

# 1 Karyotype Description and Comparative

## 2 Chromosomal Mapping of 5S rDNA in 21 species

3 Running title: Karyotyping of 64 Plants by FISH-5S rDNA

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14

15 **Abstract:** 5S rDNA is essential component of all cell types. A survey was conducted to study the 5S  
16 rDNA site number, position, and origin of signal pattern diversity in 64 plants using fluorescence *in situ*  
17 hybridization. The species used for this experiment were chosen due to the discovery of karyotype  
18 rearrangement, and for species checked in which 5S rDNA has not yet been explored. The  
19 chromosome number was 14–160, while the chromosome length was 0.63–6.88  $\mu\text{m}$ , with 37 plants  
20 (58%) had small chromosome ( $<3 \mu\text{m}$ ). The chromosome numbers of three species and 5S rDNA loci of  
21 19 species have been reported for the first time. 5S rDNA was varied and abundant in signal site  
22 number (2–18), position (e.g., interstitial, distal, and proximal position, occasionally, outside  
23 chromosome), and even as intense (e.g., strong, weak, and slight). Our results exposed modifiability in  
24 the number and location of 5S rDNA in 33 plants and demonstrated an extensive stable number and  
25 location of 5S rDNA in 31 plants. The potential origin of signal pattern diversity was probably caused  
26 by chromosome rearrangement (e.g., deletion, duplication, inversion, translocation), polyploidization,  
27 self-incompatibility, and chromosome satellites. These data characterized the variability of 5S rDNA  
28 within the karyotypes of the 64 plants that exhibited massive chromosomal rearrangements and  
29 provided anchor points for genetic physical maps. These data prove the utility of the 5S rDNA  
30 oligonucleotide as a chromosome marker in identifying plant chromosomes. This method provides a  
31 basis for developing similar applications for cytogenetic analysis in other species.

32

33 **Key words:** Chromosomes; Conservation; Diversity; FISH Oligonucleotides probes

34

35 **Article Summary**

36 A survey was exploited to study 5S rDNA signal pattern diversity in 64 plants by FISH. The  
37 chromosome number of three species and 5S rDNA loci of 19 species have been reported by the first  
38 time. 5S rDNA was really rather various and abundant in signal site number (2–18), position (interstitial,  
39 distal, proximal position, occasionally, outside chromosome), even as the intense (strong, weak, slight).  
40 These data prove the utility of 5S rDNA oligonucleotide as chromosome marker in identifying plant  
41 chromosomes.

42

## 43 Introduction

44 Ribosomal DNA (rDNA) is essential to all cell types that code rRNA. rDNAs are generally detected by exercising  
45 considerable parts of chromosomes, including both 45S and 5S rDNA (Slidler 2016). The 5S rDNA structured  
46 numerous copies in genomes and has been far and widely used in hundreds of cytogenetic investigations as an  
47 important marker for fluorescence *in situ* hybridization (FISH) analysis (Heslop-Harrison 2000, Rodríguez-González  
48 et al. 2023), not only for essential crops (Kamisugi et al. 1994, Luo et al. 2018a, b, c), but also for woody plants, such  
49 as walnut (Luo and Chen 2020), the Chinese pepper (Luo et al. 2018d, He et al. 2023, Hu et al. 2023), and wintersweet  
50 (Luo and Chen 2019).

51 The lengths of the 5S rDNA sequence ranged from 48 bp (Turner et al. 2005) to 854 bp (Liu et al. 2017) because of  
52 its high copies and variations when searching for “5S rDNA” in the database Nucleotide of National Center for  
53 Biotechnology Information. Furthermore, the lengths of 5S rDNA as a FISH probe in the PubMed database from  
54 NCBI varied considerably: 41 bp (Luo et al. 2017, Islam-Faridi et al. 2020), 94 bp (Sergeeva et al. 2017), 117 bp  
55 (Lukjanová et al. 2023), 120 bp (Taketa et al. 2001, Symonová et al. 2017, Deon et al. 2022, de Moraes et al. 2023), 124  
56 bp (Waminal et al. 2018), 131 bp (Sergeeva et al. 2017), 222 bp (Glugoski et al. 2018), 285 bp (Röser et al. 2001), 300  
57 bp (Araya-Jaime et al. 2022), 302 bp (Zhang et al. 2016), 303 bp (Mahelka et al. 2013), 320 bp (Khensuwan et al. 2023),  
58 324 bp (Kamisugi et al. 1994), 326 bp (Robledo and Seijo 2008), 329 bp (Röser et al. 2001), 347 bp, ~400 bp (Pedrosa  
59 et al. 2002), 410 bp (Kovács et al. 2023), 456 bp (Röser et al. 2001), 468 bp, 473 bp, 477 bp, 496 bp (Martins et al.  
60 2000), 497 bp (Alexandrov et al. 2022), 498 bp (Martins et al. 2000), 500 bp (de Barros et al. 2023), 556 bp  
61 (Gottlob-McHugh et al. 1990), 596 bp (Joshi et al. 2023), 702 bp (Glugoski et al. 2018), 871 bp (Amarasinghe and  
62 Carlson 1988), and 1193 bp (Glugoski et al. 2020).

63 Conversely, 5S rDNA FISH signal sites in previous research ranged from 1 to 71 (Rodríguez-González et al. 2023).  
64 Quite of species conserve 5S rDNA, including only two stable chromosomes with two 5S rDNA signal sites (Zhang et  
65 al. 2016, Moraes et al. 2022, Yurkevich et al. 2022, Alexandrov et al. 2022, Khensuwan et al. 2023). Nevertheless, a few  
66 species occupy visible 5S rDNA FISH signals, including the numbers of both major and visible dispersed sites. For  
67 example, four 5S rDNA signal sites in six in *Sinapidendron frutescens* (Aiton) Lowe (Ali et al. 2005), eight in *Prunus*  
68 *pseudocerasus* (Lindl.) G. Don (Wang et al. 2022), 8-19 in *Trifolium medium* L. (Lukjanová et al. 2023), 10 in *Brassica*  
69 *juncea* (Linnaeus) Czernajew, 12 in *Olimarabidopsis cabulica* (J. D. Hooker & Thomson) Al-Shehbaz et al., 14 in  
70 *Brassica napus* L. (Ali et al. 2005), 15 in *Paphiopedilum sukhakulii* Schoser & Senghas (Lan and Albert 2011), 16 in  
71 *Piptanthus concolor* Harrow ex Craib (Luo et al. 2017), 19 in *Paphiopedilum henryanum* Braem, 20 in *Paphiopedilum*  
72 *druryi* (Bedd.) Stein, 22 in *Paphiopedilum sangii* Braem, 23 in *Paphiopedilum tigrinum* Koop. et Haseg., 26 in  
73 *Paphiopedilum liemianum* Fowlie, 27 in *Paphiopedilum hirsutissimum* (Lindl. ex Hook.) Stein, 28 in *Paphiopedilum*  
74 *victoria-regina* (Sander) M.W.Wood, 29 in *Paphiopedilum primulinum* M. W. Wood et P. Taylor, 30 in *Paphiopedilum*  
75 *glanduliferum* (Blume) Stein, 32 in *Paphiopedilum adductum* Asher, 34 in *Paphiopedilum randsii* Fowlie, 36 in  
76 *Paphiopedilum parishii* (Rchb. F.) Stein, and 38 in *Paphiopedilum gigantifolium* Braem, M.L.Baker & C.O.Baker (Lan  
77 and Albert 2011). Notably, there were two 5S rDNA sites on the same chromosome, such as *P. concolor* (Luo et al.  
78 2017), *Brassica oleracea* L. (Ali et al. 2005), *O. cabulica* (Ali et al. 2005), *P. primulinum*, *P. liemianum*, *P. randsii*, *P.*  
79 *parishii*, *Paphiopedilum dianthum* T. Tang et F. T. Wang (Lan and Albert 2011), *Prunus cerasus* L. (Wang et al. 2022),  
80 and *Plantago maxima* Juss. ex Jacq. (Kovács et al. 2023).

81 Conversely, the 5S rDNA FISH signal position was diverse and abundant. The 5S rDNA signal has been found in  
82 the chromosome interstitial position from *Macroptilium bracteatum* (Nees & Mart.) Maréchal & Baudet (de Barros et  
83 al. 2023), *Deschampsia antarctica* E. Desv (Amosova et al. 2022), *Polemonium caeruleum* Linnaeus (Samatadze et al.  
84 2023), in the chromosome distal position from *Pinus koraiensis* Siebold et Zuccarini (Cai et al. 2006), *Cannabis sativa*  
85 L. (Alexandrov et al. 2022), *Pseudotsuga menziesii* (Mlrb.) Franco (Amarasinghe and Carlson 1988), or in the  
86 chromosome proximal position from *Brassica rapa* L. (Campomayor et al. 2021), even as far away the chromosome  
87 from *Hedysarum setigerum* Turcz. (Yurkevich et al. 2022), and *P. cerasus* (Wang et al. 2022).

88 As a consequence, the 5S rDNA as a FISH probe was an excellent marker to label plant chromosomes in species  
89 and to distinguish closely related species among more than 300 plant species (listed in Supplementary Table 1),  
90 including more than 20 woody plant species (bold type in Supplementary Table 1). For example, in six species of  
91 Fabaceae: *Amorpha fruticosa* L., *Styphnolobium japonicum* (L.) Schott, three species of *Robinia* L. (He et al. 2022a), *P.*  
92 *concolor* (Luo et al. 2017); in five *Pinus* L. species of Pinaceae (Cai et al. 2006); in four species of Oleaceae: *Fraxinus*  
93 *pennsylvanica* Marsh., two species of *Ligustrum* L., *Syringa oblata* Lindl. (Luo and Liu 2019); in two *Berberis* L. species  
94 of Berberidaceae (Liu and Luo 2019); in two species of Malvaceae: *Adansonia digitata* L. (Islam-Faridi et al. 2020),

95 *Hibiscus mutabilis* L. (Luo and He 2021); and in Rutaceae of *Zanthoxylum armatum* DC. (Luo et al. 2018d, He et al.  
96 2023).

97 However, the 5S rDNA as a FISH probe lacked discrimination (only stable two chromosomes with signal) in *Pistia*  
98 *stratiotes* L. of Araceae (Stepanenko et al. 2022); in *Chimonanthus campanulatus* R.H. Chang & C.S. Ding of  
99 Calycanthaceae (Luo and Chen 2019); in four *Citrullus* Schrad. species of Cucurbitaceae (Li et al. 2016); in  
100 cultural/wild *Hippophæ rhamnoides* ssp. *sinensis* and three *Hippophæ rhamnoides* L. cultivars of Elaeagnaceae (Luo  
101 et al. 2022a); in Fabaceae of six *Hedysarum* L. species (Yurkevich et al. 2022); in *Juglans regia* L. and *Juglans sigillata*  
102 Dode (Luo and Chen) of Juglandaceae; in four *Glechoma* L. species of Lamiaceae (Jang et al. 2016); in three *Bletilla*  
103 Rchb. f. species of Orchidaceae (Huan et al. 2022); in three *Zea* L. species of Poaceae (Han et al. 2003); in four *Citrus* L.  
104 species of Rutaceae (He et al. 2020), *Zanthoxylum bungeanum* Maxim. and *Z. armatum* (Hu et al. 2023); in four  
105 *Populus* L. species of Salicaceae (Xin et al. 2019); in five *Taxus* L. species of Taxaceae (He et al. 2022b).

