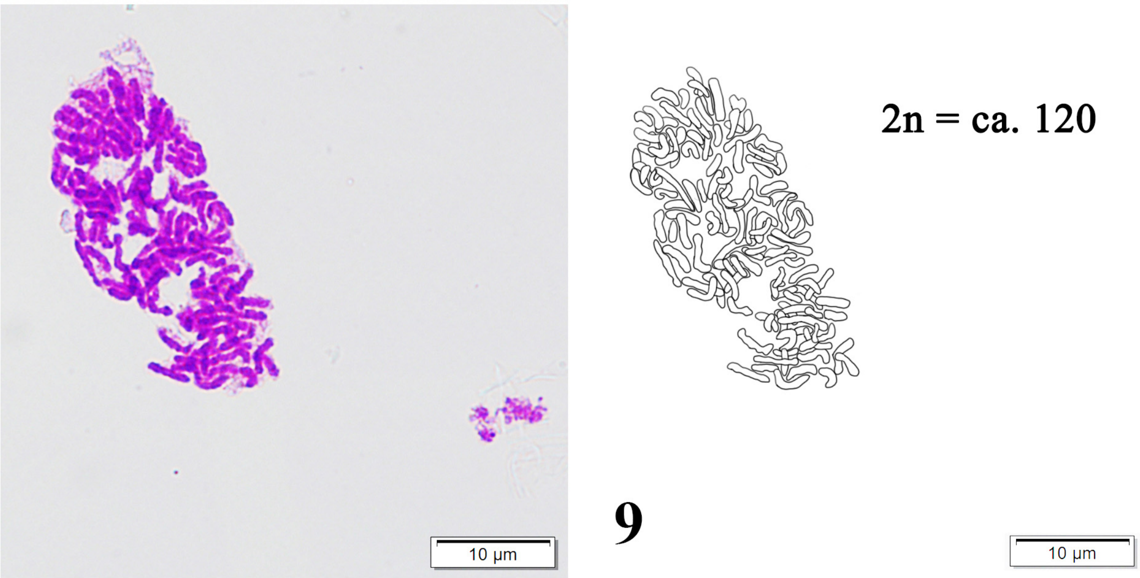

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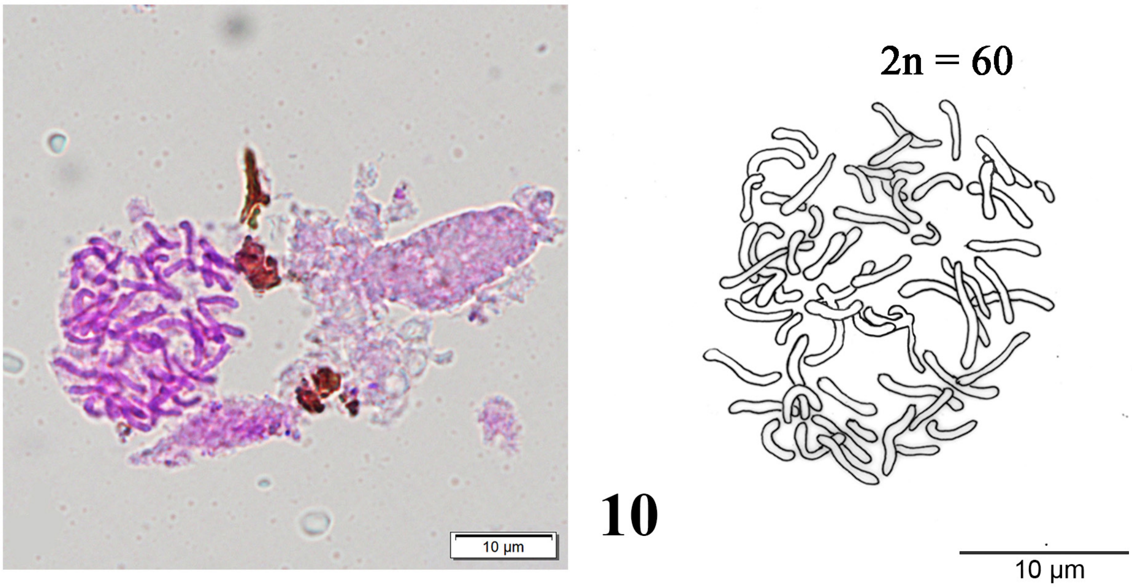

