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Cytological studies on *Primulina* taxa (Gesneriaceae) from limestone karsts in Guangxi province, China

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Primulina, a large genus restricted mainly to limestone karst areas, was chosen as a suitable subject for the study of speciation patterns in its center of diversity and endemism at the chromosomal level. We investigated the chromosome numbers and chromosome morphology for 39 species, three varieties and 11 undescribed taxa of *Primulina* collected from limestone karsts in Guangxi province, China, half of which are known so far to be endemics. The chromosome numbers for the taxa examined were found to be highly constant, all being diploid with $2n = 36$. The previously reported basic chromosome number $x = 18$ was confirmed, as were eight counts; the counts for the remaining taxa reported for the first time except for one gave different numbers from previously published counts. The chromosomes in *Primulina* are relatively small in size and slight variation occurs among taxa. One or two satellites were encountered in 18 taxa. The relative uniformity in chromosome numbers indicates that variation at the diploid level seems to be the principal feature of chromosomal evolution in this genus and it seems that polyploidization has played a minor role in the diversification and speciation of genus *Primulina* in limestone areas of SW China.

Keywords: chromosome number; limestone karsts; *Primulina*; polyploidy; speciation

Introduction

Primulina, a highly diversified genus of Tribe Didymocarpeae (Gesneriaceae, subfamily Cyrtandroideae), originally monotypic but now enormously enlarged to approximately 130 species, comprises the members of previous genera *Chiritopsis*, *Wentsaiboea* (except for *W. tiandengensis* Liu Yan & B. Pan) and *Chirita* sect. *Gibbosaccus*, based on available molecular phylogenies of *Chirita* and its related taxa and in combination with the morphological characters (Wang et al. 2011; Weber, Middleton, et al. 2011). The available information suggests that *Primulina* is essentially a calciphilous genus with typical habitats being the limestone cliffs and caves from Western and Southern China, with certain species also occurring in Vietnam. The Guangxi province is the center of diversity of *Primulina*, with more than half of the species described occurring there and with most of them endemics with narrow distribution ranges (Wang WT et al. 1998; Li and Wang 2004; Wei et al. 2010; Weber, Middleton, et al. 2011).

Limestone karsts are sedimentary rock outcrops that consist primarily of calcium carbonate. In Southeast Asia, karsts cover an area of around 400,000 km^2 (Clements et al. 2006). The Guangxi Zhuangzu Autonomous Region of southern China, residing in the heart of an immense limestone terrain stretching across the Sino-Vietnamese border (Xu 1995), is one of the most representative regions of limestone karsts, with 40% of the province covered by limestone landscapes (Zhou YY et al. 2004; Hou et al. 2010). Renowned for its picturesque karst areas, Guangxi is also one of the major biodiversity hotspots in China (López-Pujol et al. 2011), characterized by its extraordinarily high diversity and endemism of vascular plants associated with limestone ecosystems (Xu 1995; Hou et al. 2010; Xu et al. 2012), with *c*.80% of the endemic genera only occurring in its limestone areas (Lu et al. 1989). Based on the data reported by several authors (e.g. Xu 1995; Li and Wang 2004), families such as Gesneriaceae, Begoniaceae, Liliaceae, and Rubiaceae comprise a large proportion of endemics in the limestone areas of Guangxi (Hou et al. 2010). Among 25 genera and 186 species of Gesneriaceae described in Guangxi, two genera and 101 species are endemics (Wei et al. 2004; Möller et al. 2011; Weber, Middleton, et al. 2011; Weber, Wei, Puglisi, et al. 2011; Weber, Wei, Sontag, et al. 2011).

Cytological data such as chromosome numbers and their morphology are fundamental to elucidate the origin, speciation and phylogenetic relationship of plants (Stebbins 1971; Liu et al. 2001; Cai et al. 2004;

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Liu 2004; Pavlova and Tosheva 2005). Cytological studies in Gesneriaceae have been carried out over a number of decades, mainly focused on diversity in chromosome number (especially basic number), chromosome size (Ratter 1975; Skog 1984; Christie et al. 2012), and satellites (Kiehn et al. 1998; Christie et al. 2012). Up to now, nearly 1000 chromosome counts have been reported for Gesneriaceae (Möller et al. 2002 onwards; Möller and Kiehn 2004; Christie et al. 2012). In general, the chromosomes across the family are relatively small, ranging from less than 1 to 2 μm, and difficult to analyze (Möller and Kiehn 2004; Christie et al. 2012). As a result few karyotypes have been published so far, mainly in Chinese taxa (Wang YZ et al. 1998; Wang and Gu 1999; Lu et al. 2002; Zhou P et al. 2004; Ji et al. 2008; Yang et al. 2012) and two for African taxa (Möller and Kiehn 2004).