106 Summarily, 5S rDNA has been confirmed to be a significant cytogenetic symbol by tandem formatting and giving  
107 in multicopy numbers with unusual chromosomal allocation. Moreover, these discoveries may afford a responsible  
108 method to survey the locations and number of rDNA diversifications among respective species and their relational  
109 accessions. Such findings also let us further understand the developmental and phylogenetic links of exemplified  
110 species. The patent oligonucleotide FISH (Oligo-FISH) technology is widely problematic in related cytogenetic  
111 investigations compared to conventional FISH analysis because of its low cost (Beliveau et al. 2012). Moreover, based  
112 on available DNA sequencing data, oligonucleotide probes (Oligo-probe) have been developed and successfully  
113 applied to many plants (He et al. 2020, Xin et al. 2020, Luo et al. 2022a, b, He et al. 2023). Therefore, examining the 5S  
114 rDNA sites by Oligo-FISH would efficiently contribute to further cytogenetic research on the 64 plants. This study  
115 aimed to analyze and compare the polymorphism of the signal pattern of 5S rDNA among species and genera in the  
116 64 plants examined, including 5S rDNA signal number, position, and intensity, and the chromosome number of each  
117 plant. Furthermore, several contributing factors caused by the diversity of the 5S rDNA signal pattern remain to be  
118 addressed.

119

## 120 **Materials and Methods**

121 The species used for this experiment were chosen due to the discovery of karyotype realignments (Luo et al. 2017,  
122 Luo et al. 2018d, Liu and Luo 2019, Luo and Liu 2019, Luo and Chen 2019, 2020, Luo and He 2021, Luo et al. 2022a, b,  
123 He et al. 2022a, b, c, 2023). Because these species were checked, their 5S rDNA has not yet been explored.  
124 Information on the seeds or seedlings of 64 plants (52 woody plants and 12 herbaceous plants belonging to 21  
125 species, 18 genera, and 16 families) used in the present work is provided in Table 1. All 64 plants were collected from  
126 23 counties or districts of six Chinese provinces.

127

### 128 **Chromosome and Oligonucleotide Probe Preparation**

129 Converged seeds were germinated in Petri dishes with moistened filter paper and stayed at 25°C in the daytime and  
130 at 18°C in the night until the roots touched ~2 cm in length. Next, the roots were cut. The collected seedlings were  
131 grown in soil at room temperature (15–25°C) until they had new roots and then cut again. The cut root tips were  
132 served with N<sub>2</sub>O (nitrous oxide) gas for 3–5 h, with processing time according to cell wall lignification and  
133 chromosome size. Afterward, the root tips were soaked in glacial acetic acid for 5 min and then in 75% ethyl alcohol.  
134 Chromosome preparation was executed based on the steps described by Luo et al. (2017). Since these procedures  
135 have been reported elsewhere, a brief description is available here. About 1 mm of the meristematic zone of the root  
136 tip (cut root cap) was in enzymolysis at 37°C for 45 min by using pectinase and cellulase (the buffer 50 mL was  
137 covered 0.4324 g citric acid + 0.5707 g trisodium citrate, then 1 mL buffer + 0.02 g pectinase + 0.04 g cellulase). The  
138 enzymes were yielded by Kyowa Chemical Products Co., Ltd. (Osaka, Japan) and Yakult Pharmaceutical Ind. Co., Ltd.  
139 (Tokyo, Japan). The enzymes were then blended into suspension for dropping onto clean slides. These slides were air  
140 dried at room temperature and examined using an Olympus CX23 microscope (Olympus, Tokyo, Japan).

141 The oligoprobe of 5' TCAGAACTCCGAAGTTAAGCGTGCTTGGGCGAGAGTAGTAC 3' (41 bp) was initially reported in  
142 *P. nepalensis* (*P. concolor*, former name in Flora of China) of Fabaceae (Luo et al. 2017). Then, it has been steadily  
143 executed in two *Berberis* species of Berberidaceae (Liu and Luo 2019), *C. campanulatus* of Calycanthaceae (Luo and

144 [Chen 2019](#)), and in cultural/wild *H. rhamnoides* ssp. *sinensis* and three *H. rhamnoides* cultivars of Elaeagnaceae ([Luo](#)  
145 [et al. 2022a](#)), in *A. fruticose* and *S. japonicum* of Fabaceae and three *Robinia* species ([He et al. 2022a](#)), two *Juglans*  
146 species of Juglandaceae ([Luo and Chen 2020](#)), *A. digitata* and *H. mutabilis* of Malvaceae ([Islam-Faridi et al. 2020](#), [Luo](#)  
147 [and He 2021](#)), *F. pennsylvanica* of Oleaceae, two species of *Ligustrum* and *S. oblata* ([Luo and Liu 2019](#)), three *Bletilla*  
148 species of Orchidaceae ([Huan et al. 2023](#)), two *Zanthoxylum* species of Rutaceae ([Luo et al. 2018](#), [He et al. 2023](#)), and  
149 five *Taxus* species of Taxaceae ([He et al. 2022b](#)). This oligoprobe was produced by Sangon Biotech Co., Ltd. (Shanghai,  
150 China) and conducted simultaneously in a single round of FISH. The oligo-probe was 50-labeled with  
151 6-carboxy-fluorescein (6-FAM; absorption /emission wavelengths 494 nm/518 nm; green).

152

### 153 FISH Hybridization

154 Slides with well-spread chromosomes were used to hybridize. Chromosome samples first experienced a series of  
155 reinforcement (4% paraformaldehyde, room temperature, 10 min), dehydration (75%, 95%, and 100% ethanol at  
156 room temperature for 5 min), denaturation (deionized formamide at 80°C for 2 min), and once again dehydration  
157 (75%, 95%, and 100% ethanol, at -20°C for 5 min), and then hybridization (0.375  $\mu$ L of 5S rDNA, 4.675  $\mu$ L of 2 $\times$  SSC,  
158 and 4.95  $\mu$ L of ddH<sub>2</sub>O in total 10  $\mu$ L hybridization mixture) for 2 h in an incubator at 37°C. Then, hybridized spreads  
159 were rinsed with 2 $\times$  SSC and ddH<sub>2</sub>O twice for 5 min at room temperature and air-dried. Finally, the spreads were  
160 counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA, USA) for 5 min,  
161 according to the step described by [Luo et al. \(2017\)](#). Chromosomes were traced using an Olympus BX-63 microscope  
162 (Olympus Corporation, Tokyo, Japan), and FISH photographs were taken using a DP-70 CCD camera allocated to the  
163 microscope.

164

### 165 Karyotype Analysis

166 Raw data were executed using the Photoshop CC 2015 (Adobe Systems Inc., San Jose, CA, USA) and DP Manager  
167 (Olympus Corporation, Tokyo, Japan) software. More than eight slides of each plant were observed, and at least 15  
168 well-spread cells were executed to count the chromosome number and length. All chromosomes examined were  
169 assembled from longest to shortest. The chromosome ratio was controlled by the length of the longest  
170 chromosome to that of the shortest chromosome. Exhaustive and deep karyotype analysis could not be conducted  
171 because of the obscure centromere position and small chromosome size of many of the species.

172

## 173 Results

### 174 Karyotype Analysis Revealed Differences among 64 Plants

175 We performed FISH analysis to visualize the chromosomal distribution of 5S rDNA in 64 plants, as shown in [Figure 1](#)  
176 [\(A1–A16\)](#), [Figure 2 \(B1–B16\)](#), [Figure 3 \(C1–C16\)](#), and [Figure 4 \(D1–D16\)](#). We cut each chromosome distribution of the  
177 5S rDNA in [Figures 1–4](#) and aligned them for display, as shown in [Figure 5 \(A1–A16\)](#), [Figure 6 \(B1–B16\)](#), [Figure 7 \(C1–](#)  
178 [C16\)](#), and [Figure 8 \(D1–D16\)](#). For 19 species from 13 families, this is the first time that 5S rDNA testing has been  
179 analyzed (A1, A8–A11, A13–A16, B11, B14, C12, C14–C16, D6–D12, and D14–D15).

180 The chromosome numbers and lengths for the considered species are sorted in [Table 2](#). The chromosome  
181 numbers in the 64 plants ranged from 14 (*C. chinensis*, A1) to 160 (*Z. bungeanum* ‘Hanyuanhuajiao’ 3, D11). Thirteen  
182 plants possessed 24 chromosomes (one-fifth), whereas another 13 plants possessed 34 chromosomes (one-fifth).  
183 The chromosome number of the three species was analyzed for the first time here (bold type in [Table 2](#)): *P. sibiricum*  
184 ( $2n = 18$ ), *I. chinensis* ( $2n = 40$ ), and *T. sebifera* ( $2n = 88$ ). The longest chromosome length of each plant ranged from  
185 1.23  $\mu$ m (*T. sebifera*, D6) to 6.88  $\mu$ m (*T.  $\times$  media*, B2; *T. aestivum*, D1). The shortest chromosome length of each plant  
186 ranged from 0.63  $\mu$ m (*J. sigillata* ‘Maerkang,’ B16) to 3.85  $\mu$ m (*T. aestivum*, D1). Thirty-seven plants (58%) had a  
187 chromosome length of less than 3  $\mu$ m, thus falling into the small chromosome category. Detailed and deep  
188 karyotype analysis was not conducted because of the unclear position of centromeres and the small size of  
189 chromosomes in many of the exemplified plants, such as long/short arm length and karyotype formula.

190 Karyotype asymmetry (Table 2) was assessed using the longest to shortest chromosome length ratio. The largest  
191 ratio was 4.61 in *P. sibiricum* (A10), while the smallest ratio was 0.74 in *R. pseudoacacia* 'Idaho' (A5). The ratio for 35  
192 plants ranged from 2 to 3 (55%), while that of 16 plants ranged from 1 to 2 (25%), and 10 plants ranged from 3 to 4  
193 (16%). The ratio was greater than 4 for two plants: *P. sibiricum* (A10) and *B. striata* f. 'Dujiangyan' (C4), while the ratio  
194 was less than 1 for *R. pseudoacacia* 'Idaho' (A5). These results indicate that abundant differences exist among 44 of  
195 the considered species.

