Ploidy Level, Karyotype, and Genome Size of *Bletilla* Species (Orchidaceae) From China

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Abstract. *Bletilla* is an Orchidaceae genus with high medical value, including detumescence, antibacterial, and hemostasis. In this study, detailed estimates of ploidy level, karyotype, and genome size were first obtained, and a comprehensive cytological analysis was carried out to better understand the evolution of the genus. The karyotypes of *Bletilla* were mainly composed of metacentric and submetacentric chromosomes with lengths ranging from 1.25 to 4.93 μm . There was moderate cytological variation in *Bletilla* (chromosome number $2n = 32$ to 76). Diploid with $2n = 34$ and $2n = 36$ was detected in *Bletilla ochracea* and *Bletilla formosana*, respectively, whereas diploid ($2n = 32$) was dominant in *Bletilla striata*, dysploidy ($2n = 34$, $2n = 36$) and polyploid ($2n = 48$, 51, 64, 76) variations were also observed. Three species had a relatively symmetric karyotype, and which of *B. ochracea* was more asymmetry. The genome size (1C-values) varied from 2.94 pg (*B. striata*) to 3.33 pg (*B. ochracea*), of which *B. ochracea* was significantly larger than the others ($P < 0.05$). A positive correlation ($P < 0.01$) between 1Cx vs. haploid chromosome length (HCL) and asymmetry coefficient of karyotypes (AsK%) was observed.

Bletilla Reichb.f. is a small genus with five species, including *B. striata* (Thunb.) Rchb.f., *B. ochracea* Schltr., *B. formosana* (Hayata) Schltr., *Bletilla foliosa* (King & Pantl.) Tang & F.T.Wang and *Bletilla chartacea* (King & Pantl.) Tang & F.T.Wang (<http://www.theplantlist.org>), primarily distributed in Asia from Myanmar, southern and eastern China, to Japan and Korea. China is its distribution center with four species, except for *B. chartacea* (Chen X. et al., 2009). Among the four species, *B. striata* is the main traditional medicinal resource recorded in the Shen-nong's Classic of Materia Medica and is also the only one recognized in the Chinese Pharmacopoeia (2020 edition). Its pharmacological values include detumescence, antibacterial, hemostasis, and antitumor properties, among others (Chen et al., 2020; Zhou et al., 2020). However, wild resources of *Bletilla* have sharply decreased due to the limited natural reproduction, increased utilization, and natural habitat destruction. Consequently, *Bletilla* has been included in the List of National Key

Protected Wild Plants (Zhang et al., 2019). On the other hand, breeding of cultivars for high yields and quality is critical for resource conservation and sustainable utilization.

Karyotype and genome size encompass the hereditary elements of the whole nuclear genome, which can provide important

information for species identification and diversity studies (Guerra, 2012; Jang and Weiss-schneeweiss, 2018). Furthermore, they also play a key role in establishing phylogenetic relationships between species and inferring evolutionary trends (Sharma and Mukai, 2015; Stace, 2000). Despite being one of the largest families of angiosperm, cytological analysis has been carried out on no more than 10% of Orchidaceae (orchids) species due to the relatively high diversity in chromosome numbers and comparatively small or even minute chromosome size (Myo et al., 2011; Tanaka and Kamemoto, 1984). Their patterns of speciation and evolution of the entire Orchidaceae family are still debated because of lack of information on valuable chromosome landmarks (Sharma and Mukai, 2015).

As a genus of Orchidaceae, classification status of *Bletilla* has also been uncertain for many years (Goldman et al., 2001). Tan (1969) concluded that *Bletilla* was more closely associated with *Bletia purpurea*, and *Bletilla* was placed in the same subtribe (Bletiinae, Arethuseae) with *Bletia* (Dressler, 1981, 1993) or subtribe Bletiinae (tribe Epi-dendreae) (van den Berg et al., 2005). With DNA phylogenetic analysis, genus *Bletilla* is now placed in the updated subtribe Coelogy-ninae (Chase et al., 2003; Li et al., 2015; van den Berg et al., 2005).

Previous cytological investigations of *Bletilla* provided the chromosome counts for only two species, without detailed karyotype information. The main chromosome number of *B. striata* was $n = 16$ ($2n = 32$), and intraspecific variation had also been reported, that is, *B. striata* ($2n = 64$ and 76). *B. formosana* has a

Fig. 1. Geographic distribution and ploidy level of the *Bletilla* plants collected from China. All population samples were diploid, except for the HBYL, HBLC, and HBEX populations, which had a small proportion of triploid individuals. For location details, see Supplemental Table 1. This map is provided by the International Potato Center (CIP) public domain and is drawn by DIVA-GIS (<https://research.cip.cgiar.org/confluence/display/divagis/Home>).

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uniform chromosome number $n = 18$ ($2n = 36$) (Felix and Guerra, 2010; Li and Chen, 1987; Tan, 1969). Based on the reported chromosome number variations, a basic chromosome number of $x = 16$ was suggested for the genus *Bletilla* (Felix and Guerra, 2010).

Genome size is another karyotypic character that can vary considerably among species with difference in repetitive DNA amount (Flavell et al., 1974). Species in the orchid family present the second largest genome size variation among plants (168-fold variation) (Leitch et al., 2009) following Melanthiaceae (230-fold variation) (Pellicer et al., 2014). So far, the genome size of only one species of *Bletilla* has been estimated (*B. striata*, $1C = 2.95$ pg), and its patterns of interspecific and intraspecific variations of DNA contents and genome size evolution of the genus are still unclear (Zhang et al., 2013; Zonneveld et al., 2005).