Although more than 130 species are now included in the newly delimited genus *Primulina*, and chromosome data for 15 taxa have recently been published (Christie et al. 2012), the total number of counted taxa is only 18 (Ratter and Prentice 1964; Cao et al. 2003; Zhou P et al. 2004; Yang et al. 2012). In *Primulina*, the basic chromosome number has been found to be $x = 18$, with 16 out of all counts uniformly possessing $2n = 36$ chromosomes (excluding two somewhat dubious counts) and only one polyploid determined as $2n = 72$. However, there are many gaps remaining to be filled before any definite conclusions can be drawn about chromosome evolution in the genus. Therefore, in the present study, we examined the chromosome numbers and chromosome morphology for 53 *Primulina* taxa sampled from limestone karsts of Guangxi province, China, half of which are known so far to be endemic to Guangxi province. The main aims of this study are: (1) to contribute more chromosome data to the genus *Primulina*; (2) to confirm whether the somatic chromosome number of $2n = 36$ is prevailing; and (3) to determine what degree of polyploidy occurs within this genus.

Materials and methods

The names and localities of taxa studied are listed in Table 1. The plant samples were collected from wild populations growing on limestone karsts of Guangxi Province, China and were cultivated in the Guangxi Institute of Botany, Guangxi Zhuangzu Autonomous Region and the Chinese Academy of Sciences. Actively growing root tips were harvested from cuttings grown in vermiculite in a culture room and usable roots were produced within 2–3 weeks. The roots were pretreated with distilled water at 0°C for 24 h, fixed with Carnoy's fluid (absolute alcohol:glacial acetic acid 3:1) at 4°C for at least 30 min, then stored in 70% aqueous ethanol until required. After being macerated in a 1:1 mixture of 1N HCl and 45% acetic acid at 60°C for 3 minutes, the root tips were rinsed in distilled water, stained and squashed in carbol fuchsin.

The best metaphase plates were photographed using an Olympus BX51 microscope with Olympus DP71 camera attachment. The chromosome numbers were determined in at least 20 cells with well-spread chromosomes of 10 different root tips. Voucher specimens are deposited at the Guangxi Institute of Botany, Guangxi Zhuang Autonomous Region and the Chinese Academy of Sciences.

Results

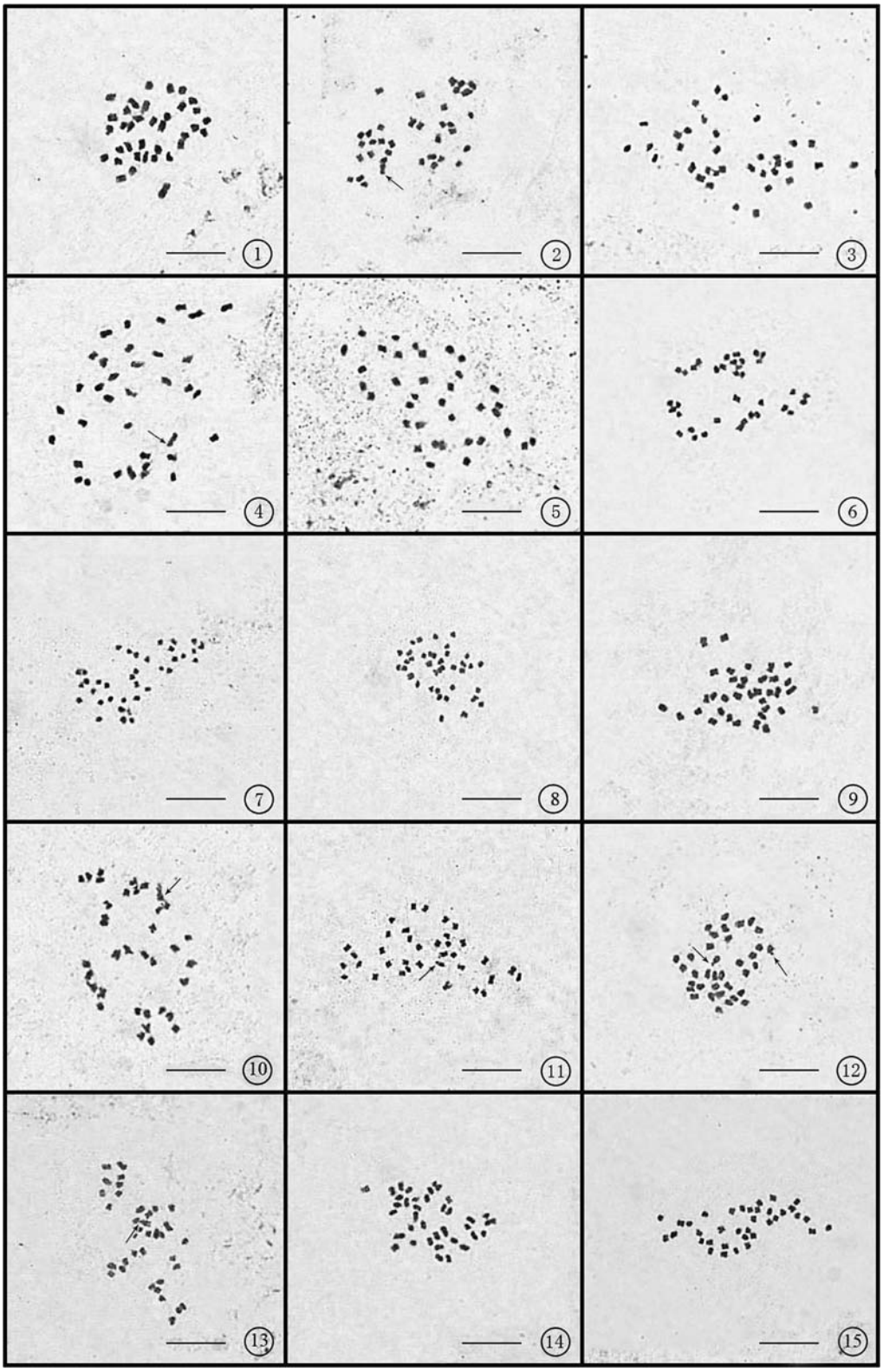
A survey of somatic chromosome numbers and chromosome morphology of 39 species, three varieties and 11 unnamed taxa was carried out in this study. The somatic chromosomes of the 53 taxa are illustrated in Figures 1–53 and the chromosome counts are listed in Table 1. All examined taxa were consistently determined as diploid with $2n = 36$ chromosomes. Apart from eight confirmatory counts and one new somatic chromosome number for previously investigated species, all other counts are reported here for the first time. Chromosomes in *Primulina* are small in size according to the classification of Lima-de-faria (1980), with a range of 0.6 to 2.4 μm, showing only slight size variation. Frequently, one or two satellites were observed in the 18 taxa studied. It was most common that one satellite was found, although *P. langshanica* and *P.* sp. nov. 4 were found with two satellites. The satellites in several taxa, residing on the terminal of relatively large chromosomes, were very small and difficult to detect without careful observation. However, the satellites present in *P. linglingensis*, *P. nandanensis*, *P. eburnea* and *P.* sp. nov. 8 were relatively large. Because of the relatively small size of the chromosomes for most observed taxa, the position of their centromere could not be determined, which prevented a detailed karyotype analysis. From photographs of taxa with well-spread and somewhat larger chromosomes it was possible to observe that their karyotypes were mainly composed of metacentric and submetacentric chromosomes, as for example for *P. guihaiensis*, *P. lungzhouensis* and *P. subrhomboidea* (Figures 9, 23, 47).