196

## 197 The Diverse Signal Patterns of 5S rDNA Reveal the Complex Genome Architecture

198 Different types of ideograms for the 64 plants were drawn based on the FISH karyograms shown in Figures 5–8 to  
199 better investigate the diversity of 5S rDNA (Figures 9–12). The first diversity is signal location. Proximal signals were  
200 observed at several chromosomes in 39 plants (61%), while distal signals were observed at several chromosome  
201 terminus in 30 plants (47%). Interstitial signals were observed at several chromosomes in 21 plants (33%), while  
202 distal signals deviated from the chromosome in 4 plants (6%, the fourth class): *C. chinensis* (A1), *S. japonicum* (A6), *K.*  
203 *paniculata* (B14), and *Z. nitidum* (D7). The second diversity is the signal number. The largest number of  
204 chromosomes with a 5S rDNA signal was 18 in *T. sebifera* (D6), while the smallest number was 2 in 31 plants (48%).  
205 The number of chromosomes with a 5S rDNA signal for 11 plants ranged from 10 to 16 (17%), while that of 21 plants  
206 ranged from 4 to 8 (33%). The ratio of chromosomes with a 5S rDNA signal to total chromosome was assessed to  
207 signal cover. The largest ratio was 0.89 in *P. nepalensis* (A2), while the smallest ratio was 0.03 in *Z. nitidum* (D7) and *Z.*  
208 *armatum* 'Putaoqingjiao' (D16). The ratio for 50 plants ranged from 0.03 to 0.20 (78%), while the ratio for nine plants  
209 ranged from 0.20 to 0.50 (14%). The ratio for only two plants ranged from 0.50 to 0.89 in *R. pseudoacacia* (A3) and *T.*  
210 *wallichiana* var. *mairei* (B5).

211 Furthermore, we summarized the results in Figures 9–12 to produce the 5S rDNA signal pattern in Figure 13. The  
212 results for 64 plants belonging to 20 families are shown, including 10 plants in Rutaceae (gray), nine in Orchidaceae  
213 (light blue), eight in Fabaceae (cyan), six in Elaeagnaceae (light yellow), five in Taxaceae (light pink), four in  
214 Juglandaceae (green), four in Oleaceae (orange), two in Asparagaceae (yellow), two in Berberidaceae (blue), two in  
215 Euphorbiaceae (red), two in Malvaceae (magenta), two in Lauraceae (pink), and one in each of Aquifoliaceae,  
216 Calycanthaceae, Cupressaceae, Podocarpaceae, Fagaceae, Poaceae, Salicaceae, and Sapindaceae, respectively.  
217 Except for Aquifoliaceae, Asparagaceae, Calycanthaceae, Cupressaceae, Elaeagnaceae, Juglandaceae, Orchidaceae,  
218 Podocarpaceae, and Salicaceae, which each presented a single signal pattern type, the other families (Berberidaceae,  
219 Euphorbiaceae, Fagaceae, Fabaceae, Lauraceae, Malvaceae, Oleaceae, Poaceae, Rutaceae, Sapindaceae, and  
220 Taxaceae) all presented at least two sites.

221 As shown in Figure 13, there were six 5S rDNA signal pattern types in total: type I, chromosome includes the  
222 proximal signal location; Type II, chromosome includes the distal signal location; Type III, chromosome includes the  
223 proximal and distal signal locations; Type IV, chromosome only includes signal outside of the chromosome; Type V,  
224 chromosome includes the distal signal location and signal outside of the chromosome; and Type VI, satellite  
225 chromosome includes distal signal location. These types of signal patterns indicate that there is abundant diversity  
226 in the 5S rDNA signal arrangement.

227 All 64 plants had the 10 signal pattern types or type combinations shown in Figure 13. The 26 plants only  
228 possessed signal pattern type I; nine plants only possessed signal pattern type II; 16 plants had a combination of  
229 type I + type II; six plants only had type III; *R. pseudoacacia* f. *decaisneana* had a combination of type I + type III; *T.*  
230 *wallichiana* var. *mairei* had a combination of type I + type II + type III; *S. japonicum* only had type IV; *C. chinensis* and  
231 *K. bipinnata* had a combination of type II + type IV; *Z. nitidum* only had type V; and *P. nepalensis* had a combination  
232 of type I + type II + type III + type VI.

233 There were diverse signal patterns of 5S rDNA among 44 species, indicating a complex genome architecture. For  
234 example, considering (i) in Rutaceae, four varieties of *Zanthoxylum* had type I, but five varieties of *Zanthoxylum* had  
235 a combination of type I + type II, and *Z. nitidum* had type V. (ii) In Fabaceae, *A. fruticosa* and *E. crista-galli* had type I,  
236 but *R. pseudoacacia* and *R. pseudoacacia* 'Idaho' had type III, *R. pseudoacacia* f. *decaisneana* had combination of  
237 type I + type III, *S. japonicum* only had type IV, *C. chinensis* had a combination of type II + type IV, and *P. nepalensis*  
238 had a combination of type I + type II + type III + type VI. (iii) In Taxaceae, four species of *Taxus* had type III, but *T.*  
239 *wallichiana* var. *mairei* had a combination of type I + type II + type III. (iv) In Oleaceae, *F. pennsylvanica* had type II,  
240 but *L. lucidum*, *L. × vicaryi* and *S. oblata* had a combination of type I + type III. (v) In Berberidaceae, *B. diaphana* had  
241 type II, but *B. soulieana* had a combination of type I + type II. (vi) In Malvaceae, *F. simplex* had type I, but *H. mutabilis*

242 had a combination of type I + type II. (*vii*) In Lauraceae, *L. baviensis* had type I, but *L. baviensis* had a combination of  
243 type I + type II.

244

## 245 Proposed Origin of 5S rDNA Signal Diversity

246 Based on Figures 9–13, the proposed origin of the 5S rDNA signal diversity is illustrated in Figure 14. There are three  
247 major groups. (*i*) Signal number: signal number increases probably caused by the insertion of transposable elements,  
248 inversion, or translocation, and transposition events. Signal number decrease probably by elimination from the  
249 genome or conversion into pseudogenes and then loss, fusion event, and polyploidization followed by DNA  
250 sequence loss. (*ii*) Signal location on distal chromosome: polyploidization-related tendency toward the terminal  
251 location from an interstitial location. Signal location on proximal chromosome: interstitial or centromeric, a massive  
252 trend apparent in species with a single locus. The end signal deviation from the chromosome is probably caused by  
253 chromosome satellite, while transposon-mediated transpositional events and gene silencing probably cause end  
254 signal loss. (*iii*) Signal size: Signal size increase is probably caused by artificial selection, environmental induction,  
255 unequal crossing over recombination, illegitimate recombination, and duplication. The normal signal size indicates  
256 chromosome conservation. The signal size decrease is probably caused by self-incompatibility non-allelic  
257 homologous recombination. In brief, the signal number, location, and size variations were probably caused by  
258 chromosome rearrangement (deletion, duplication, inversion, and translocation), polyploidization,  
259 self-incompatibility, and chromosome satellites.

260

## 261 Discussion

### 262 Karyotype Analysis of the 64 Plants

263 Traditional karyotype analysis involves counting chromosome numbers, determining centromere location, and  
264 measuring chromosome length and long/short arm ratio. Based on this, the karyotype formula and cytotype are  
265 obtained to compare whether the species is evolving (Alberts et al. 2002). In this study, the chromosome numbers in  
266 the 64 plants ranged from  $2n = 14$  to 160. Most chromosome numbers are consistent with previous studies (Sun  
267 1996, Chen and Zhou 2005, Luo et al. 2017, Sergeeva et al. 2017, Liu and Luo 2019, Luo and Liu 2019, Wang et al.  
268 2019, He et al. 2022a, b, c, Yang et al. 2023). Only nine species had different chromosome numbers. In addition, the  
269 chromosome number of three species was the first time to be analyzed here: *P. sibiricum* ( $2n = 18$ ), *I. chinensis* ( $2n =$   
270  $40$ ), and *T. sebifera* ( $2n = 88$ ) in this study. More related information on *T. sebifera* was found in the chromosome  
271 number of the previous research. A stable chromosome number was found in the genus *Ilex*: *Ilex crenata* Thunb. ( $2n$   
272  $= 40$ ), *I. makinoi* ‘Hara’ ( $2n = 40$ ), *I. leucoclada* ( $2n = 40$ ), and *I. yunnanensis* var. *gentilis* Franch. ( $2n = 160$ ) (Geukens  
273 et al. 2023). Conversely, chromosome number varied greatly in four genera: *i*) *Polygonatum anhuiense* D. C. Zhang et  
274 J. Z. Shao ( $2n = 24$ ), *Polygonatum langyaense* D. C. Zhang et J. Z. Shao ( $2n = 18$ ), *Polygonatum odoratum* (Mill.)  
275 Druce ( $2n = 18$ ), *Polygonatum zanscianense* Pamp. ( $2n = 28$ ), *P. cyrtoneuma* ( $2n = 22$ ) (Sun 1996, Chen and Zhou  
276 2005); *ii*) *Zanthoxylum acanthopodium* Candolle ( $2n = 64$ ), *Zanthoxylum dimorphophyllum* Hemsley ( $2n = 36/68$ ),  
277 *Zanthoxylum scandens* Blume ( $2n = 68$ ), *Zanthoxylum oxyphyllum* Edgeworth ( $2n = 72$ ), *Zanthoxylum tomentellum*  
278 J.D. Hance ( $2n = 72$ ), *Zanthoxylum simulans* Hance ( $2n = \sim 132$ ), *Z. nitidum* ( $2n = 68$ ), *Z. armatum* ( $2n =$   
279  $66/98/128/132/136$ ), *Z. bungeanum* ( $2n = 132/136$ ) (Zhang and Hartley 2008, Chen et al. 2009, Yu et al. 2010, Luo et  
280 al. 2018d, Luo et al. 2022b, He et al. 2023, Hu et al. 2023); *iii*) *Bletilla formosana* ( $2n = 32/36$ ), *B. striata* ( $2n =$   
281  $32/34/36/48/51/64/76$ ), *B. ochracea* ( $2n = 34/36$ ) (Miduno 1954, He et al. 2022c, Huan et al. 2022, Yang et al. 2023);  
282 *iv*) *J. regia* and *J. sigillata* ( $2n = 34$ ) (Luo and Chen 2020), *Juglans* ( $2n = 32$ ) (Woodworth 1930, Hans 1970, Tulecke et  
283 al. 1988, Mu and Xi 1988, Mu et al. 1990). In this study, *P. cyrtoneuma* ( $2n = 18$ ), *Z. bungeanum* ( $2n = 76/134/136/160$ ),  
284 *Z. armatum* ( $2n = 96/100/102/104/132$ ), *Z. nitidum* ( $2n = 66$ ), *B. formosana*, *B. striata*, *B. ochracea*, *J. regia*, and *J.*  
285 *sigillata* (all  $2n = 34$ ). These results contradict previous studies. The possible causes of inconsistency may be *i*)  
286 improper chromosome count in the small and high chromosomes, *ii*) root lignification limiting their chromosome  
287 preparation, *iii*) hybridization between closely related species, *iv*) natural or artificial polyploidization, and *v*)  
288 apomixis (polyembryo).