Considering the scarce cytological information available for *Bletilla*, we carried out a study on the ploidy level, chromosome number, and genome size. Our research aims to provide important information for the further reservation and development of the important medicinal resource of *Bletilla* plants. In addition, the investigation patterns of DNA content variation and variation in chromosome number can also provide meaningful and essential information to further explore the evolution of genome size and the main mechanisms of chromosome evolution in a phylogenetic context of *Bletilla*.

Materials and Methods

Plant materials. Living collections of 1082 plants, including *B. striata*, *B. ochracea*, and *B. formosana*, were obtained from 62 locations in China (Fig. 1, Table 1). Due to their extremely narrow distribution and endangered situation, *B. foliosa* (synonymy *B. sinensis*) could not be collected, and only one population of *B. formosana* was collected in this study. The representative specimens of plants from each population were deposited in the herbarium (HIB) of Wuhan Botanical Garden, Chinese Academy of Sciences. A small number of young roots and leaves were sampled from seedlings for chromosome counting and nuclear DNA content estimation. The ploidy level of all the collected plants was analyzed by flow cytometry (FCM). Chromosome counts, karyotypes, and DNA content analyses were performed on three individuals from each of 25 populations of three species.

Ploidy levels and genome size estimation. Nuclear DNA content was assessed by FCM with minor modifications (Doležel et al., 2007). All procedures were performed at 4 °C or on ice. Approximately 50 to 100 mg of newly expanding leaf tissue from each sample and/or calibration standard sample was placed in a plastic 60-mm petri dish standing on ice. Ice-cold LB01 buffer [15 mM Tris; 2 mM Na_2EDTA ; 0.5 mM spermine.4HCl; 80 mM KCl; 20 mM NaCl; 15 mM β -mercaptoethanol; 0.1% (v/v) Triton X-100; pH 7.5] was added in the proportion of 1.5 mL buffer per 100 mg tissue. Tissues were chopped using a sharp

Table 1. Karyomorphometric data for the representative populations of *Bletilla* were obtained in this study.

Population code	Species	2n	Karyotypic formula	Chromosome size			Inter index		Intrachromosomal index				1Cx (pg)	cv (%)
				HCL (μm)	RLR	Mean size (μm)	A ₂	AsK%	Sy _i	A ₁	2C (pg)	mean ± sd		
<i>B. striata</i>	HBYL	48	2n = 3x = 36m + 12sm	34.42	2.00	2.24	1.54–3.10	0.09	63.8	0.37	6.21 ± 0.12	3.11 ± 0.06	2.83	
	HBLC	32	2n = 2x = 24m + 8sm	35.86	2.16	2.15	1.45–3.09	0.09	65.1	0.36	8.97 ± 0.33	2.99 ± 0.11	2.97	
		48	2n = 3x = 39m + 9sm	35.79	2.27	2.01	1.33–3.14	0.10	64.9	0.36	6.01 ± 0.20	3.00 ± 0.10	2.93	
		32	2n = 2x = 24m + 8sm	32.17	2.45	2.24	1.41–3.45	0.09	64.7	0.36	8.32 ± 0.33	2.77 ± 0.11	3.75	
	HBXE	51	2n = 3x = 36m + 15sm	40.06	2.51	2.34	1.50–3.76	0.08	60.9	0.40	6.41 ± 0.43	3.21 ± 0.22	3.44	
		34	2n = 2x = 24m + 10sm	39.74	2.20	2.38	1.53–3.38	0.08	65.4	0.35	8.89 ± 0.77	2.97 ± 0.26	3.42	
	HNLS	32	2n = 2x = 22m + 10sm	32.08	2.03	1.99	1.38–2.80	0.10	66.1	0.34	5.79 ± 0.15	2.89 ± 0.08	2.51	
		GSCX	32	2n = 2x = 24m + 8sm	34.73	2.24	2.17	1.40–3.17	0.09	66.6	0.34	5.77 ± 0.13	2.88 ± 0.07	2.63
	SXXX	32	2n = 2x = 24m + 8sm	32.25	2.26	2.02	1.32–2.86	0.09	66.1	0.36	5.65 ± 0.06	2.82 ± 0.03	2.56	
	GZGY	32	2n = 2x = 24m + 6sm + 2st	34.96	2.23	2.19	1.43–3.41	0.09	65.1	0.36	5.67 ± 0.30	2.83 ± 0.15	3.62	
<i>B. ochracea</i>	YNBS	32	2n = 2x = 22m + 8sm + 2st	31.57	2.11	1.79	1.32–2.80	0.11	65.3	0.36	5.94 ± 0.13	2.97 ± 0.07	2.48	
	GXHZ	32	2n = 2x = 24m + 8sm	34.55	2.03	2.16	1.55–3.05	0.09	63.5	0.37	6.06 ± 0.05	3.03 ± 0.03	2.75	
	JSNJ	32	2n = 2x = 28m + 4sm	38.38	2.32	2.40	1.54–3.66	0.08	66.6	0.34	5.78 ± 0.06	2.89 ± 0.05	2.60	
	ABHZ	32	2n = 2x = 24m + 6sm + 2st	38.01	2.09	2.38	1.54–3.11	0.08	69.6	0.30	5.70 ± 0.02	2.85 ± 0.01	2.75	
	CQJF	32	2n = 2x = 24m + 8sm	30.36	2.10	1.90	1.39–2.74	0.10	67.9	0.31	5.82 ± 0.09	2.91 ± 0.05	2.65	
	ZJAJ	32	2n = 2x = 24m + 8sm	33.91	2.05	2.12	1.48–2.90	0.09	64.0	0.37	5.85 ± 0.21	2.93 ± 0.11	2.82	
	HNSC	32	2n = 2x = 24m + 8sm	34.70	2.04	2.15	1.50–3.04	0.09	65.0	0.35	5.88 ± 0.13	2.94 ± 0.07	3.00	
	SCLS	36	2n = 2x = 28m + 8sm	43.18	2.19	2.34	1.52–3.60	0.08	67.8	0.32	5.87 ± 0.16	2.97 ± 0.08	3.09	
	HN SZ	36	2n = 2x = 26m + 10sm	38.68	2.65	2.15	1.41–3.61	0.09	63.7	0.37	5.70 ± 0.02	2.85 ± 0.01	2.80	
	HB DJ	34	2n = 2x = 20m + 14sm	37.75	2.83	2.22	1.25–3.46	0.09	60.4	0.42	7.00 ± 0.20	3.50 ± 0.10	3.03	
<i>B. formosana</i>	HBXJ	34	2n = 2x = 18m + 16sm	39.79	2.99	2.34	1.30–3.68	0.08	59.1	0.42	7.16 ± 0.01	3.59 ± 0.01	3.00	
	HBXS	34	2n = 2x = 24m + 10sm	37.77	2.51	2.22	1.38–3.54	0.09	61.2	0.40	6.94 ± 0.10	3.47 ± 0.05	3.10	
	SXNT	34	2n = 2x = 24m + 10sm	40.80	2.87	2.40	1.39–3.88	0.08	62.4	0.39	6.51 ± 0.23	3.26 ± 0.12	2.88	
	GSWX	34	2n = 2x = 24m + 10sm	39.63	3.44	2.33	1.25–4.25	0.08	65.2	0.36	6.76 ± 0.38	3.28 ± 0.19	2.44	
	HBWH	34	2n = 2x = 24m + 10sm	40.29	2.97	2.65	1.68–4.93	0.07	62.0	0.39	6.37 ± 0.30	3.19 ± 0.15	2.97	
	HNBS-1	34	2n = 2x = 28m + 6sm	39.66	2.75	2.33	1.41–4.00	0.08	67.8	0.33	6.52 ± 0.28	3.26 ± 0.14	3.06	
	COLF	34	2n = 2x = 22m + 12sm	40.96	2.93	2.41	1.43–4.18	0.08	58.4	0.42	6.31 ± 0.09	3.16 ± 0.05	2.87	
	NNNE	36	2n = 2x = 24m + 12sm	42.69	2.49	2.59	1.72–3.77	0.07	64.1	0.36	6.03 ± 0.10	3.01 ± 0.05	2.70	