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FIGURES 8–10. Photomicrographs (left) and explanatory diagrams (right) of chromosomes of *A. dentatum* and *A. mariesii*. 8: sample of *A. dentatum* (2n = ca. 60) collected in HB2C (*wah2020052604*); 9: sample of *A. mariesii* (2n = ca. 120) from the SBSX (*wah20191113002*); 10: sample of *A. mariesii* complex (2n = 60) collected in HMMX (*wah2020060609*). All short names of HMMX, SBSX and HB2C were listed in Table 1.

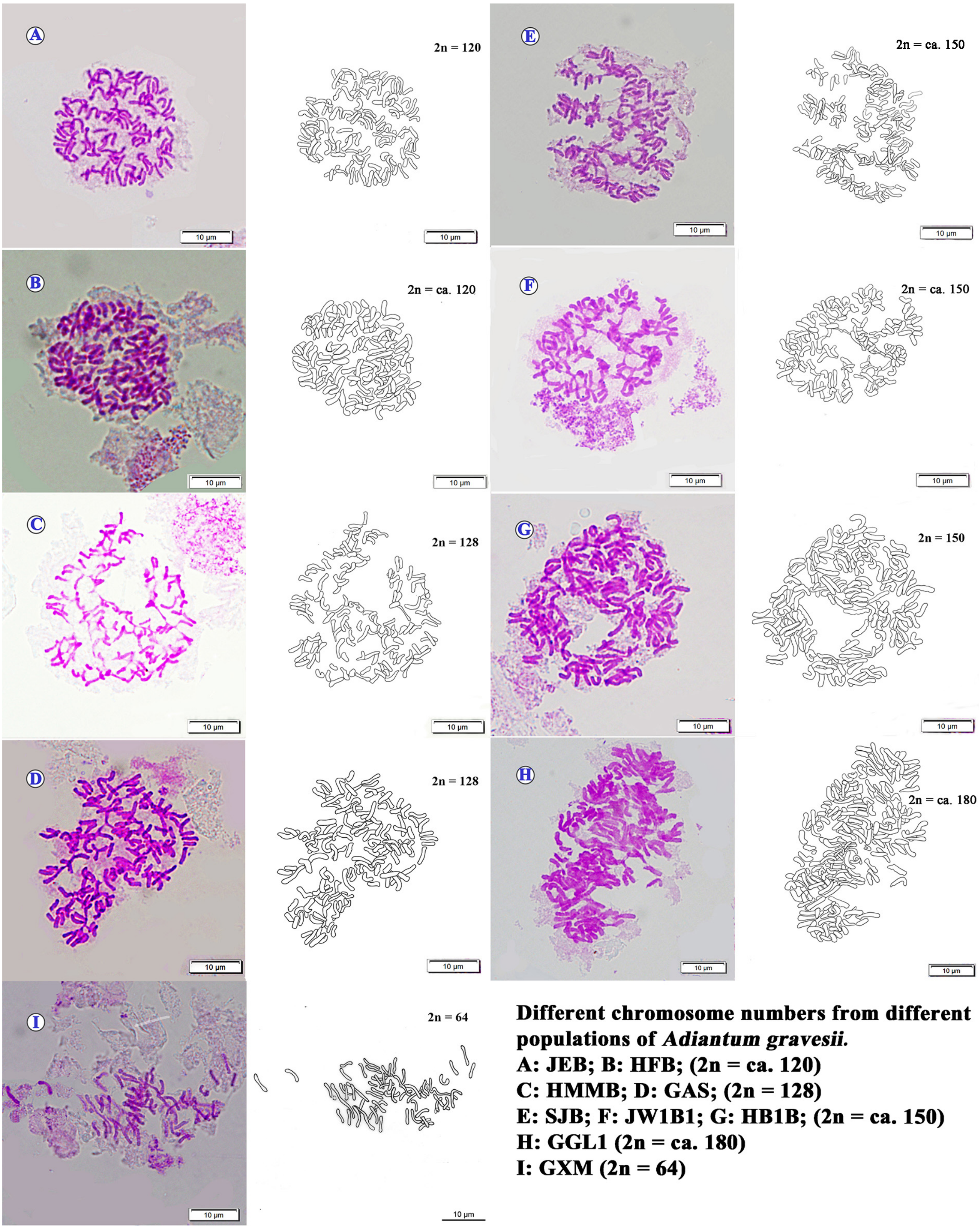

FIGURE 11. Photomicrographs and explanatory diagrams of chromosomes of *A. gravesii* complex from different populations. A: individuals collected in JEB (2n = 120) (*wah2020052609*); B: individuals collected in HFB (2n = ca. 120) (*WAH055*); C: individuals collected in HMMB (2n = 128) (*wah2020060608*); D: individuals collected in GAS (2n = 128) (*wah2020062102*); E: individuals collected in SJB (2n = ca. 150) (*wah20191113003*); F: individuals collected in JW1B1 (2n = ca. 150) (*wah2020060611*); G: individuals collected in HB1B (2n = 150) (*wah2020052605*); H: individuals collected in GGL1 (2n = ca. 180) (*wah2020062003*); I: individuals collected in GXM (2n = 64) (*wah2020062202*) . All short names of populations were listed in Table 1.