289 Intrasppecific chromosome number variation, even as a population, has also been found in species such as  
290 *Cuscuta epithymum* (L.) L. and *Cuscuta planiflora* Ten. (García and Castroviejo, 2003). In this case, most variation is

291 attributable to auto- or allopolyploidy. The additional numbers can be explained by ascending or descending  
292 dysploidy. Thus, the accumulation of repetitive DNA can lead to an increase in chromosomes and, consequently, to  
293 an increase in genome size, especially in subgenus *Monogynella* (Ibiapino et al. 2022). In the present study,  
294 chromosome numbers varied in interspecific and intraspecific populations of the genus *Zanthoxylum*. The cause of  
295 the variation is probably similar to that of *Cuscuta* and *Monogynella*. Furthermore, the stable differentiation in the  
296 5S rDNA FISH pattern between the subgenera suggests that chromosomal rearrangements played a role in splitting  
297 the two subgenera. Rather than major structural changes, transpositional events are likely responsible for the  
298 variable rDNA distribution patterns among species of the same subgenus with conserved karyotypes (Cai et al.  
299 2006). *Zanthoxylum* genomes have complex chromosome rearrangements, such as chromosomal fission, reversal,  
300 and translocations (Hu et al. 2023). This result also further rationalizes the chromosome number variation in this  
301 genus. Chromosome polymorphisms within species in natural populations of vertebrates are far less common and  
302 are believed to be temporary transitions during chromosomal evolution (Damas et al. 2021, 2022). Likewise, the  
303 *Zanthoxylum* may be experiencing chromosomal evolution.

304 In this study of 64 plants evaluated, the longest chromosome length of each plant was 1.23–6.88  $\mu\text{m}$ , while the  
305 shortest chromosome length of each plant was 0.63–3.85  $\mu\text{m}$ , exhibiting strikingly different among the examined  
306 species. Previous research accumulated chromosome lengths of hundreds of plant species (Luo et al. 2022a, b, He et  
307 al. 2022a, b, d, Luo and He 2021, Liu and Luo 2019, Luo and Chen 2019, Luo et al. 2018d, Luo et al. 2017, Xing et al.  
308 1989, Liu and Sheng 2011). By analyzing these data, it is not difficult to find that chromosome length was slightly  
309 different, even for the same accession of the same species. For example, *R. pseudoacacia* 1.12–1.74  $\mu\text{m}$  (Luo et al.  
310 2022b), 0.94–1.67  $\mu\text{m}$  (He et al. 2022a). Nonetheless, chromosome length in the former two literatures was small for  
311 both chromosomes ( $<3 \mu\text{m}$ ). Hence, chromosome length was more suitable for qualitative than for quantitative  
312 analyses. Thirty-seven plant species analyzed (more than half) had chromosome lengths lower than 3  $\mu\text{m}$  in this  
313 study, consequently dividing them into the small chromosome rank. Because of the hazy centromere mark and tiny  
314 chromosomes in many plants investigated, the chromosome size was determined by metaphase and the  
315 measurement method. Still, a more delicate karyotype analysis (e.g., arm length, karyotype, and cytotype) was  
316 unavailable and limited.

317

### 318 Occurrence and Diversity of 5S rDNA in Plants

319 5S rDNA, which occurs in all cellular life forms, is a highly stable tandem repeat sequence that ubiquitously exists in  
320 plants (Said et al. 2018). With the evolution and development of the plant, 5S rDNA also underwent simultaneous  
321 changes. The length of 5S rDNA in the NCBI database Nucleotide of NCBI was 48–854 bp (Turner et al. 2005, Liu et al.  
322 2017), while its length as a FISH probe in the PubMed database of NCBI was 41–1193 bp (Luo et al. 2017, Islam-Faridi  
323 et al. 2020, Glugoski et al. 2020). This study is the first time 5S rDNA testing has been analyzed for 19 species from 13  
324 families. Overall, 5S rDNA occurred in at least two chromosomes in all 64 plants. With advances in science and  
325 technology, the occurrence of 5S rDNA has been confirmed in an increasing amount of species (de Barros et al. 2023,  
326 de Moraes et al. 2023, Kroupin et al. 2023, Rodríguez-González et al. 2023). Whether the reported length of 5S rDNA  
327 is a complete or partial sequence, there is no doubt there is a big difference among these 5S rDNA, including the  
328 length and base pair (Röser et al. 2001, Kulak et al. 2002, Moraes et al. 2022).

329 Because of this, it is quite reasonable that the chromosomally diverse distribution of 5S rDNA is visualized by  
330 FISH. Numerous previous studies showed that the numbers of 5S rDNA FISH signal sites ranged from 1 to 71 (Ali et  
331 al. 2005, Lan and Albert 2011, Luo et al. 2017, Kovács et al. 2023, Rodríguez-González et al. 2023). Their signal  
332 position was found in the chromosome interstitial position, distal position, proximal position, and far away from the  
333 chromosome (Amarasinghe and Carlson 1988, Cai et al. 2006, Campomayor et al. 2021, Wang et al. 2022,  
334 Rodríguez-González et al. 2023). In this study, 5S rDNA was rather diverse and abundant in signal site number (2–18),  
335 position (e.g., interstitial, distal, proximal position, occasionally, outside chromosome), and even as intense (e.g.,  
336 strong, weak, slight). This is consistent with previous studies of the 5S rDNA signal pattern of *A. fruticosum*, *B.*  
337 *formosana* ‘Leshan’, *C. campanulatus*, *H. mutabilis*, *P. nepalensis*, *S. oblata*, two species of *Berberis*, two varieties of *J.*  
338 *regia*, two varieties of *J. sigillata*, two species of *Ligustrum*, two types of *Robinia*, and two varieties of *Z. armatum*,  
339 wild/cultural *H. rhamnoides* ssp. *sinensis*, three varieties of *H. rhamnoides*, four species of *Taxus*, and six types of *B.*  
340 *striata* (Luo et al. 2017, Luo et al. 2018d, Liu and Luo 2019, Luo and Liu 2019, Luo and Chen 2019, 2020, Luo et al.  
341 2022a, b). On the contrary, it was different from the 5S rDNA signal pattern of *F. pennsylvanica*, *R. pseudoacacia*, *S.*  
342 *japonicum*, *T. wallichiana* var. *mairei*, *Z. bungeanum* ‘Hanyuanhuajiao’, two types of *B. ochracea*, and two varieties of



343 *Z. armatum* (Luo and Liu 2019, He et al. 2022c, Huan et al. 2022, He et al. 2023) from previous studies. The possible  
344 causes of the 5S rDNA signal pattern discrepancy are *i*) lost satellite chromosome with signal and *ii*) variation in  
345 different batches of materials in the same species (i.e., intraspecific variation).

346

### 347 Potential Origin of 5S rDNA Diversity in Plants

348 Due to the diversity of signal patterns, 5S rDNA was used as an excellent and dynamic marker in the species of *F.*  
349 *pennsylvanica*, *Iris versicolor* L., *L. × vicaryi*, *L. lucidum*, *P. nepalensis* (*P. concolor*, former name in Flora of China), *R.*  
350 *pseudoacacia*, *S. oblata* (Lim et al. 2007, Luo et al. 2017, Luo and Liu 2019, He et al. 2022a). After comparing the 5S  
351 rDNA in previous studies and in ours, both perfectly reflect the extensive diversity in *P. nepalensis*, which can  
352 distinguish all 18 chromosomes according to the signal position, signal intensity, and signal number. Nevertheless,  
353 5S rDNA was quite conserved and dormant in numerous species, such as *C. campanulatus*, *C. sativa*, *H. rhamnoides*,  
354 *J. regia*, *M. atropurpureum*, *P. stratiotes*, *P. trichocarpa*, and *Sanguisorba* L. (Mishima et al. 2002, Luo and Chen 2019,  
355 2020, Xin et al. 2020, Alexandrov et al. 2022, Luo et al. 2022a, Stepanenko et al. 2022, de Barros et al. 2023). Why is  
356 there such a big difference in 5S rDNA among the above species? There are several plausible hypotheses under  
357 investigation, such as *i*) chromosome rearrangement (e.g., deletion, duplication, inversion, translocation), *ii*)  
358 polyploidization, *iii*) self-incompatibility, and *iv*) chromosome satellites.

359 The 5S rDNA position is a hot issue for chromosomal realignment because of its system into long reaches of the  
360 standpat tandem repetition unit and its active transcription. This feature implies they are impressionable to  
361 chromosomal destruction or non-allelic homologous recombination, thus raising the feasibility of chromosomal  
362 realignments, such as fissions, inversions, and fusions (Rosa et al. 2012; Barros et al. 2017; Potapova and Gerton 2019;  
363 Warmerdam and Wolthuis 2019; Deon et al. 2020). The 5S rDNA diversification was regarded as variable genomic  
364 areas, compliant with double-strand break and chromosomal realignment, facilitating karyotypic reconstruction  
365 (Glugoski et al., 2018; Deon et al., 2020, 2022). The 5S rDNA position was also diversified by translocation or  
366 transposition events of repeats in those chromosomes (Venancio Neto et al. 2022). An interstitial 5S rDNA position  
367 with a diverse location is current, presumably because of a thin inversion. A plus 5S rDNA likely implies the presence  
368 of a replication (Coluccia et al. 2020). The 5S rDNA site of one parent was either excluded from the chromosome or  
369 shifted into gene silencing and then disappeared, which could decrease the 5S rDNA site number (de Melo and  
370 Guerra 2003, Volkov et al. 2017). These studies demonstrate that chromosome rearrangement causes variation in 5S  
371 rDNA diversity.

372 The 5S rDNA has a polyploidization-relevant preference to the distal from a proximal position but keeps a stable  
373 loci number (Zhang et al. 2016). The 5S rDNA sites are largely proximal, a highly transparent direction in  
374 chromosomes with a single site (Garcia et al. 2016). Consequently, the presence of the 5S rDNA site in the distal  
375 chromosomes and the abundance of microsatellites in adjacent areas provide friendly conditions for adding  
376 realignments. These results emphasize the effect of variable chromosomal 5S rDNA loci in generating assignments  
377 (Gkugoski et al. 2022). No associations between the number of 5S loci and chromosome number, but  
378 correspondence with ploidy level and genome size (Adams et al. 2000, Lan and Albert 2011, Garcia et al. 2016), but  
379 disputes still occurred (Hasterok et al. 2005, Said et al. 2018, Chai et al. 2022), such as the genus *Cuscuta* L. (Garcia et  
380 al., 2017). These studies demonstrate that polyploidization causes variation in 5S rDNA diversity.

381

### 382 Conclusion

383 This study conducted FISH-based chromosomal mapping of 5S rDNA markers to provide valid karyotype landmarks  
384 to reveal the chromosome number and 5S rDNA signal pattern distribution in 64 plants. Furthermore, we  
385 established chromosome physical mapping of each species. Finally, we discuss the proposed origin of 5S rDNA  
386 diversity in plants. We are devoted to developing universal oligosequence markers (GAA)<sub>6</sub>, (TTG)<sub>6</sub>, (ACT)<sub>6</sub>, 45S, ITS,  
387 and combinatory analysis of additional plant species, particularly woody plants. Altogether, the results reported here  
388 enhance the assumption that cytogenetic characteristics (conventional and molecular) could be regarded as  
389 excellent markers for chromosome distinction and the presentation and profile of the existing biodiversity in woody  
390 plants.

### 391 Data availability

392 The authors affirm that all data necessary for confirming the conclusions of the article are present within the article,

393 figures, and supplemental figures.