2n = diploid chromosome number; HCL = haploid chromosome length; RLR = relative length (longest/shortest); m = metacentric; sm = submetacentric; st = subtelocentric; A₂ = interchromosomal asymmetry index; A₁, Syi, and AsK% = intrachromosomal asymmetry index; cv = coefficients of variation; sd = standard deviation; 1Cx-value = monoploid genome size.

razor blade for 2 to 3 min and then filtered through a 30- μm nylon filter to remove large debris and cell fragments. The homogenate was treated with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ RNase A (DNase free) and stained with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ propidium iodide (Sigma, St. Louis, MO). Homogenate was incubated for 30 min in the dark on ice and then measured using a BD Accuri C6 FCM (BD Biosciences, San Jose, CA) equipped with software BD Accuri C6. The known diploid *B. striata* ($2n = 2x = 32$) was used as a standard to define the ploidy levels. A total of 1082 individuals from 62 populations of three species were examined.

FCM was also used to estimate genome size following the method as described previously. *Solanum lycopersicum* var. ‘Stupiché’ with a DNA content of $2C = 1.96$ pg (Doležel et al., 1992) was used as an internal standard. Genome sizes of 25 populations of three species were estimated (Table 1), and one or two individuals were analyzed for each population. For each individual, three leaf samples were prepared and each sample was run three times. The fluorescence intensity of more than 10,000 particles was recorded in each run. Based on the peak of internal standard and *Bletilla* species, experimental genome size was calculated using the following equation: $2C = (\text{G1 peak value of sample/G1 peak value of standard}) \times 2C$ genome size of standard (pg DNA). The term “monoploid genome size” (1Cx) was used to represent the DNA content in a basic chromosome set (x) of a somatic cell (Greilhuber et al., 2005), whereas 2C referred to the whole genome size of a somatic cell. Data collection was performed using software BD Accuri C6.

Chromosome preparation. Active root tips were sampled from representative individuals and immediately placed in a saturated solution of P-dichlorobenzene for 8 h at 4 °C in the dark. Roots were fixed for a minimum of 2 h in fresh absolute ethanol/glacial acetic acid (3:1, v/v) at 4 °C and then transferred to a 70% ethanol solution. To prepare slides, the fixed root tips were hydrolyzed at 60 °C in 1N HCl for 6 to 8 min and stained in carbol-fuchsin solution. Finally, root tips were squashed for cytological observation. Prepared materials were analyzed under an Olympus Microscope (BX53/Flex; Tokyo, Japan), and chromosome images with well-spread metaphases were captured using a computer-assisted cooled charge-coupled device camera using cellSens software. Chromosome counts were performed on one to two representative individuals from the preceding 25 populations.