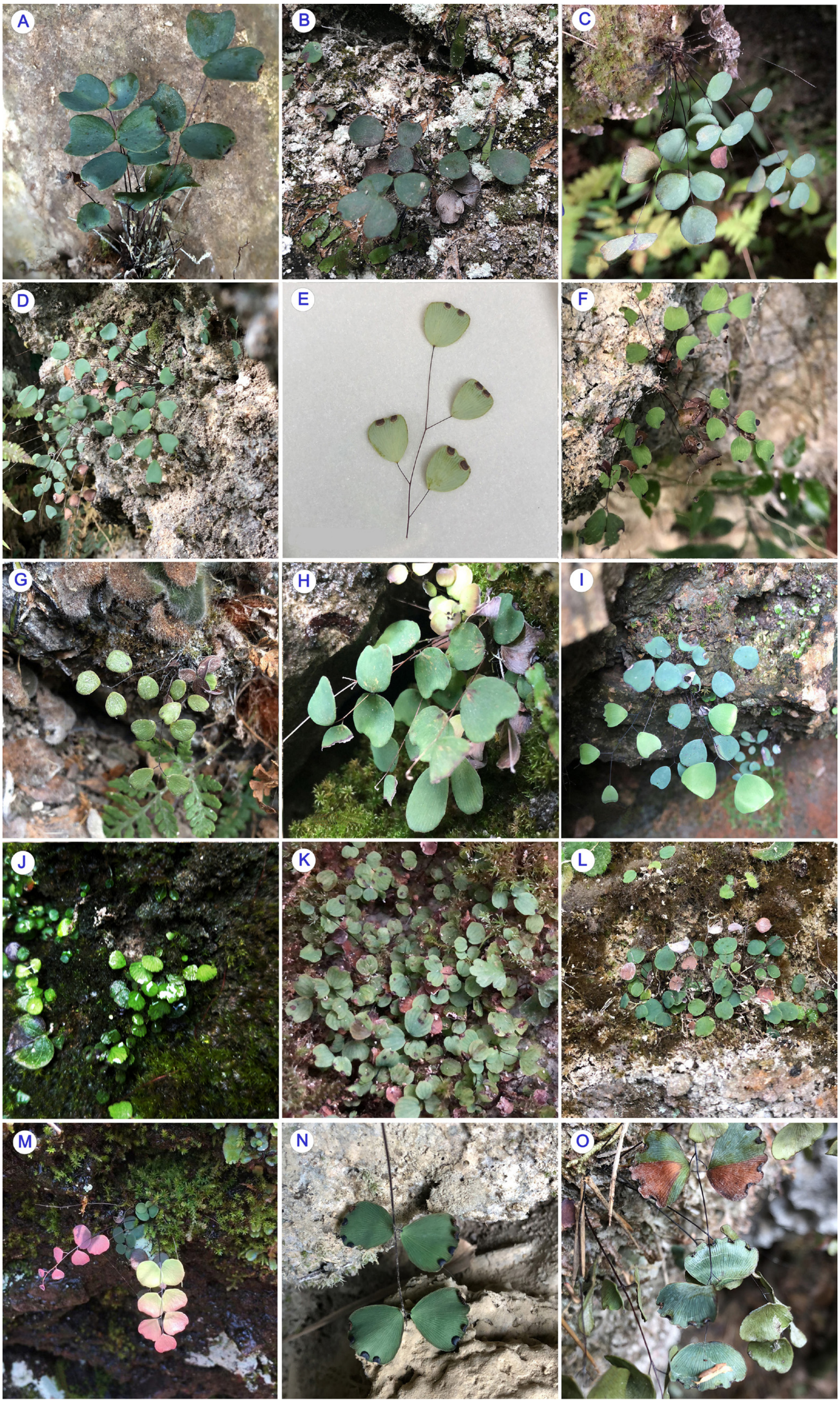

FIGURE 12. Morphology of individuals responding to Figs 1–11 and Table 1. A–I: *A. gravesii* complex; J: *A. dentatum*; K–L: *A. mariesii* complex; M: *A. juxtapositum*; N-O: *A. longzhouensis*. A: JEB, B: GAS; C: HFB; D: HMMB; E: JW1B1; F: HB1B; G: SJB; H: GGL1; I: GXM; J: HB2C; K: HMMX; L: SBSX; M: GSD; N: DX; O: DB. Among the *A. gravesii* complex (A–I), D and G were typical *A. gravesii*, the others were intermediate morphology. In *A. mariesii* complex, L is the typical *A. mariesii* and K was the *A. mariesii* complex. All short names of populations and their morphological characters were listed in Table 1.

TABLE 1 Chromosome numbers of different populations from different species in series *Gravesiana*, including their collected localities and voucher specimens.

Scientific names	Populations	Chromosome numbers (2n)	**Morphological characters**	**Localities**	**Latitude, longitude**
A. juxtapositum	GSD	64	Plants tall >5 cm, pinna often opposite, with white powder on abaxial surface; veins flabellate at base > 8; sori 1 to many, false indusium terminal transversally linear, truncate.	Danxia mountain in Renhua County, Guangdong Province, China.	25.016667N, 113.733333E
A. juxtapositum	HCZ	64	same as above GSD.	Cheng-jiang-kou in Zixing County, Hunan Province, China	25.02725271N, 113.1717517E
A. longzhouensis	DX	60	Plants tall > 5 cm, pinna often opposite, without white powder on abaxial surface, veins flabellate at base > 8; sori 1 to many, reniform.	Around Ming-shi-tian-yuan in Daxin County, Guangxi Province, China.	22.71479461N, 106.90579142E
A. longzhouensis	DB	60	same as above DX.	Jing-yang-cun primary school, Daxin County, Guangxi Province, China.	22.65344013N, 106.95694744E
A. dentatum	HB2C	ca. 60	plants small <5 cm, pinnae dentate with 2-4 veins, alternative, sori 1, see Fig 12J	Gan-hong-dong in Daluo Village, Bama County, Guangxi Province, China.	24.30998788N, 106.99668612E
A. mariesii	SBSX	ca. 120	plants small <5 cm, pinnae round with 4 or < 4 veins, alternative, each pinna has 1 sori, see Fig 12L.	The cave near Shui-tian-ba in Ba-mao-xi Township, Sangzhi County, Zhangjiajie City, Hunan Province, China.	29.66275615N, 110.04385259E