394 Supplemental material available at GENETICS online.

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## 399 Conflicts of interest statement

400 The authors declare no conflict of interest.

401

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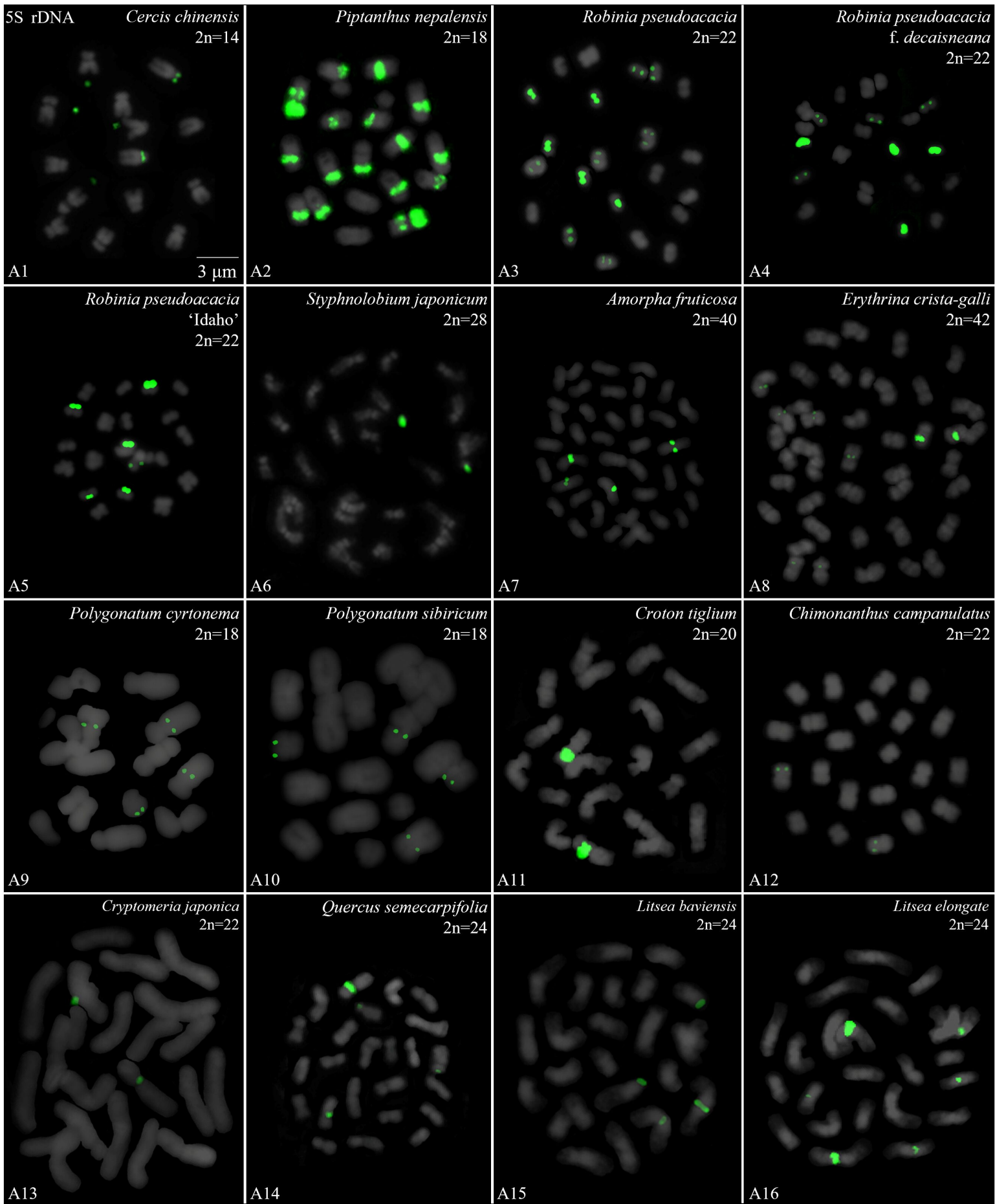