Chromosome measurement. At least five spread metaphases with clear and separate chromosomes were examined for chromosome number and karyotypic asymmetry. Karyotype features included chromosome number ($2n$), HCL, and Stebbins karyotype (SK) (Medeiros-Neto et al., 2017; Stebbins, 1971), and the asymmetric indexes were selected as follows: interchromosomal asymmetry indexes A_2 , intra-chromosomal asymmetry indexes A_1 (Zarco, 1986), Sy_i (Venora et al., 2002), and asymmetry coefficient of karyotypes $AsK\%$ (Arano, 1963). The arm ratio ($r = \text{length of the long arm/length of the short arm}$) was used to classify the

chromosomes as metacentric (m , $r = 1.0$ to 1.7), submetacentric (sm , $r = 1.7$ to 3.0), subtelocentric (st , $r = 3.0$ to 7.0), and acrocentric (t , $r = 7.0$ to ∞) (Levan et al., 1964).

Phylogenetic analysis. A phylogenetic analysis based on its internal transcribed spacer (ITS), *matK* and *trnL* refers to the sequence information of (Li et al., 2015) and the detailed information is listed in Supplemental Table 2. *Arethusa bulbosa* was used as outgroup. The analyses were performed using the maximum likelihood (ML) criterion implemented in MEGA-X.

Conflicts were explored through visual examination of resulting ML trees and comparison of nodes with >70% bootstrap support.

Statistical analysis. Analysis of variance (ANOVA) and Student’s *t* test at 5% probability level were conducted through SPSS software (SigmaStat for Windows Version 20.1; SPSS Inc., Richmond, CA). Duncan’s multiple-range test was also used to identify the significant differences among the means via the statistical program.

Fig. 2. Metaphase chromosomes of *Bletilla striata* representative samples obtained from 13 populations in China. S1–S13: All samples were diploid with $2n = 2x = 32$; S1 to S13 display GSCX, AHBZ, GXHZ, GZGY, HNLS, JSNJ, SXXX, YNBS, HNLC, CQJF, ZJAJ, HBYL-14, and HBLC-21 in order. S14–S15: Triploid samples of HBYL and HBLC populations with $2n = 3x = 48$ (HBYL-65 and HBLC-15). Arrows represent satellites. Scale bar: 5 μm .

Results and Discussion

DNA ploidy level and chromosome counts.

The DNA ploidy levels of 1082 individuals from 62 locations by FCM and the Cx nuclear DNA contents of all populations are shown in Supplemental Table 1. The Cx-value was defined as the DNA content of a monoploid genome with chromosome number x (Greilhuber et al., 2005). Results showed that all the populations of *B. striata* had 2 Cx nuclear DNA content except for three populations in Western Hubei (HBYL, HBLC, and HBXE), which showed two DNA ploidy levels, and 5.8%, 18.3%, and 6.3% of individuals in these populations, respectively, had 3 Cx nuclear DNA content. For *B. ochracea* and *B. formosana*, all individuals had 2 Cx nuclear DNA content, and no tetraploid or higher ploidy was found among the *Bletilla* samples.

Chromosome counting of representative individuals from 25 populations confirmed that most populations of *B. striata* were diploid with $2n = 2x = 32$, whereas small proportions of individuals were triploids ($2n = 3x = 48$) from HBYL and HBLC populations (Figs. 2 and 4A). Individuals of the HBXE population demonstrated dysploidy $2n = 2x = 34$, and 6.3% individuals were triploid ($2n = 3x = 51$). Dysploidy was found in a small number of individuals ($2n = 2x = 36$) from SCLS and HNSZ populations (Figs. 3 and 4C). *B. ochracea* and *B. formosana* were all diploid with $2n = 2x = 34$ and $2n = 2x = 36$, respectively (Figs. 3, 4B, and 4D). *Bletilla* had moderate chromosomal variation levels, with basic chromosome numbers of $x = 16$, 17, and 18. *B. striata* showed more chromosome diversity than other species.

Karyotype. Metaphase chromosome morphologies of the 25 populations are shown in Figs. 2–4, and their karyotype formulae and related parameters are listed in Table 1. All chromosomes of *Bletilla* were small with lengths ranging from 1.25 μm to 4.93 μm in *B. ochracea*. Their chromosomes consisted of three types, including metacentric (m), submetacentric (sm), and subtelocentric (st). HCL ranged from 30.36 μm to 43.18 μm in *B. striata*. All individuals of *B. striata* possessed m and sm chromosomes with percentages of 88% and 10%, respectively, and three of these individuals exhibited m, sm, and st chromosomes (Table 1). The karyotypes of *B. striata* were mainly characterized by $2n = 2x = 32 = 24m + 8sm$ and $2n = 2x = 32 = 24m + 6sm + 2st$. For individuals demonstrating dysploidy, karyotypes were characterized by $2n = 2x = 34 = 24m + 10sm$ and $2n = 2x = 36 = 28m + 8sm$. For *B. ochracea* and *B. formosana*, their karyotypes were mainly characterized by $2n = 2x = 34 = 24m + 10sm$ and $2n = 2x = 36 = 24m + 12sm$. There was one pair of satellites located on the short arms of two *B. ochracea* and two *B. striata* (Figs. 3 O3, 3 O4, 3 D3, and 2 S10).