A. mariesii complex	HMMX	60	similar to *A. mariesii* but its pinnae nearly opposite	Ming-li-tun, Mulun National Nature Reserve, Huanjiang County, Guangxi Province, China.	25.10056593N, 107.96536935E
A. gravesii	SJB	ca. 150	Plants tall > 5 cm, pinna alternative, without white powder abaxially, veins flabellate at base > 4, sori 1, reniform.	Moist limestones near the Jiashi river in Sangzhi County, Zhangjiajie City, Hunan Province, China.	29.47835826N, 109.93279799E
A. gravesii	HMMB	128	Plants tall > 5 cm, pinna alternative, without white powder abaxially, veins flabellate at base > 4, sori 1, reniform.	Ming-li-tun, Mulun National Nature Reserve, Huanjiang County, Guangxi Province, China	25.10052896N, 107.96531981E
A. gravesii complex	JEB	120	similar to *A. gravesii* but pinnae opposite, with white powder abaxially and short stipes.	Cliffs near the cave at E-quan Village, Jinxi County, Guangxi Province, China.	23.10341593N, 106.38306309E
A. gravesii complex	HFB	ca. 120	similar to *A. gravesii* but pinnae opposite, with white powder abaxially and short stipes	Yang-zi-dong, Fenshan County, Guangxi Province, China.	24.39477005N, 107.06247795E
A. gravesii complex	GAS	128	similar to *A. gravesii* but pinnae opposite, with white powder abaxially and short stipes	The Guan-ying-dong at Xuantang in Anshun City, Guizhou Province, China.	26.11320649N, 105.87850965E
A. gravesii complex	JW1B1	ca. 150	similar to *A. gravesii* but pinnae alternative, with long stipes, plant much slimmer.	Nong-wei-tun in Nongguang Village, Jinxi County, Guangxi Province, China.	23.19056141N, 106.5370083E
A. gravesii complex	GGL1	ca. 180	pinnae alternative, without white powder abaxially but pinna much rounder.	Lei-da-yan near the Sanjiang farm in Guiyang City, Guizhou Province, China	26.70183582N, 106.81966804E
A. gravesii complex	HB1B	150	pinnae alternative, without white powder abaxially but much thinner and rounder.	The small cave near Gan-hong-dong in Daluo Village, Bama County, Guangxi Province, China.	24.30960055N, 106.99468704E
A. gravesii complex	GXM	64	pinnae alternative, with white powder abaxially and long stipes.	Grand canyon in Maling river, Xingyi City, Guizhou Province, China.	25.13439608N, 104.95497429E