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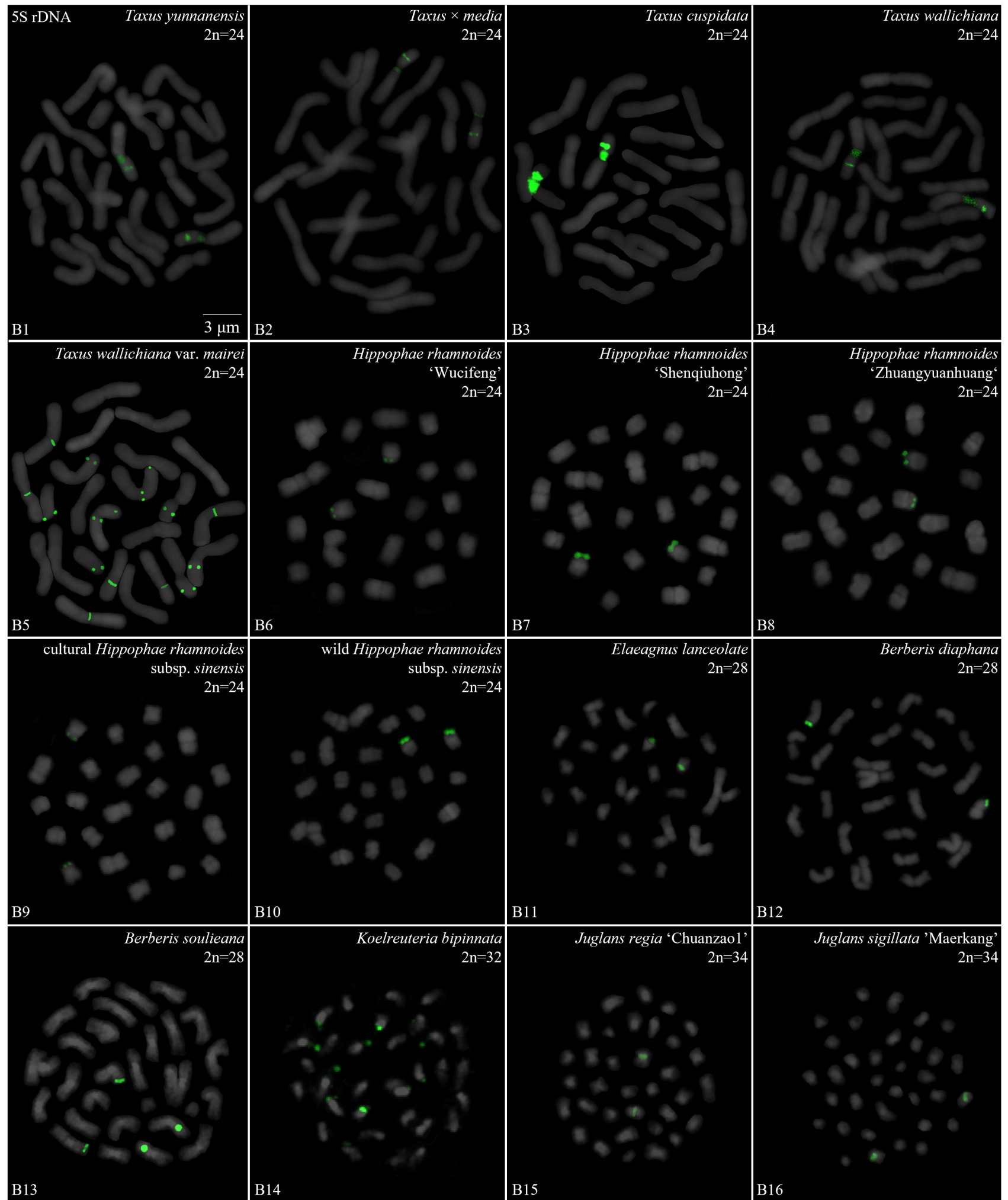
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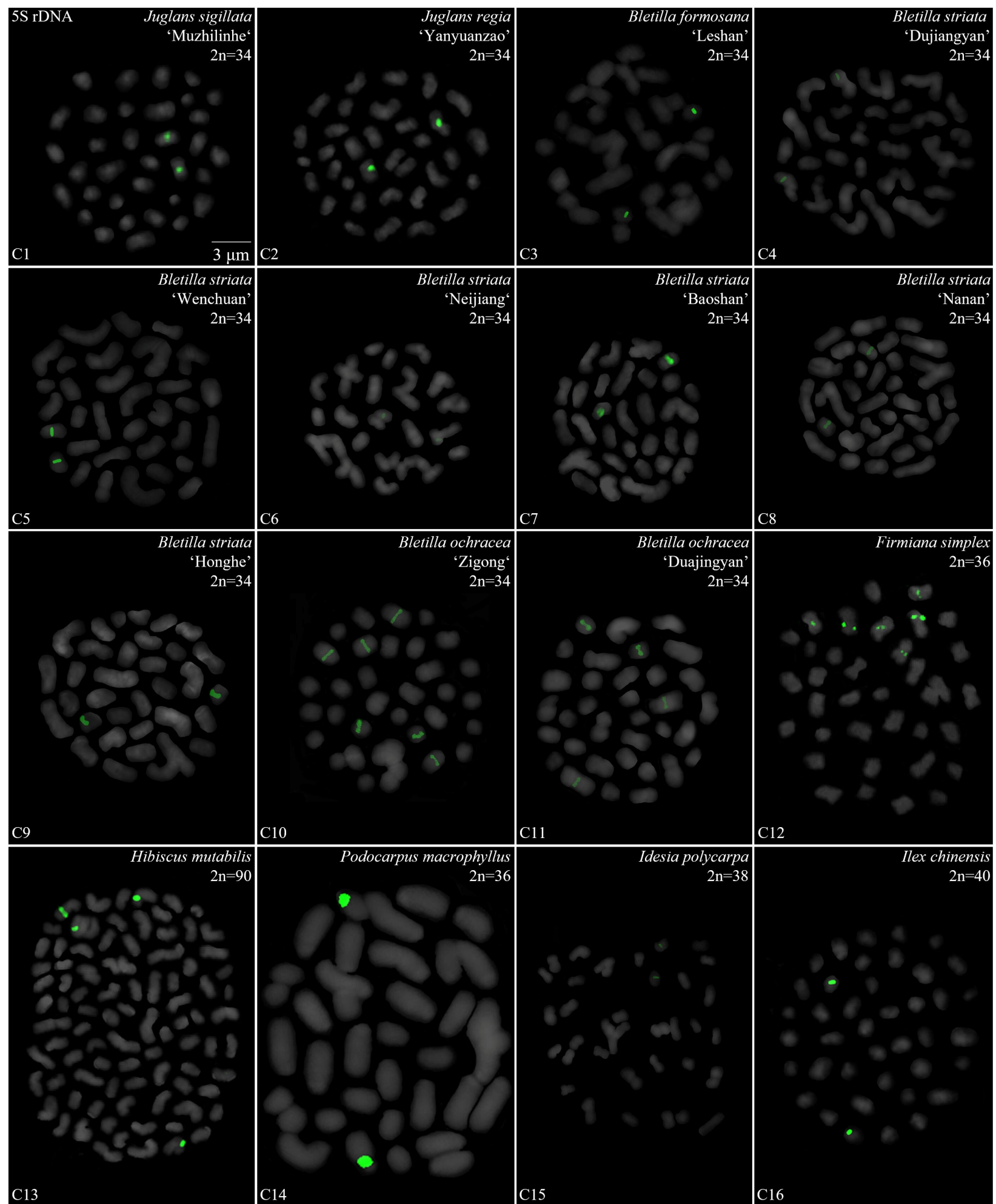
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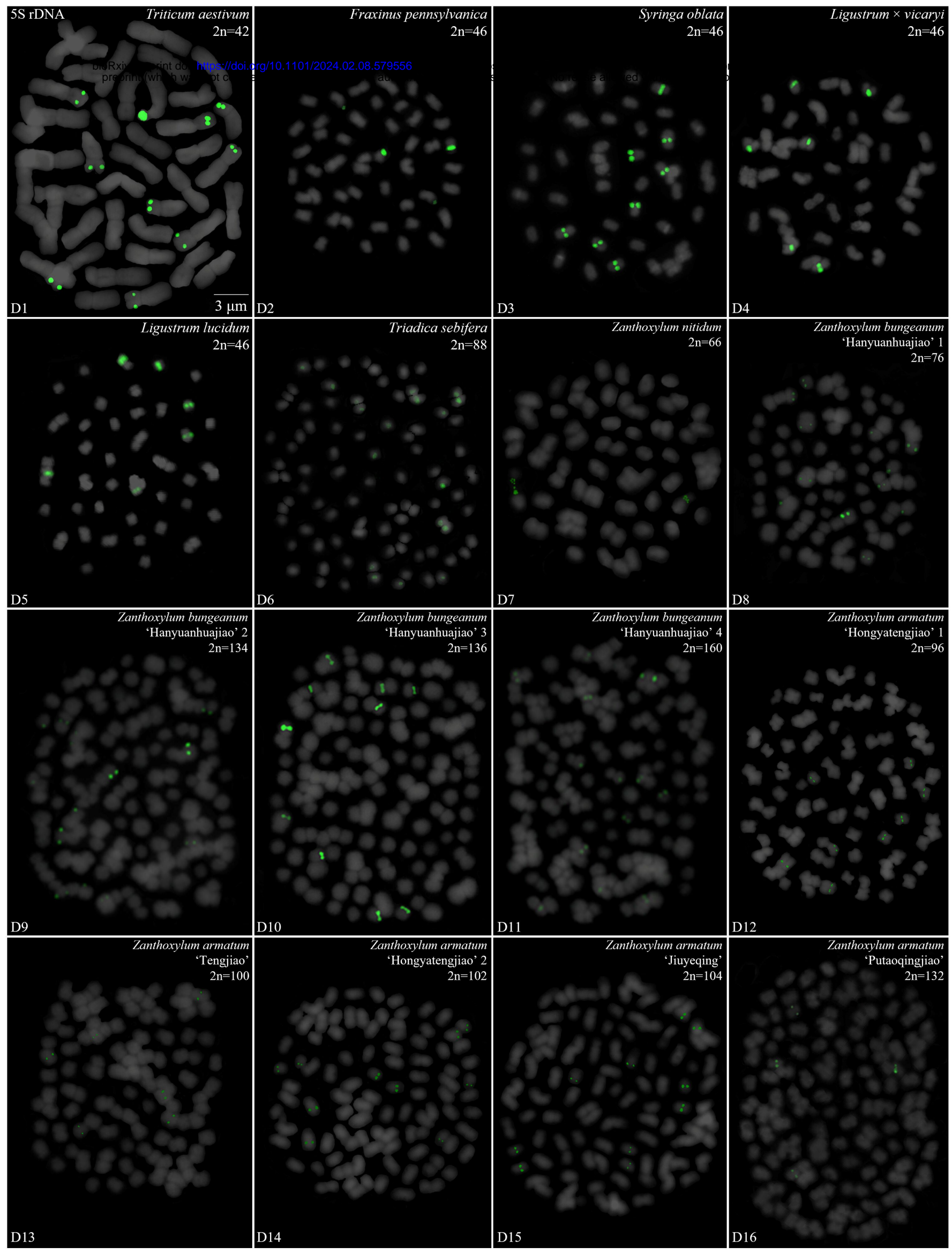
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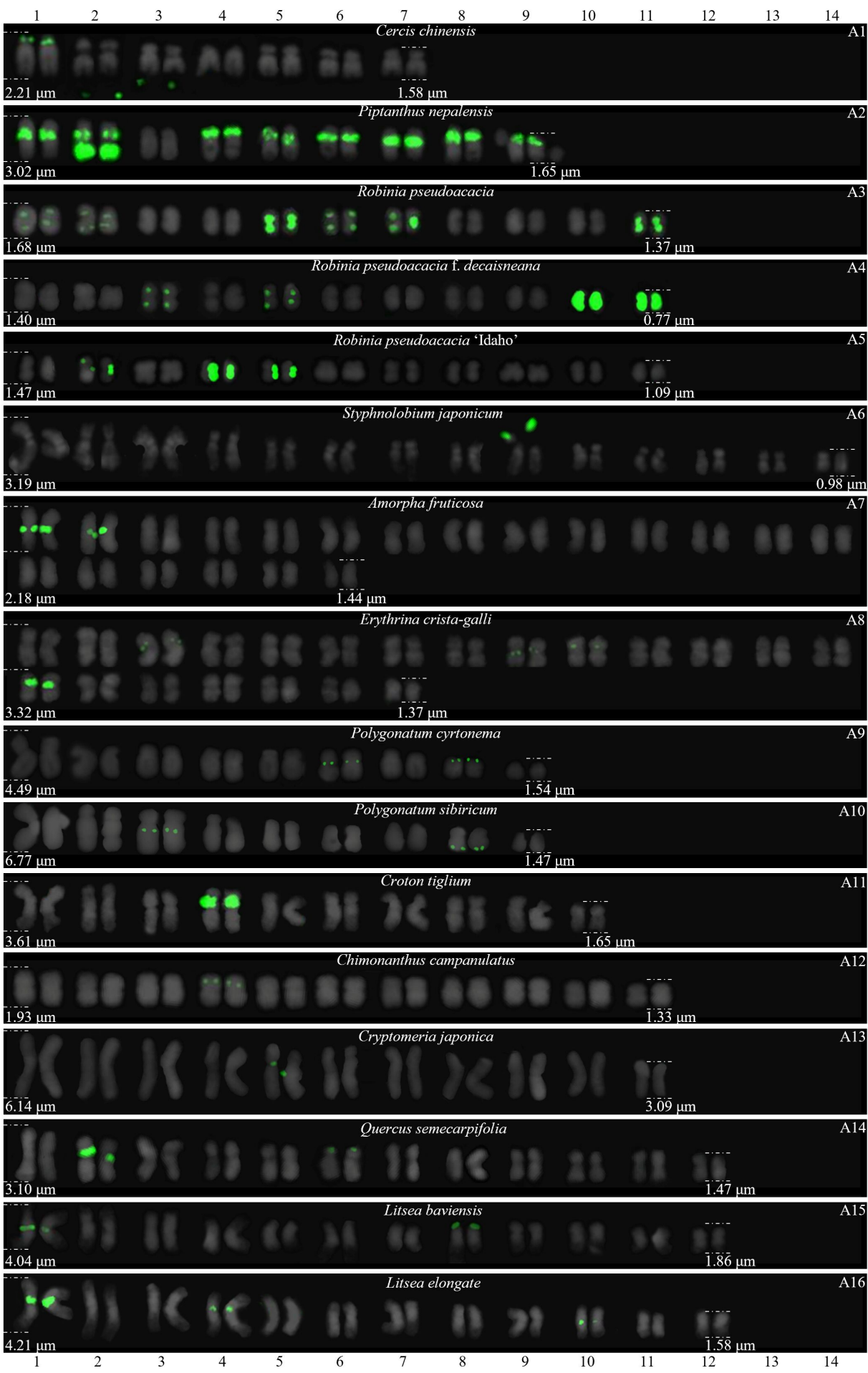