Regarding interchromosomal asymmetry (A_2), the most symmetric karyotype was found in *B. formosana* and *B. ochracea* ($A_2 = 0.07$), whereas the most asymmetrical was found in *B. striata* ($A_2 = 0.11$; Table 1). For

Fig. 3. Metaphase chromosomes of other samples obtained from another 13 populations. O1–O8: All the *Bletilla ochracea* samples were diploid with $2n = 2x = 34$ (CQLF, HBDJ, HBXJ, GSWX, HBJS, SXNT, HNLS, and HBWH). D1–D2: dysploidy samples and their triploids with $2n = 2x = 34$, $2n = 3x = 51$ (HBXE-10 and HBXE-20); D3–D4: dysploidy $2n = 2x = 36$ (SCLS, HNSZ). F1: *Bletilla formosana* $2n = 2x = 36$ (YNNE). Arrows represent satellites. Scale bar: 5 μm .

intrachromosomal asymmetry indexes, AsK% and A_1 showed that *B. striata* had the minimum mean values (0.61 and 0.35), whereas the maximum mean values (0.62 and 0.39) were found in *B. ochracea*. Analysis of karyotype symmetry showed that *B. striata* had the maximum Syi mean value (65.18), and the minimum mean value (62.06) was found in *B. ochracea*. In addition, the AsK%, A_1 and Syi karyotype parameters of *B. formosana* were 0.61, 0.36, and 64.10, respectively. The most symmetric karyotype was found in *B. striata* and the most asymmetric karyotype was found in *B. ochracea* (Fig. 5, Table 1).

Nuclear DNA content. Nuclear DNA contents were determined for the 25 selected populations (Fig. 6A, Table 1). The mean 1Cx (monoploid genome size) nuclear DNA content of *B. striata* was 2.94 pg. *B. striata* had a diploid cytotype ($2n = 2x = 32$) and a triploid ($2n = 3x = 48$) cytotype, with mean 1Cx nuclear DNA contents of 2.94 pg and

2.91 pg, respectively. The nuclear DNA contents within the diploid cytotype of *B. striata* varied from 2.82 to 3.11 pg, whereas the variation in the triploid cytotype was from 2.77 to 2.99 pg. The variation of the mean 1Cx nuclear DNA content in triploid samples was smaller than that in diploid samples but not significant. For the dysploidy of *B. striata*, the mean 1Cx nuclear DNA contents of its diploid cytotype ($2n = 2x = 34$) and triploid ($2n = 3x = 51$) cytotype were 2.97 pg and 3.21 pg, respectively. The highest mean 1Cx of *B. ochracea* ($2n = 2x = 34$) with nuclear DNA content of 3.33 pg was determined, which varied by 1.14-fold among different populations (from 3.16 to 3.59 pg). For *B. formosana* ($2n = 2x = 36$), the mean 1Cx nuclear DNA content was 3.01 pg. The cv ranged from 2.11% to 4.13%.

The ANOVA and Student's t test showed that there were significant differences in nuclear DNA content between *B. ochracea* and *B. striata*,

Fig. 4. Karyotypes of wild *Bletilla* samples from 25 populations in China. (A) *Bletilla striata* $2n = 2x = 32$, $2n = 3x = 48$. (B) *Bletilla ochracea* $2n = 2x = 34$. (C) Dysploidy of *B. striata* $2n = 2x = 34$ and $2n = 3x = 51$, $2n = 2x = 36$. (D) *Bletilla formosana* $2n = 2x = 36$. There were two satellites on the short arms of chromosome 2 of *B. striata* (codes AS10 and CD3). For *B. ochracea*, the two satellites are located on the short arms of chromosome 3 and chromosome 1 of codes BO3 and BO4, respectively. Scale bar: 5 μ m.

and *B. ochracea* and *B. formosana* ($P < 0.05$), whereas the difference between *B. striata* and *B. formosana* was not significant (Fig. 6B, Table 1).

Relation between genome sizes and karyological characteristics. No obvious correlation between monoploid genome sizes (1Cx)

Fig. 5. Intrachromosome asymmetry values obtained by karyotype asymmetry index (AsK%) (blue), Syi (red), and A1 (green) indexes for *Bletilla*. The numeric scale at the right side indicates the mean value for the four indexes. The Syi value was divided by 100. Species indicated by the same letters are not significantly different (Duncan test, $P < 0.05$).

vs. ploidy levels and chromosome numbers was showed by a linear regression model. However, a positive correlation ($r = 0.49$; $P = 0.013$, $F = 0.013$) between 1Cx and HCL in *Bletilla* (Fig. 7A) and between 1Cx and AsK% ($r = 0.58$; $P = 0.0014$, $F = 0.0014$) was found (Fig. 7B).

Evolution of chromosome numbers and genome size. Phylogenetic analyses based on ITS, *matK* and *trnL* were made following Li et al. (2015). The strict consensus tree from parsimony ratchet analysis with bootstrap support values for individual branches is shown in Fig. 8. Combining these chromosome numbers and genome size data with those from the literature and superimposing them on the phylogenetic tree (Fig. 8) showed that most of the species with counts were diploid with different chromosome numbers (i.e., *B. ochracea*, $2n = 2x = 34$; *B. formosana*, $2n = 2x = 36$). In addition to the diploids ($2n = 2x = 32$), the triploid ($2n = 3x = 48, 51$) and dysploidy ($2n = 34, 36$) arisen by chromosome fission were detected in the *B. striata*.

B. ochracea showed an increase in 2C DNA content with an increase in the chromosome number, whereas *B. formosana* with the largest chromosome number had a relatively small 2C value ($2C = 6.02$). An obvious correlation between the chromosome number and nuclear DNA content could not be drawn from the present investigation.