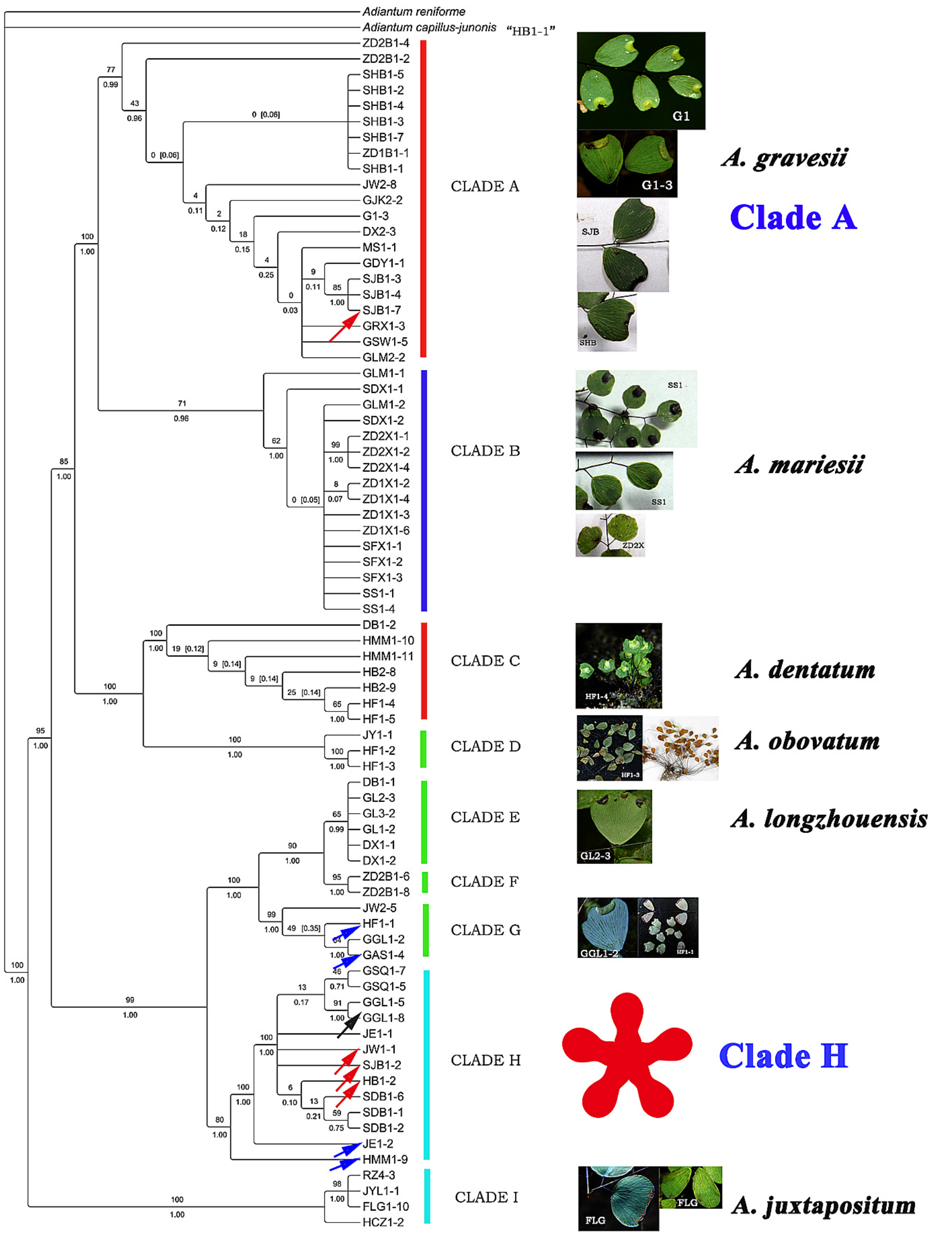

FIGURE 13. Strict consensus tree of six maximally parsimonious trees for series *Gravesiana* obtained from *atpA*, *atpB*, *rbcL*, *trnL-F*, *rps4-trnS* and *matK* sequences in Wang *et al.* 2017. The bootstrap values were shown above the lines, and the Bayesian posterior probabilities were shown below the lines.

A. mariesii (one plant from one population "SBSX") was tetraploid with 2n = ca. 120 (Fig 9), while some individuals collected in the locality "HMM" (short for "HMMX") were diploids with 2n = 60 (Fig 10). These individuals from "HMMX" are small, with rounded fronds and 2-4 obvious veins spreading from the base to apex of blade; they are very similar to *A. mariesii*, but can be easily distinguished from *A. mariesii* for their opposite leaves.

Chromosomes were observed in nine samples from nine populations of *A. gravesii* (including individuals whose morphology were intermediate between *A. gravesii* and *A. mariesii*). The plant from "JEB" was 2n = 120 (Fig 11A), the sample of "HFB" was 2n = ca. 120 (Fig 11B), samples from both the "HMMB" and "GAS" were 2n = 128 (Fig 11C, D). Plant from the "SJB" was 2n = ca. 150 (Fig 11E), that of "JW1B1" was 2n = ca. 150 (Fig 11F) and sample of "HB1B" was 2n = 150 (Fig 11G). The sample of "GXM" was 2n = 64 (Fig 11I) and that of "GGL1" was 2n = ca. 180 (Fig 11H). So, there were in total four cytotypes, namely, diploid [2n = 64 (ca. 60)], tetraploid [2n = 120 or 128 (ca. 120)], pentaploid [2n = 150 (ca. 150)] and hexaploid (2n = ca. 180) in *A. gravesii*. Responding morphology of above individuals from above populations were shown in Figure 12.

Discussion

Classification of series *Gravesiana* has been confused although Wang *et al.* (2017) recently made a revision based on phylogenetic and morphological analyses, and confirmed six species. The fact we found a series of intermediate morphologies between species *A. mariesii* and *A. gravesii*, confirms that there remain many unknown factors to be explored in *Gravesiana*.