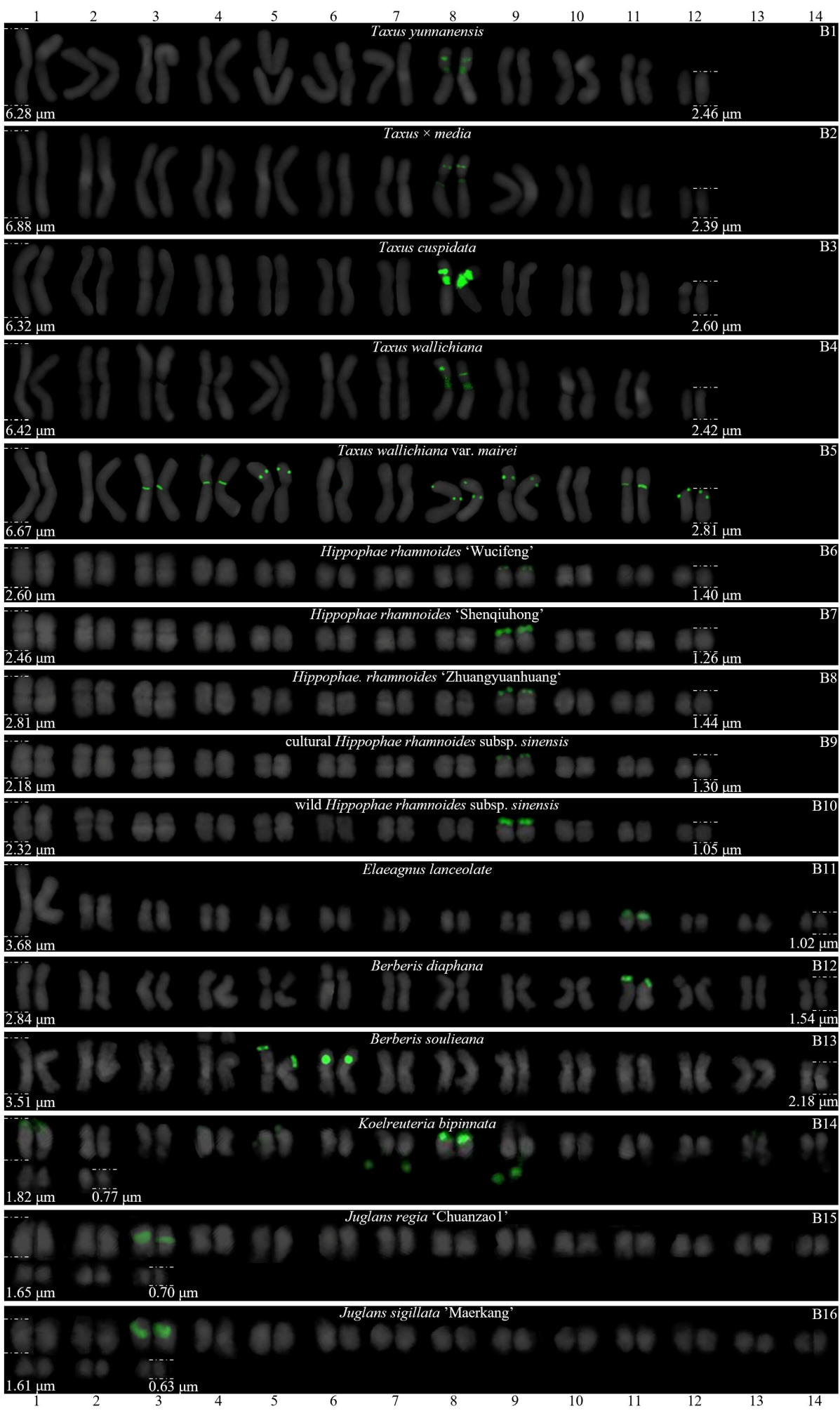


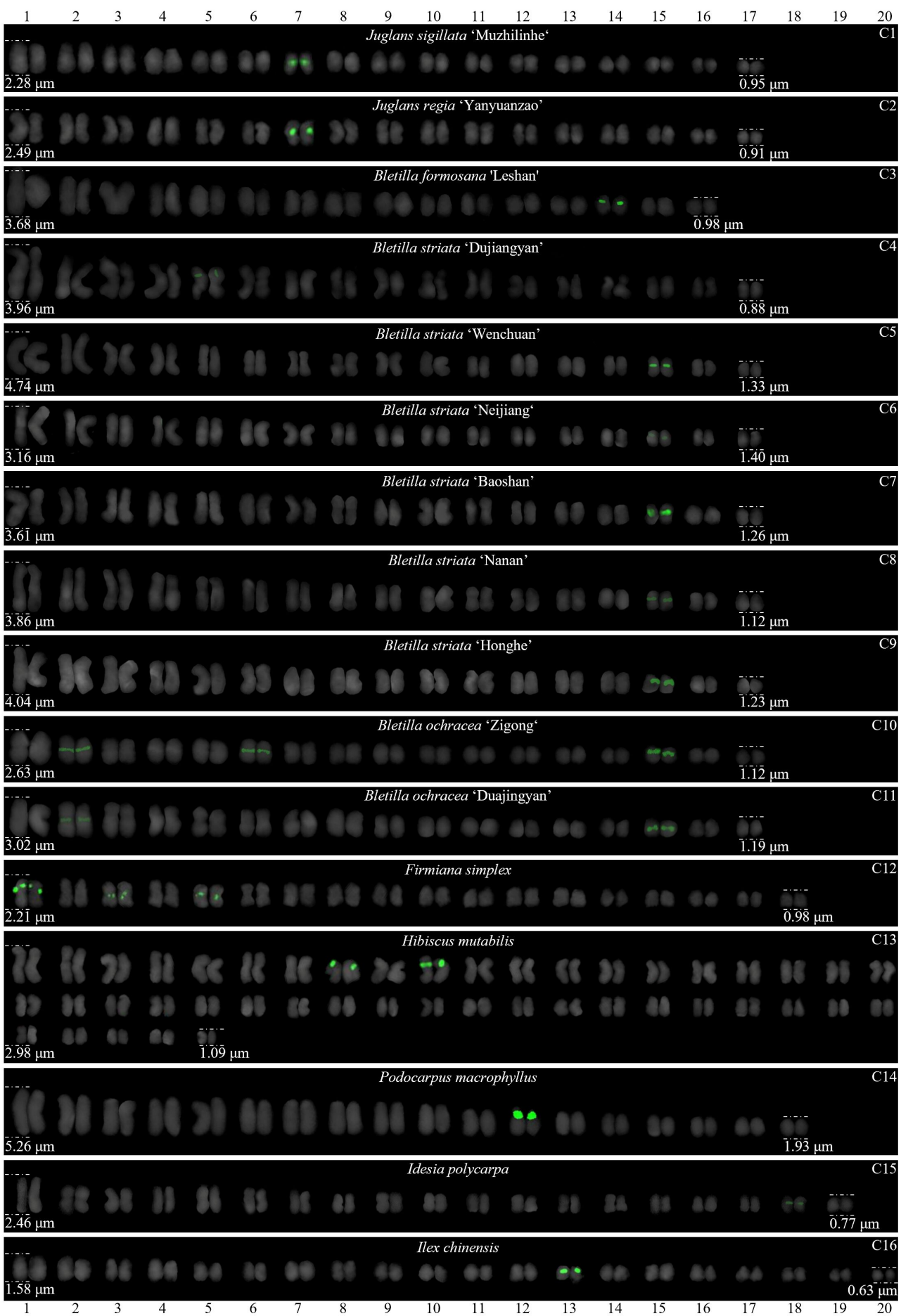


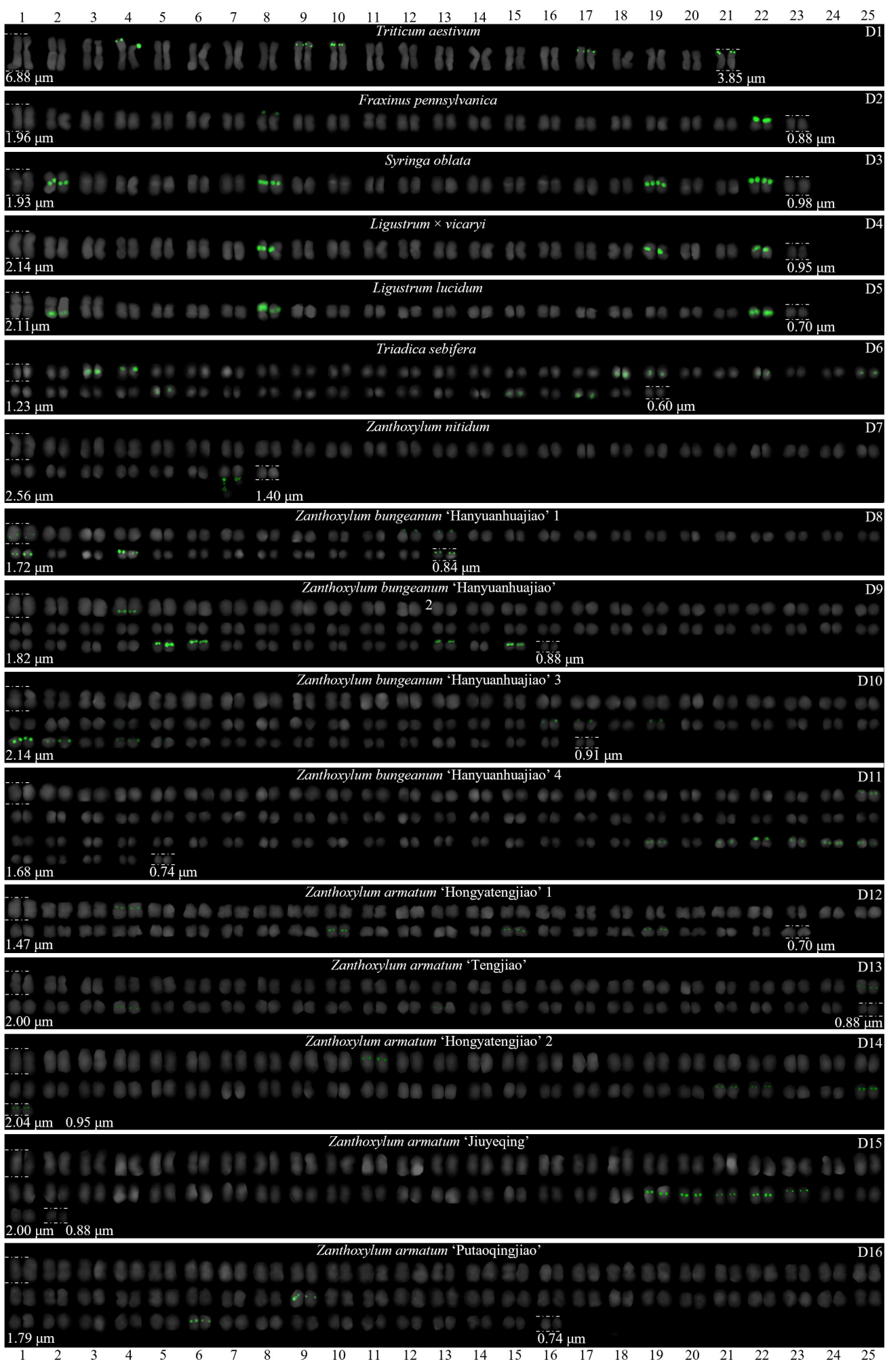


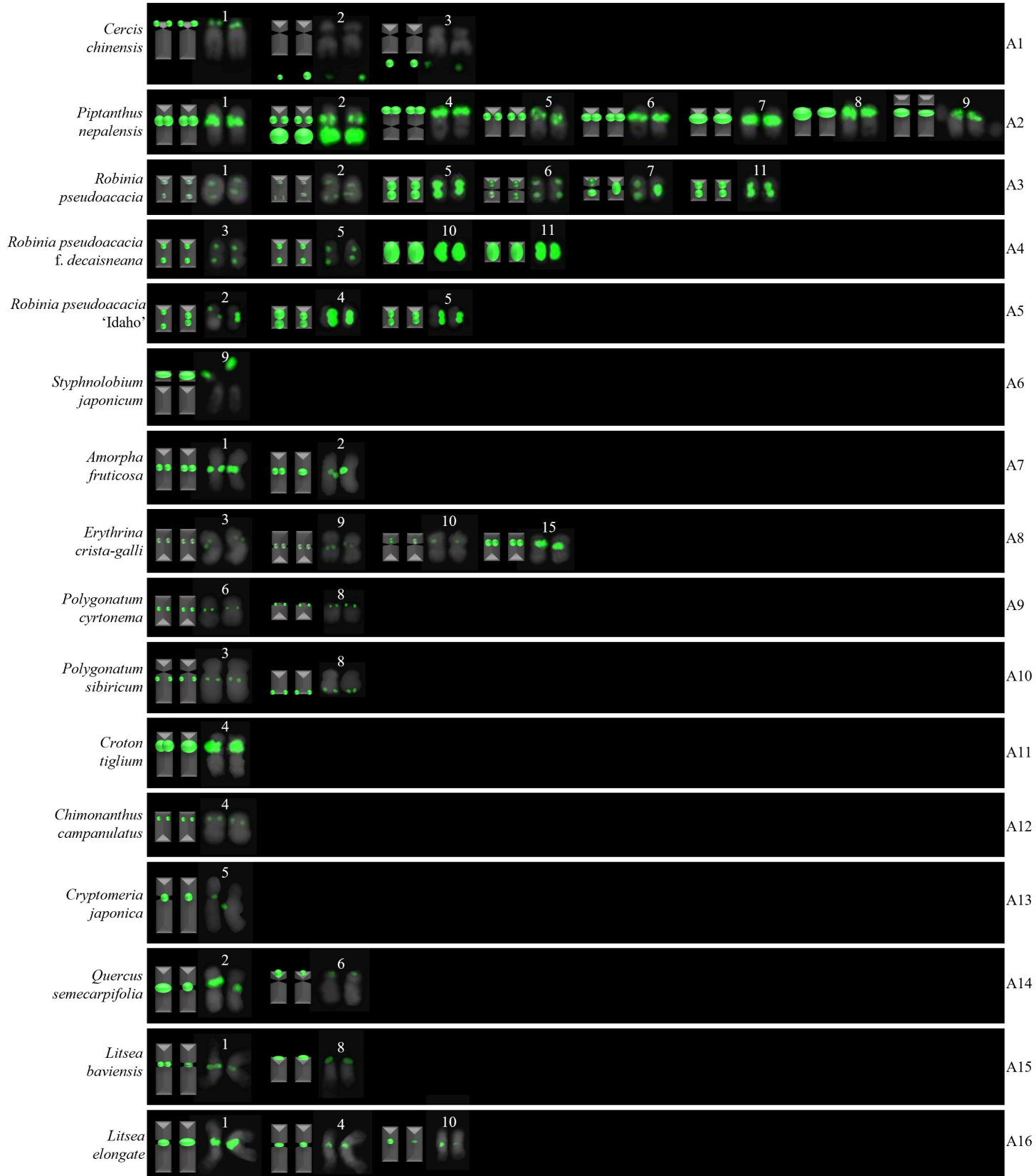