Discussion

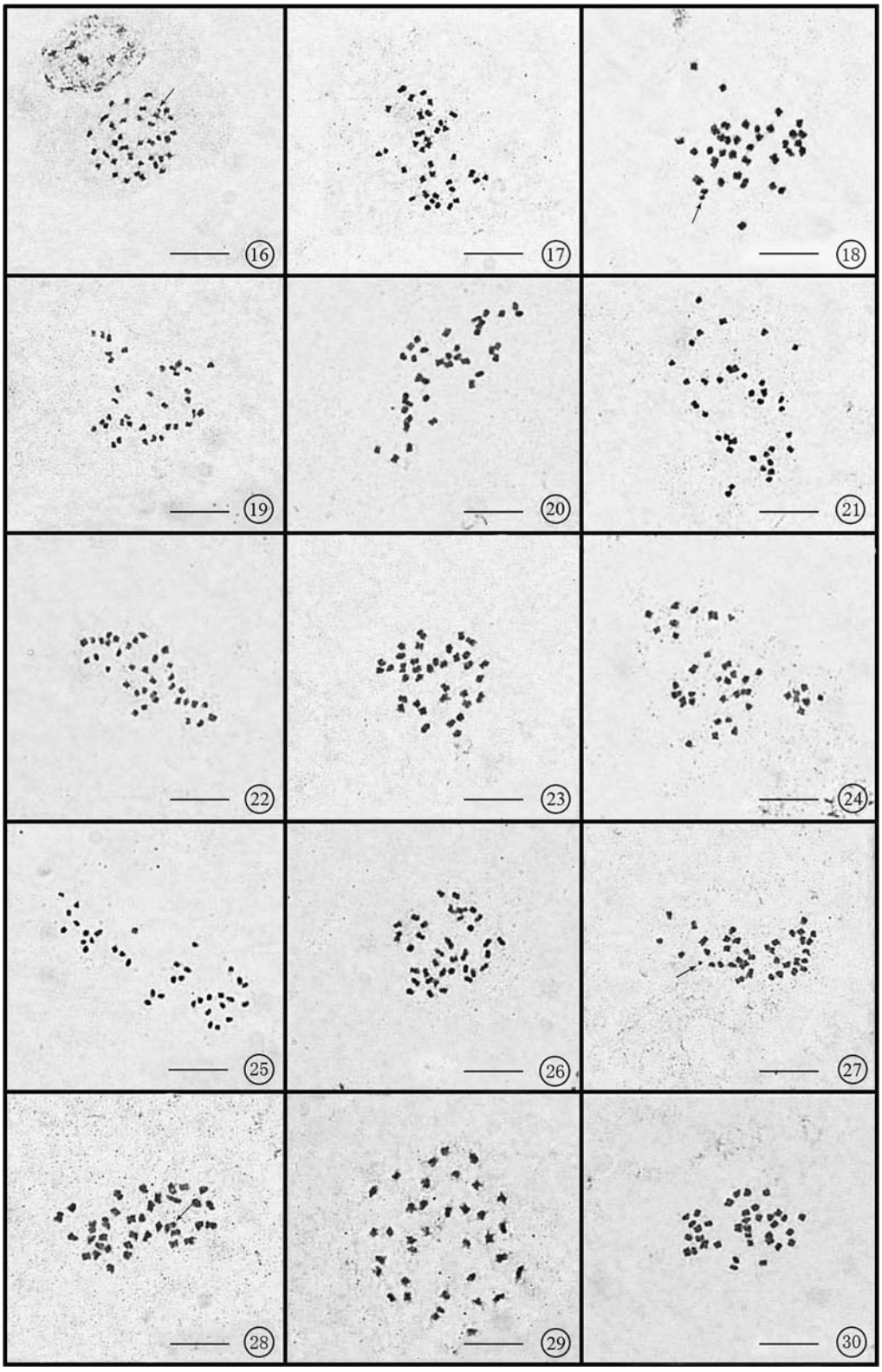
The present study is an extensive investigation on chromosome numbers and chromosome morphology for the genus *Primulina*, with 44 new counts and eight confirmatory ones as well as one new count adding to previously investigated taxa. Our counts of $2n = 36$ for *Primulina* taxa investigated here fall well in line with the previously published basic chromosome number $x = 18$ for this genus (Ratter and Prentice 1964; Zhou P et al. 2004; Christie et al. 2012; Yang et al. 2012). The somatic chromosome number seems to be constant in *Primulina*, as all described and undescribed taxa examined in the present study uniformly show $2n = 36$, identical with previous reports for 16 out of 18 species. The only exception was observed for *P. longgangensis* var. *longgangensis* which was determined as $2n = 36$