Our results supported the earlier views that *B. striata* mainly has a karyotype of $2n = 2x = 32$, and *B. formosana* mainly has a karyotype of $2n = 2x = 36$ (Figs. 2 and 3, Table 1) (Felix and Guerra, 2010; Li and Chen, 1987; Li et al., 1992; Tan, 1969; Tanaka, 1971), but we did not find the previously reported chromosome numbers of *B. striata* ($2n = 64$ and 76) (Felix and Guerra, 2010; Li and Chen, 1987; Li et al., 1992). Karyotype data for *B. ochracea* ($2n = 2x = 34$) was reported for the first time in the present study. All the samples of *B. ochracea* and *B. formosana* were diploid, which may be due to the small num-

Fig. 6. Estimation of genomic size in the genus *Bletilla*. (A) DNA histogram showing G0/G1 peaks resulting from simultaneous processing and analysis of nuclear suspensions of tissue from young leaves stained with propidium iodide. *Bletilla striata* ($2C = 5.88$ pg), *Bletilla ochracea* ($2C = 6.66$ pg), and *Bletilla formosana* ($2C = 6.03$ pg). *Solanum lycopersicum* L. ($2C = 1.96$ pg, marked with triangles) was used as an internal standard. (B) Box plot of $1Cx$ values (pg) of *Bletilla*. Genome size estimate range for each species; asterisks indicate that there is a significant difference (least significant difference test, $P < 0.05$). The ends of the horizontal lines indicate the minimum and maximum data values. Circles indicate outliers. The mean $1Cx$ nuclear DNA contents of *B. striata*, *B. ochracea*, and *B. formosana* are 2.94, 3.33, and 3.01 pg, respectively. The right side corresponds to the flower characteristics of each species.

bers of samples collected. *B. striata* showed a relatively high chromosome number diversity, which is consistent with the view that the species characterizes by a wide geographic

distribution and shows greater karyomorphological variation than species with restricted distributions (Oliveira et al., 2010; Turco et al., 2018).

In nature, odd polyploidy usually originates from fusion of unreduced gametes with regularly reduced gametes and is maintained by apomictic or vegetative propagation (Faehrich et al., 2019; Ramsey and Schemske, 1998; Wang et al., 2018). The capability of *Bletilla* to multiply asexually through rhizomes would enhance the likelihood that a low frequency ($\approx 5.8\%$ to 18.3%) of triploids originates and is maintained in natural populations. Polyploidization, a widespread phenomenon among plants, is considered a major speciation mechanism (Kohler et al., 2010). In particular, autotetraploids may originate from matings involving viable triploids (unilateral polyploidization), which in turn results from the fusion of n and $2n$ gametes produced by diploids (triploid bridge hypothesis) (Harlan, 1975; Kovalsky et al., 2018). Our discovery of considerable proportion of triploid plants in the diploid population implied that unilateral polyploidization through a triploid bridge

Fig. 7. Relationship between genome size ($1Cx$) and karyotype features, namely, (A) chromosome total haploid length (HCL) and (B) karyotype asymmetry index (AsK%).

Fig. 8. A phylogenetic maximum likelihood (ML) tree constructed using MEGA-X based on the internal transcribed spacer (ITS), *matK* and *trnL* sequence of four *Bletilla* species including *Arethusa bulbosa* as an outgroup. Numbers on the branches indicate bootstrap. The chromosome and genome size of the corresponding species are marked on the left side of the phylogenetic tree. Chromosome numbers from Felix and Guerra (2010) are marked with a rectangle and the others obtained in this study. *Indicates no chromosomal and genomic data have been obtained, currently.

might be an alternative mechanism of polyploid formation in complexes, which was supported by our observation that triploids of *B. striata* are not completely sterile. In addition, all the detected triploids, dysploids, and mixed-distribution populations of *B. striata* and *B. ochracea* were mainly distributed in southeast Sichuan, northwest Hunan, and southwest Hubei, which are the main areas of the important biodiversity center of east Sichuan–west Hubei. The results showed that this area of east Sichuan–west Hubei is likely the genetic diversity center of *Bletilla*.

The variations in chromosome numbers also reflected frequent dysploidy events detected in Orchidaceae (Felix and Guerra, 2010; Mandakova and Lysak, 2018; Moraes et al., 2017). Dysploids with $2n = 2x = 36$ and $2n = 2x = 34$ in *B. striata* were found in this study. Dysploidy is an increase or decrease in the chromosome number as a result of structural rearrangements (Shan et al., 2003; Yang et al., 2014; Yeh et al., 2017). Our information on the nuclear DNA contents of *B. striata* showed that the 2C values in populations with dysploidy ($2n = 2x = 34$; $2n = 2x = 36$) were not significantly different from those in other *B. striata* populations with $2n = 2x = 32$ (Table 1). Indeed, dysploidy events are considered the most important chromosome evolutionary mechanism in Orchidaceae (Grabiele et al., 2013; Moraes et al., 2017), and the increase from $2n = 32$ to $2n = 36$ in *B. striata* was consistent with this hypothesis. Therefore, to fully support polyploidy and dysploidy as important evolutionary mechanisms in the genus *Bletilla*, chromosome banding techniques and/or in situ hybridization need to be used, which will enable particular chromosome rearrangements to be tracked. A moderate level of chromosomal variation of the genus *Bletilla* was shown in our article, with basic chromosome numbers of $x = 16, 17$, and 18 , and further determination of the original basic number will be very meaningful for exploring

the ancestral types, evolutionary directions, and pathways of *Bletilla*.