We squashed chromosomes of most representative populations of series *Gravesiana* for the first time. According to x = 29 or 30 in *Adiantum* (Lovis 1977, Tryon & Tryon 1982, Walker 1984, Nakato & Kato 2005), we confirm that *A. longzhouensis* is diploid (2n = 60, x = 30) because no other cytotype was observed in the two populations we sampled (three samples each population). *Adiantum juxtapositum* is also confirmed to be diploid (2n = 64, x = 32), but its base number (x = 32) is quite different from the previously reported x = 29 or 30, which may be related to its exclusive Danxia habitat. Alternatively, this could be related to its phylogenetic position, since *A. juxtapositum* was sister to the rest of series *Gravesiana* in the phylogenetic tree of Wang *et al.* (2017) (Fig 13). The base chromosome number of the remaining species (except *A. juxtapositum*) could have resulted from the loss of one or two chromosomes from the base chromosome number of *A. juxtapositum.*

Although chromosome number of *A. mariesii* (2n = ca. 120, tetraploid, Fig 9) is greater than that of *A. juxtapositum* (2n = 64, diploid, Figs 1-2), its chromosome size seems smaller than that of the latter. Chromosome size of *A. dentatum* (2n = ca. 60, diploid) seems equal to that of the "HMMX" (2n = 60, diploid) (Figs 8, 10). Interestingly, *A. dentatum* is the smallest plant in series *Gravesiana* (see Fig 12). So, polyploidization and change of chromosome size may be two different pathways for them to survive the stress of their extreme habitat (extremely shallow soil, lack of water and nutrients; Liu *et al*. 2020), which is in accordance with view of de Peer *et al.* (2021).

In the phylogeny of series *Gravesiana* based on chloroplast sequence (Wang *et al.* 2017), samples from the same population like "*SJB1-2*" and "*SJB1-7*" clustered into two different clades (clade H and clade A, respectively, Fig 13), which makes sense given the four cytotypes of *A. gravesii* [2n = 64 (ca. 60), 120 or 128 (ca. 120), 150 (ca. 150), ca. 180]. Among *A. gravesii* (including individuals whose morphology were intermediate between *A. gravesii* and *A. mariesii*), plants from populations JEB, GAS and HFB were tetraploids [2n = 120 or 128 (ca. 120)] and they have same morphological characters—white powder on the abaxial surface of the pinna, obdeltoid opposite pinna, short stipe and one sorus per pinna (Fig 12). While plants without white powder on the abaxial leaf and alternate pinna from populations SJB, JW1B1 and HB1B were pentaploids [2n = 150 (ca. 150)], from which, we can infer hybridization must have been happened in *A. gravesii* or between *A. gravesii* and other species. Besides, plants like GGL1, which have alternate pinna but short petioles (Fig 12), were hexaploid (2n = ca. 180). Individuals of HB1B (2n = 150), whose morphology were intermediate between *A. gravesii* and *A. mariesii* (Fig 12), may be hybrids from *A. gravesii* × *A. mariesii*. Especially, the individual from population GXM with 2n = 64, whose fronds were similar to plants from HFB (2n = ca. 120) but with long petioles, make it seems reasonable that the latter is the result of diploidization of the former.

Since *A. mariesii* (include HMMX) had two cytotypes (2n = 60, ca. 120) and *A. gravesii* had four cytotypes [2n = 64 (ca. 60), 120 or 128 (ca. 120), 150 (ca. 150), ca. 180], and the plants with different cytotypes have different morphological characteristics, we treated both *A. mariesii* and *A. gravesii* as species complexes. Polyploidization and hybridization maybe the key factors leading to their variation in morphological characteristics.

Conclusion

In series *Gravesiana*, *A. longzhouensis* is diploid with 2n = 60 and x = 30, *A. dentatum* is diploid with 2n = ca. 60, *A. juxtapositum* is also diploid (2n = 64) but with x = 32. *A. mariesii* has two cytotypes (2n = 60, ca. 120) and *A. gravesii* has four cytotypes [2n = 64 (ca. 60), 120 or 128 (ca. 120), 150 (ca. 150), ca. 180], and the plants with different cytotypes have different morphological characteristics, so we treated both *A. mariesii* and *A. gravesii* as species complexes. Furthermore, polyploidization was confirmed in the *A. mariesii* complex and *A. gravesii* complex, and hybridization has occurred in the *A. gravesii* complex. However, it is not clear how polyploidy and hybridization occurred within these two complex groups, so more research is needed to explore parents of their hybrids in future. Young crosiers of these species are more suitable for chromosome study than root tips.

Acknowledgements

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