Table 1. The details of *Primulina* taxa studied cytologically. The numbers (No.) refer to Figures 1–53. Taxa in bold were conformed ones. Asterisks (*) indicates taxa endemic to Guangxi, China.

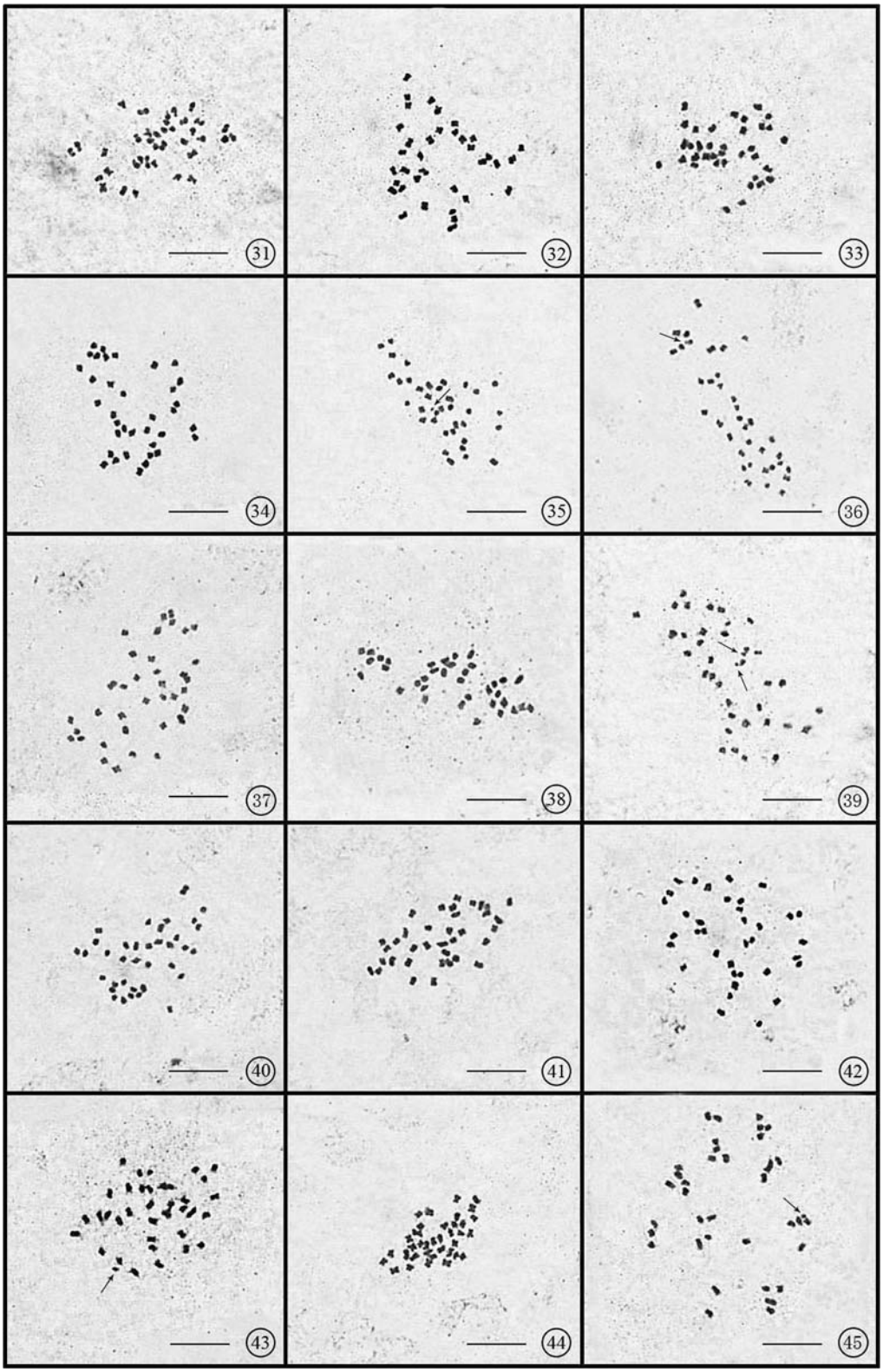
No.	Taxon	Locality	Voucher specimen	2*n*	Maximum number of satellites detected
1	*P. baishouensis* (Y.G. Wei, H.Q. Wen & S.H. Zhong) Y.Z. Wang*	Yongfu, Guangxi	P068	36	
2	*P. brassicoides* (W.T. Wang) Mich. Möller & A. Weber*	Longzhou, Guangxi	P214	36	1
3	*P. cordata* (W.T. Wang) Mich. Möller & A. Weber*	Yangshuo, Guangxi	P077	36	
4	*P. eburnea* (Hance) Y.Z. Wang	Guilin, Guangxi	P101	36	1
5	*P. fimbrisepala* var. *fimbrisepala* (Hand.-Mazz.) Y.Z. Wang	Lingchuan, Guangxi	P089	36	
6	*P. fimbrisepala* var. *mollis* (W.T. Wang) Mich. Möller & A. Weber*	Shangsi, Guangxi	P088	36	
7	***P. flavimaculata* (W.T. Wang) Mich. Möller & A. Weber**	Longzhou, Guangxi	P080	36	
8	*P. gueilinensis* (W.T. Wang) Y.Z. Wang	Guilin, Guangxi	P071	36	
9	*P. guihaiensis* (Y.G. Wei, B. Pan & W.X. Tang) Mich. Möller & A. Weber*	Guilin, Guangxi	P056	36	
10	***P. hedyotidea* (Chun) Y.Z. Wang***	Ningming, Guangxi	P013	36	1
11	*P. hochiensis* (C.C. Huang & X.X. Chen) Mich. Möller & A. Weber*	Hechi, Guangxi	P038	36	1
12	*P. langshanica* (W.T. Wang) Y.Z. Wang	Ziyuan, Guangxi	P047	36	2
13	*P. laxiflora* (W.T. Wang) Y.Z. Wang	Longzhou, Guangxi	P053	36	1
14	*P. leiophylla* (W.T. Wang) Y.Z. Wang*	Longzhou, Guangxi	P003	36	
15	***P. liboensis* (W.T. Wang & D.Y. Chen) Mich. Möller & A. Weber**	Nandan, Guangxi	P044	36	
16	***P. liguliformis* (W.T. Wang) Mich. Möller & A. Weber**	Hechi, Guangxi	P052	36	1
17	*P. linearifolia* (W.T. Wang) Y.Z. Wang*	Wuming, Guangxi	P038	36	
18	*P. linglingensis* (W.T. Wang) Mich. Möller & A. Weber	Quanzhou, Guangxi	P087	36	1
19	*P. longgangensis* var. *longgangensis* (W.T. Wang) Y.Z. Wang*	Longzhou, Guangxi	P041	36	
20	*P. longicalyx* (J.M. Li & Y.Z. Wang) Mich. Möller & A. Weber*	Guilin, Guangxi	P103	36	
21	*P. longii* (Z. Yu Li) Y.Z. Wang*	Yongfu, Guangxi	P069	36	
22	*P. lunglinensis* (W.T. Wang) Mich. Möller & A. Weber	Longlin, Guangxi	P213	36	