This study revealed nuclear DNA content values for three species of *Bletilla*, especially first time for *B. ochracea* and *B. formosana*. *B. striata*, with 1Cx-values of 2.94 pg ($2n = 2x = 32$), showed obviously smaller nuclear DNA than *B. ochracea* ($2n = 2x = 34$), with 1Cx-values of 3.33 pg, and *B. formosana* ($2n = 2x = 36$), with 1Cx-values of 3.01 pg (Table 1, Fig. 6). Despite substantial karyotypic differences and base chromosome number variation, there was no significant difference in genome sizes between *B. striata* and *B. formosana*, which proved that the genome size is not necessarily positively correlated with the number of chromosomes (Leitch et al., 2009). Previous studies have found that a strong correlation exists between the 1Cx value and HCLs in many plants (Levin, 2002). In this study, a positive correlation ($r = 0.56$; $P = 0.0048$) was identified between 1Cx and HCL in *Bletilla* (Fig. 7A), and the positive correlation ($r = 0.58$; $P = 0.0014$) found between 1Cx and AsK% indicated that additional DNA was mainly added to the long arm in *Bletilla* (Fig. 7B) (Du et al., 2017; Peruzzi et al., 2009).

The production of a phylogeny provides a useful framework to examine the evolution of genome size and chromosome number in *Bletilla*. The different karyotype characters observed in *Bletilla* were mapped directly onto the molecular phylogenetic tree proposed for this genus (Li et al., 2015) (Fig. 8). The genus showed a variation in chromosome number from $2n = 32$ to 76 and genome size from $1C = 2.94$ pg to 3.33 pg. Based on the reported chromosome number variation, a basic chromosome number of $x = 16$ has been suggested for genus *Bletilla* (Felix and Guerra, 2010). The ascension from $2n = 32$ to $2n = 36$ in the genus *Bletilla* is consistent with this previous research that dysploidy has been suggested to be the main cause of chromosome number variation in Orchidaceae (Felix and Guerra, 2010). In addition, two

different mechanisms of dysploidy ($2n = 34, 36$) and polyploidy ($2n = 48, 51, 64, 76$) were found in the *B. striata*. In *Bletilla*, the variation of chromosome number is predominantly a result of dysploidy, and polyploidy also plays a role in its chromosomal evolution. In summary, our research proved that dysploidy and polyploidy was involved in the diversity generation of the chromosome numbers, and there was no clear correlation between chromosome numbers and genome size.

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Supplemental Table 1. Detailed location information and ploidy levels of the *Bletilla* plants collected from China.