23	*P. lungzhouensis* (W.T. Wang) Mich. Möller & A. Weber	Longzhou, Guangxi	P085	36	
24	***P. lutea* (Y. Liu & Y.G. Wei) Mich. Möller & A. Weber***	Hezhou, Guangxi	P100	36	
25	*P. macrodonta* (D. Fang & D.H. Qin) Mich. Möller & A. Weber*	Lingchuan, Guangxi	P036	36	
26	*P. medica* (D. Fang) Y.Z. Wang*	Pingle, Guangxi	P076	36	
27	***P. minutimaculata* (D. Fang & W.T. Wang) Y.Z. Wang**	Longzhou, Guangxi	P048	36	1
28	*P. nandanensis* (S.X. Huang, Y.G. Wei & W.H. Luo) Mich. Möller & A. Weber	Nandan, Guangxi	P042	36	1
29	*P. pinnatifida* (Hand.-Mazz.) Y.Z. Wang	Xingan, Guangxi	P054	36	
30	*P. pseudoeburnea* (D. Fang & W.T. Wang) Mich. Möller & A. Weber*	Tiandong, Guangxi	P006	36	
31	*P. pseudoheterotricha* (T.J. Zhou, B. Pan & W.B. Xu) Mich. Möller & A. Weber	Zhongshan, Guangxi	P073	36	
32	*P. pungentisepala* (W.T. Wang) Mich. Möller & A. Weber*	Ningming, Guangxi	P050	36	
33	*P. sclerophylla* (W.T. Wang) Y.Z. Wang*	Yizhou, Guangxi	P092	36	
34	*P. shouchengensis* (Z.Y. Li) Y.Z. Wang*	Yongfu, Guangxi	P065	36	
35	***P. spinulosa* (D. Fang & W.T. Wang) Y.Z. Wang***	Fusui, Guangxi	P010	36	1
36	*P.* sp. nov. 1	Jingxi, Guangxi	P140	36	1
37	*P.* sp. nov. 2	Hechi, Guangxi	P045	36	
38	*P.* sp. nov. 3	Yulin, Guangxi	P109	36	
39	*P.* sp. nov. 4	Wuming, Guangxi	P091	36	2
40	*P.* sp. nov. 5	Liuzhou, Guangxi	P090	36	
41	*P.* sp. nov. 6	Teng county, Guangxi	P041	36	
42	*P.* sp. nov. 7	Ningming, Guangxi	P093	36	
43	*P.* sp. nov. 8	Mashan, Guangxi	P075	36	1
44	*P.* sp. nov. 9	Yongfu, Guangxi	P063	36	
45	*P.* sp. nov. 10	Rongshui, Guangxi	P066	36	1
46	*P.* sp. nov. 11	Yangshuo, Guangxi	P102	36	
47	***P. subrhomboidea* (W.T. Wang) Y.Z. Wang***	Yangshuo, Guangxi	P072	36	1
48	*P. swinglei* (Merr.) Mich. Möller & A. Weber	Yulin, Guangxi	P041	36	
49	*P. tribracteata* (W.T. Wang) Mich. Möller & A. Weber*	Fengshan, Guangxi	P023	36	1
50	*P. varicolor* (D. Fang & D.H. Qin) Y.Z. Wang*	Napo, Guangxi	P002	36	
51	*P. weii* Mich. Möller & A. Weber*	Jingxi, Guangxi	P008	36	
52	*P. wentsaii* (D. Fang & L. Zeng) Y.Z. Wang*	Longzhou, Guangxi	P020	36	1
53	*P. yungfuensis* (W.T. Wang) Mich. Möller & A. Weber*	Guilin, Guangxi	P018	36	



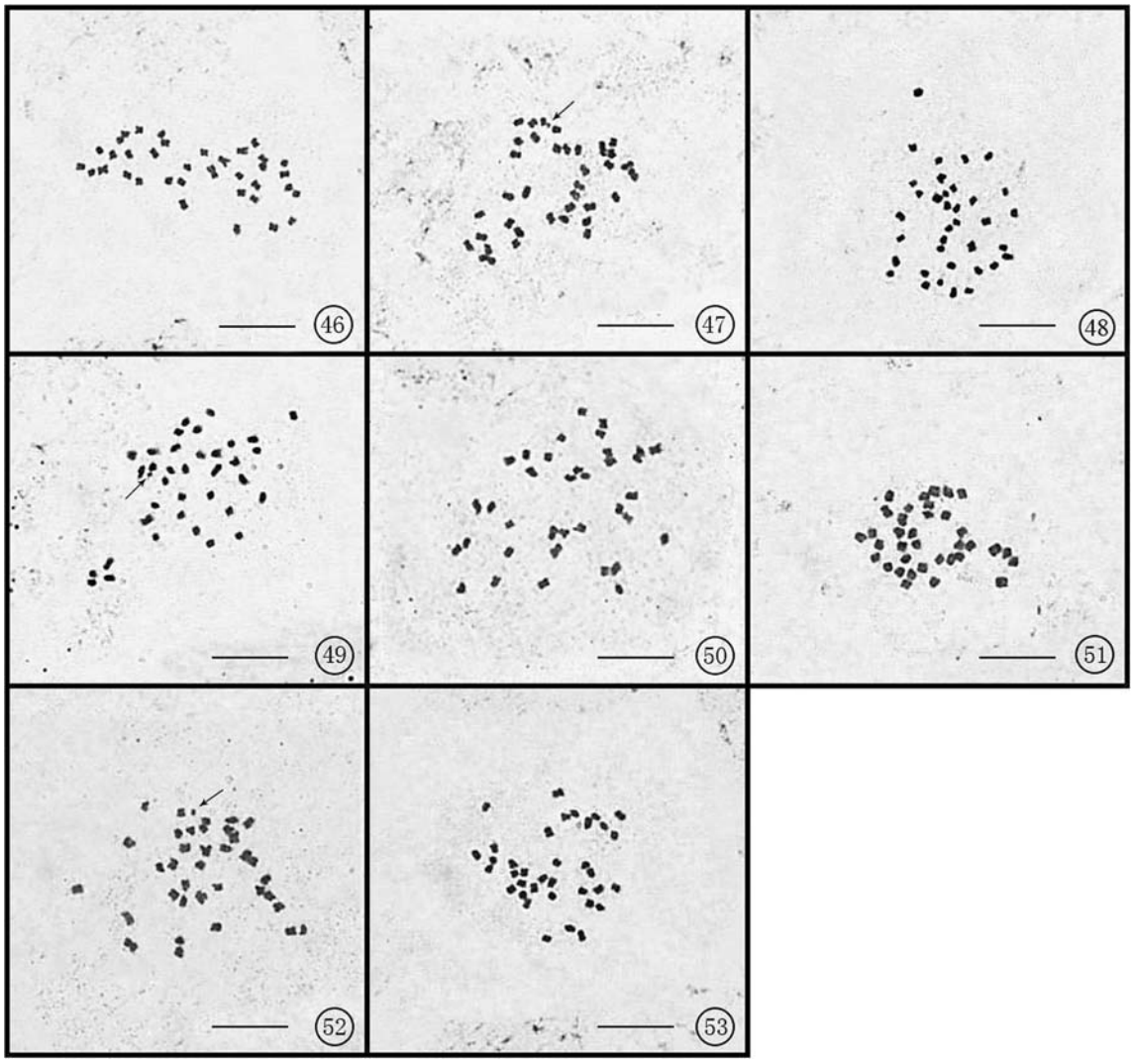
Figures 1–15. Somatic chromosomes of *Primulina* taxa studied. (1) *P. baishouensis* (Y.G. Wei, H.Q. Wen & S.H. Zhong) Y.Z. Wang. (2) *P. brassicoides* (W.T. Wang) Mich. Möller & A. Weber. (3) *P. cordata* (W.T. Wang) Mich. Möller & A. Weber. (4) *P. eburnea* (Hance) Y.Z. Wang. (5) *P. fimbrisepala* var. *fimbrisepala* (Hand.-Mazz.) Y.Z. Wang. (6) *P. fimbrisepala* var. *mollis* (W.T. Wang) Mich. Möller & A. Weber. (7) *P. flavimaculata* (W.T. Wang) Mich. Möller & A. Weber. (8) *P. gueilinensis* (W.T. Wang) Y.Z. Wang. (9) *P. guihaiensis* (Y.G. Wei, B. Pan & W.X. Tang) Mich. Möller & A. Weber. (10) *P. hedyotidea* (Chun) Y.Z. Wang. (11) *P. hochiensis* (C.C. Huang & X.X. Chen) Mich. Möller & A. Weber. (12) *P. langshanica* (W.T. Wang) Y.Z. Wang. (13) *P. laxiflora* (W.T. Wang) Y.Z. Wang. (14) *P. leiophylla* (W.T. Wang) Y.Z. Wang. (15) *P. liboensis* (W.T. Wang & D.Y. Chen) Mich. Möller & A. Weber. Arrows indicate satellites. Scale bars = 10 μm.