Population code	Species	Locality	Latitude	Longitude	Ploidy levels	Voucher
GSCX	<i>B. striata</i>	Chengxian County, GanSu Province	33.848066	105.680743	2x	Y. He 025 (HIB)
GZGY		Guiyang City, GuiZhou Province	26.627329	106.725522	2x	L. Qing 032 (HIB)
GZXZ		Xiazi Town, Zunyi City, GuiZhou Province	27.688086	107.187981	2x	L. Qing 033 (HIB)
GZZY		Zunyi City, GuiZhou Province	27.725440	106.927208	2x	L. Qing 034 (HIB)
AHBZ		Bozhou City, Anhui Province	30.278632	117.298478	2x	Y. He 035 (HIB)
ZJAJ		Anji County, Zhejiang Province	30.500504	119.525626	2x	Y. He 036 (HIB)
JSNJ		Nanjing City, Jiangsu Province	32.052702	118.834225	2x	Y. He 037 (HIB)
SHBG		Shanghai Botanical garden, Shanghai	31.146156	121.443803	2x	L. Qing 038 (HIB)
HNSC		Shangcheng Town, Henan Province	31.623593	115.330269	2x	Y. He 039 (HIB)
JXWZ		Wanzai County, Jiangxi Province	27.828233	114.400038	2x	L. Qing 040 (HIB)
JXYZ		Yuanzhou County, Jiangxi Province	28.000965	114.019911	2x	L. Qing 041 (HIB)
JXFY		Fenyi County, Jiangxi Province	27.055761	114.037439	2x	L. Qing 042 (HIB)
JXSG		Shanggao County, Jiangxi Province	28.242334	114.942889	2x	L. Qing 043 (HIB)
JXTG		Tonggu County, Jiangxi Province	28.523902	114.365817	2x	L. Qing 044 (HIB)
JXYF		Yifen County, Jiangxi Province	28.398564	114.835871	2x	L. Qing 045 (HIB)
CQJF		Jinfo Mountain, Chongqing Municipality	29.043092	107.135798	2x	Y. He 046 (HIB)
SCXJ		Xiangjiaping County, Sichuan Province	32.727511	108.743623	2x	Y. He 047 (HIB)
SCSF		Shuangfu Town, Leshan, Sichuan Province	29.403512	103.292725	2x	L. Qing 048 (HIB)
SCLS		Leshan County, Sichuan Province	29.670529	103.493227	2x	L. Qing 049 (HIB)
GXLY		Leye County, Guangxi Province	24.362146	106.281023	2x	Y. He 057 (HIB)
GXLL		Longlin County, Guangxi Province	24.770659	105.343881	2x	Y. He 058 (HIB)
GXHZ		Hezhou City, Guangxi Province	24.403474	111.566551	2x	Y. He 059 (HIB)
GXXJ		Jinxi, Guangxi Province	22.871872	106.327213	2x	Y. He 060 (HIB)
YNDQ		Diqing, Yunnan Province	27.189219	100.113174	2x	L. Qing 061 (HIB)
YNBS		Baoshan City, Yunnan Province	25.112043	99.1618010	2x	L. Qing 062 (HIB)
HBLC		Lichuan County, Hubei Province	29.581638	108.59585	2x, 3x	Y. He 001 (HIB)
HBXE		Xuanen County, Hubei Province	30.013261	109.043137	2x, 3x	Y. He 002 (HIB)
HBZG		Zigui County, Hubei Province	30.827845	110.938533	2x	Y. He 003 (HIB)
HBYL		Yiling District, Hubei Province	31.381432	111.129463	2x, 3x	L. Qing 004 (HIB)
HBSB		Songbai Town, Shenlongjia, Hubei Province	31.740776	110.678701	2x	L. Qing 005 (HIB)
HBFC		Fang County, Hubei Province	31.837410	110.605121	2x	Y. He 006 (HIB)
HBJM		Jingmen City, Hubei Province	31.035476	112.199462	2x	Y. He 007 (HIB)
HBXG		Xiaogan City, Hubei Province	30.924826	113.916436	2x	L. Qing 008 (HIB)
HBHP		Huangpi, Wuhan, Hubei Province	31.103821	114.395311	2x	Y. He 009 (HIB)
HBZS		Zhangshan Village, Hongan County, Hubei	31.247068	114.639631	2x	L. Qing 010 (HIB)
HBXJ		Xiangjiaping Village, Hongan County, Hubei	31.537247	114.538459	2x	Y. He 011 (HIB)
HBMC		Macheng County, Hubei Province	31.173319	115.008664	2x	L. Qing 012 (HIB)
HBLT		Luotian County, Hubei Province	31.097299	115.740163	2x	L. Qing 013 (HIB)
HBYS		Yingshan County, Hubei Province	30.989324	116.040803	2x	Y. He 014 (HIB)
GSWX	<i>B. striata</i> and <i>B. ochracea</i>	Wen County, Gansu Province	32.749451	105.246421	2x	Y. He 026 (HIB)
GSHX		Hui County, Gansu Province	33.697112	106.200907	2x	Y. He 027 (HIB)
HNCL		Cili County, Hunan Province	29.429720	111.139219	2x	L. Qing 051 (HIB)
HNSZ		Sangzhi County, Hunan Province	29.3997242	110.164288	2x	L. Qing 052 (HIB)
HNLS		Longshan County, Hunan Province	29.4576748	109.443939	2x	Y. He 053 (HIB)
HBJS		Jianshi County, Hubei Province	30.399723	110.005104	2x	L. Qing 015 (HIB)
HBXH		Xinhua Town, Shenlongjia, Hubei Province	31.525285	110.860645	2x	Y. He 016 (HIB)
HBHF		Hefeng County, Hubei Province	30.186543	110.198615	2x	L. Qing 017 (HIB)
HBWF		Wufeng County, Hubei Province	30.454826	110.324350	2x	L. Qing 018 (HIB)
HBXS		Xingshan County, Hubei Province	31.344837	110.508185	2x	L. Qing 019 (HIB)
HBZS		Zhushan County, Shiyan, Hubei Province	32.224692	110.228794	2x	L. Qing 020 (HIB)
HBMJ		Maojian County, Shiyan, Hubei Province	32.525404	110.752812	2x	L. Qing 021 (HIB)
SXXX		Micang Mount, Xixiang, Shanxi Province	32.335321	107.271221	2x	Y. He 054 (HIB)
HBWH	<i>B. ochracea</i>	Wuhan City, Hubei Province	30.545334	114.423711	2x	Y. He 022 (HIB)
HBDJ		Danjiangkou City, Hubei Province	32.748276	111.179125	2x	L. Qing 023 (HIB)
HBXJ		Xijiadian Town, Hubei Province	32.801606	111.196174	2x	L. Qing 024 (HIB)
SXNT		Niutoudian Town, Shanxi Province	32.525468	109.327434	2x	Y. He 055 (HIB)
CQLF		Lianfeng Village, Chengkou, Chongqing	32.412354	108.438462	2x	L. Qing 050 (HIB)
GSLC		Li County, Gansu Province	33.8520781	104.782628	2x	Y. He 028 (HIB)
GSXL		Xiaolong Mountain, Gansu Province	34.2430017	106.409681	2x	Y. He 029 (HIB)
GSXH		Xihe County, Gansu Province	33.7217471	105.340664	2x	Y. He 030 (HIB)
GSKX		Kang County, Gansu Province	33.0977697	105.792183	2x	Y. He 031 (HIB)
YNNE		Ninger County, Yunnan Province	22.7914421	100.988515	2x	L. Qing 056 (HIB)
	<i>B. formosana</i>					

HIB = herbarium of Wuhan Botanical Garden, Chinese Academy of Sciences.

Supplemental Table 2. A list of species sampled, vouchers and GenBank accession number. Markers noted ##### are sequences not available.

Species name	Vouchers	ITS	<i>matK</i>	<i>trnL</i>
<i>B. striata</i> (Thunb.) Rechb. f. –1	Chase O-556 (K)	AF461466	AF263630	AF519939
<i>B. striata</i> (Thunb.) Rechb. f. –2	KFBG3167	KY966421	KY966713	####
<i>B. ochracea</i> Schltr. –1	BS02MT02	MH520969	####	####
<i>B. ochracea</i> Schltr. –2	L. Li 06 (IBSC)	KR857328	KR857335	KR857342
<i>B. sinensis</i> (Rolfe) Schltr. –1	BSMZ02 (NJNU)	KP866835	####	####
<i>B. sinensis</i> (Rolfe) Schltr. –2	BSMZ03 (NJNU)	KP866836	####	####
<i>B. formosana</i> (Hayata) Schltr. –1	AHBZ03 (NJNU)	KF698629	####	####
<i>B. formosana</i> (Hayata) Schltr. –2	BFBZ01 (NJNU)	KP866832	####	####
<i>Listera smallii</i> Wiegand	Cameron 1001 (NCU)	AF521058	AF263668	AF519920

ITS = internal transcribed spacer.