Figures 16–30. Somatic chromosomes of *Primulina* taxa studied. (16) *P. liguliformis* (W.T. Wang) Mich. Möller & A. Weber. (17) *P. linearifolia* (W.T. Wang) Y.Z. Wang. (18) *P. linglingensis* (W.T. Wang) Mich. Möller & A. Weber. (19) *P. longgangensis* var. *longgangensis* (W.T. Wang) Y.Z. Wang. (20) *P. longicalyx* (J.M. Li & Y.Z. Wang) Mich. Möller & A. Weber. (21) *P. longii* (Z. Yu Li) Y.Z. Wang. (22) *P. lunglinensis* (W.T. Wang) Mich. Möller & A. Weber. (23) *P. lungzhouensis* (W.T. Wang) Mich. Möller & A. Weber. (24) *P. lutea* (Y. Liu & Y.G. Wei) Mich. Möller & A. Weber. (25) *P. macrodonta* (D. Fang & D.H. Qin) Mich. Möller & A. Weber. (26) *P. medica* (D. Fang) Y.Z. Wang. (27) *P. minutimaculata* (D. Fang & W.T. Wang) Y.Z. Wang. (28) *P. nandanensis* (S.X. Huang, Y.G. Wei & W.H. Luo) Mich. Möller & A. Weber. (29) *P. pinnatifida* (Hand.-Mazz.) Y.Z. Wang. (30) *P. pseudoeburnea* (D. Fang & W.T. Wang) Mich. Möller & A. Weber. Arrows indicate satellites. Scale bars = 10 μm.



Figures 31–45. Somatic chromosomes of *Primulina* taxa studied. (31) *P. pseudoheterotricha* (T.J. Zhou, B. Pan & W.B. Xu) Mich. Möller & A. Weber. (32) *P. pungentisepala* (W.T. Wang) Mich. Möller & A. Weber. (33) *P. sclerophylla* (W.T. Wang) Y.Z. Wang. (34) *P. shouchengensis* (Z.Y. Li) Y.Z. Wang. (35) *P. spinulosa* (D. Fang & W.T. Wang) Y.Z. Wang. (36) *P.* sp. nov. 1. (37) *P.* sp. nov. 2. (38) *P.* sp. nov. 3. (39) *P.* sp. nov. 4. (40) *P.* sp. nov. 5. (41) *P.* sp. nov. 6. (42) *P.* sp. nov. 7. (43) *P.* sp. nov. 8. (44) *P.* sp. nov. 9. (45) *P.* sp. nov. 10. Arrows indicate satellites. Scale bars = 10 μm.



Figures 46–53. Somatic chromosomes of *Primulina* taxa studied. (46) *P.* sp. nov. 11. (47) *P. subrhomboidea* (W.T. Wang) Y.Z. Wang. (48) *P. swinglei* (Merr.) Mich. Möller & A. Weber. (49) *P. tribracteata* (W.T. Wang) Mich. Möller & A. Weber. (50) *P. varicolor* (D. Fang & D.H. Qin) Y.Z. Wang. (51) *P. weii* Mich. Möller & A. Weber. (52) *P. wentsaii* (D. Fang & L. Zeng) Y.Z. Wang. (53) *P. yungfuensis* (W.T. Wang) Mich. Möller & A. Weber. Arrows indicate satellites. Scale bars = 10 μm.

here but $2n = 28$ by Cao et al. (2003) and $2n = 72$ by Christie et al. (2012). Since the image published in Cao et al. (2003) is poorly reproduced and it is difficult to determine the definite number, the count of $2n = 28$ is somewhat dubious, needing further verification. In this view, *P. longgangensis* var. *longgangensis* might represent a species with two ploidy levels. Compared to the count of $2n = 36$ for *P. longgangensis* var. *longgangensis* obtained in this study, $2n = 72$ by Christie et al. (2012) appears to represent a tetraploid number with a basic number of $x = 18$.

In general, *Primulina* possesses relatively small chromosomes and somatic metaphase chromosomes, with clearly visible and well-spread metaphase chromosomes that are rather hard to find. The chromosome size for *Primulina*, 0.6–2.4 μm, is mostly within the range reported for Gesneriaceae. Nevertheless, it is notable that some species with relatively large chromosomes and centromeres consist mainly of metacentrics and submetacentrics. This is consistent with the report for *P. tabacum* [with a karyotype formula of $2n = 36 =$ 24m (1SAT) + 12sm (Yang et al. 2012)], and with other species in this genus (Christie et al. 2012). The chromosomes of *P. eburnean*, *P. hedyotidea*, *P. lutea*, *P. subrhomboidea* and *P. minutimaculata* are larger in size and different in shape compared with the previously published counts; this may be due to the different pretreatment or staining techniques used by us (distilled water at 0°C and carbol fuchsin) and by Zhou P et al. (2004) (8-hydroxyquinoline and aceto-orcein) or by Christie et al. (2012) (saturated aqueous 1-bromonaphthalene or 0.002 M 8-hydroxyquinoline and Feulgen).

Satellites appear to be common in Gesneriaceae (Kiehn et al. 1998; Rashid et al. 2001; Zhou P et al. 2004; Ji et al. 2008; Christie et al. 2012; Yang et al. 2012). In the present study, we detected 1–2 satellites in 18 taxa; one satellite in 16 taxa and two in two taxa. In some cases the satellites are very small, but relatively

large and distinct satellites were encountered in *P. eburnean*, *P. linglingensis*, *P. nandanensis*, and *P.* sp. nov. 8, and may tend to detach; the stalk linking the relatively large satellites to the not very much larger chromosome is clearly visible (Figures 18, 28, 39, 49). The maximum number of satellites detected varies between this study and previous reports. Two satellites were observed for *P. hedyotidea* and *P. subrhomboidea* and one for *P. liboensis* by Christie et al. (2012), but we only observed one satellite for the former two taxa.

If we disregard the differences in the presence or absence and the number of satellites, the chromosome numbers are highly constant in *Primulina*, with only one polyploidy event. Our results together with previously published data indicate that numerical changes via polyploidization may have played a minor role in the diversification and speciation of *Primulina*. However, structural changes at diploid level involving chromosomal rearrangements, reciprocal translocations or parametric inversions with segments of equal size, which contributed little to changes of chromosomal morphology, might have contributed to chromosome evolution of *Primulina* in limestone karsts (Bonierbale et al. 1988; Seijo and Aveliano 2003). This presumption needs to be substantiated by further investigations.

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