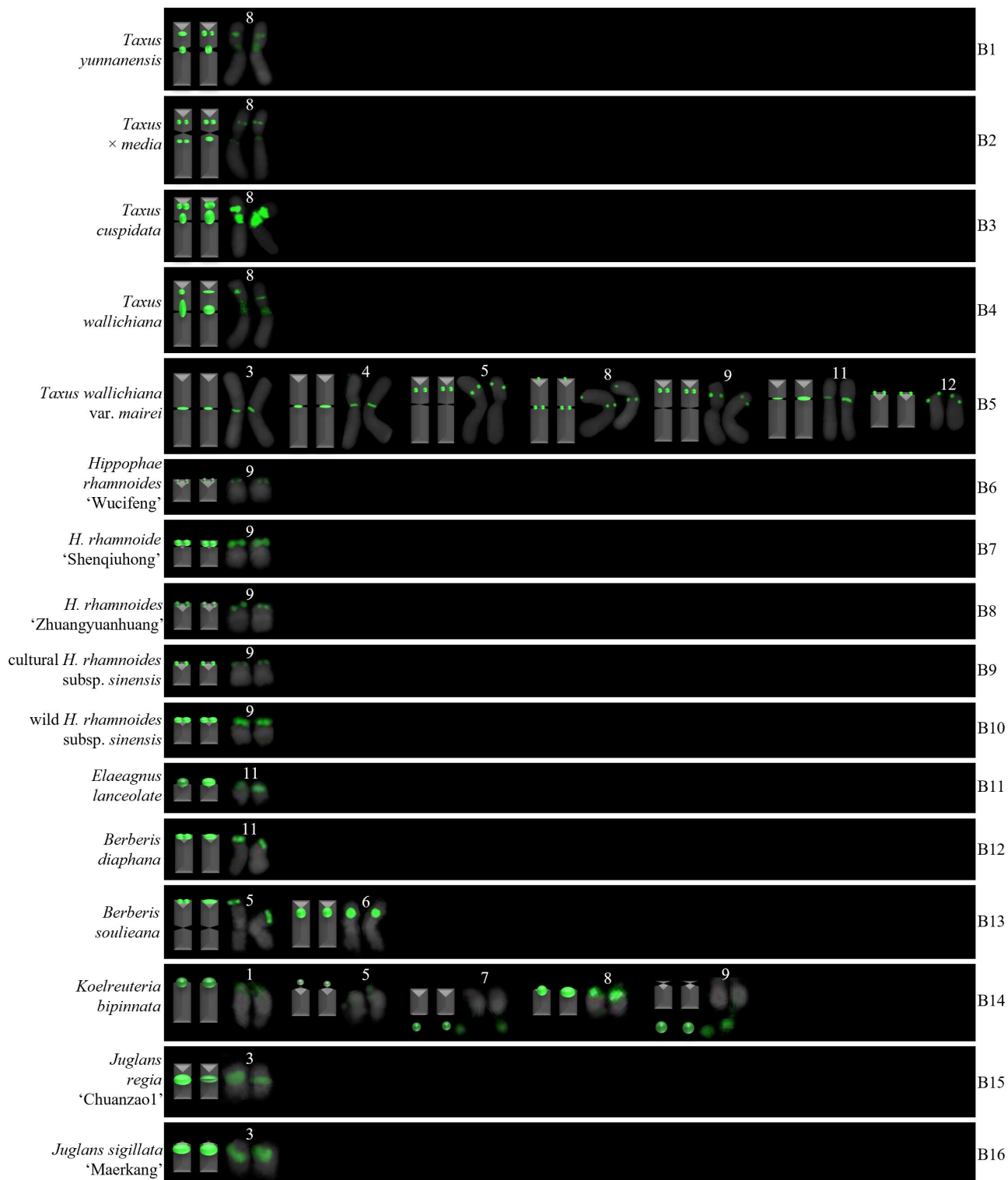


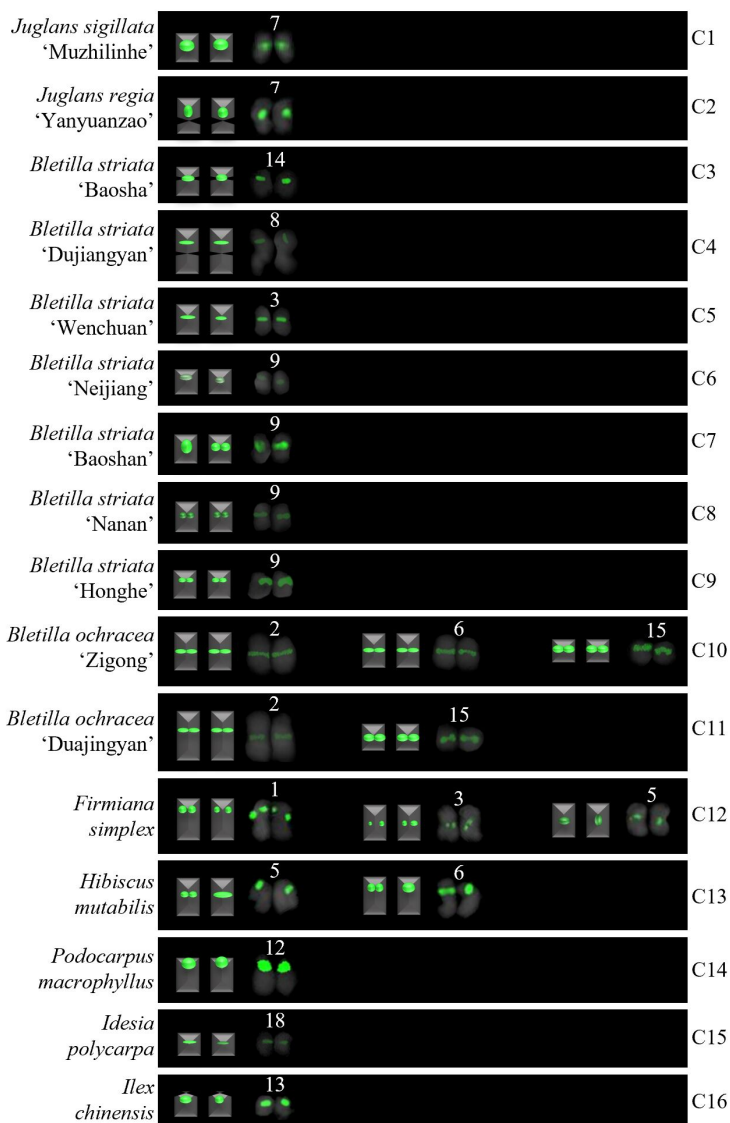


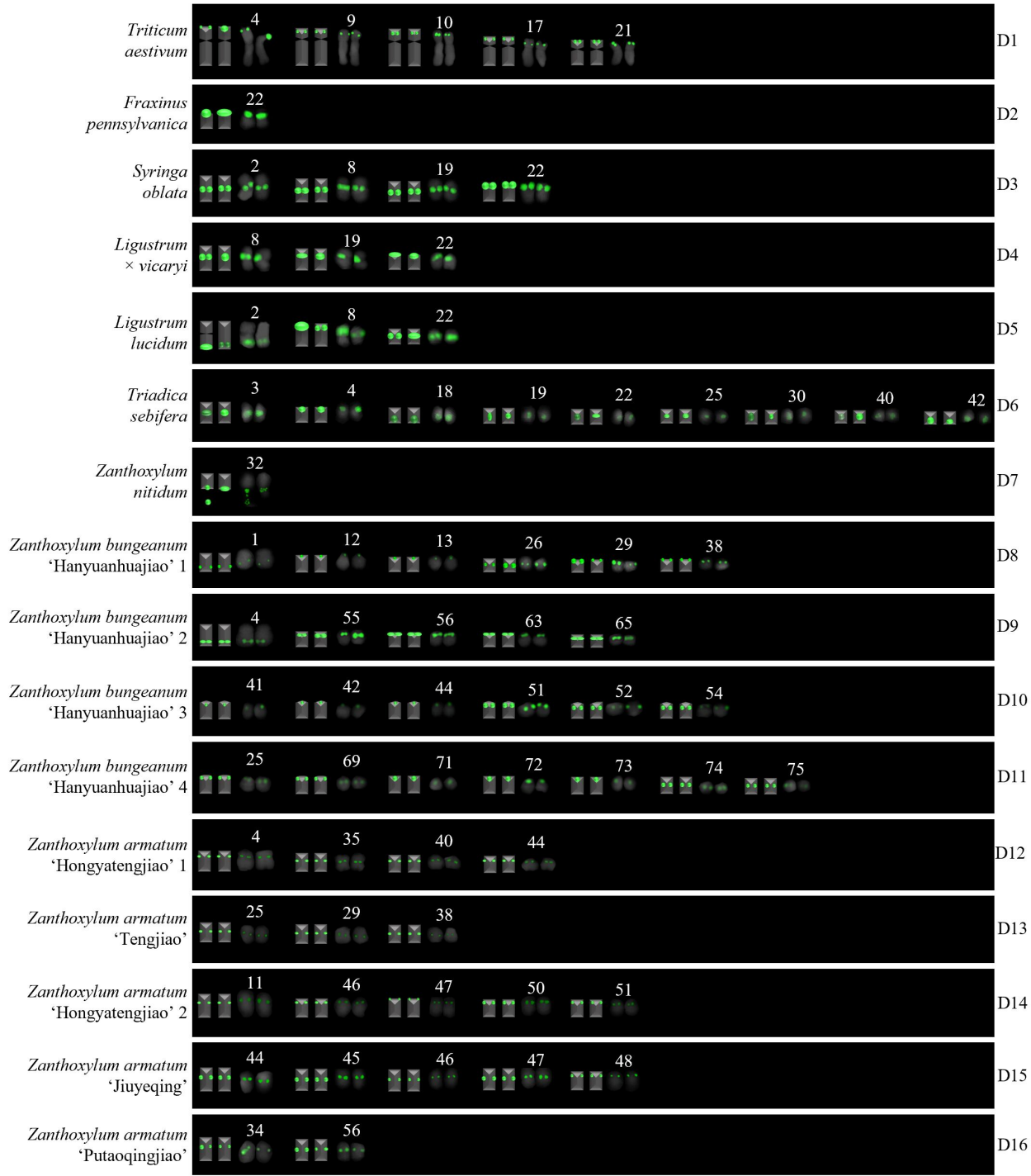




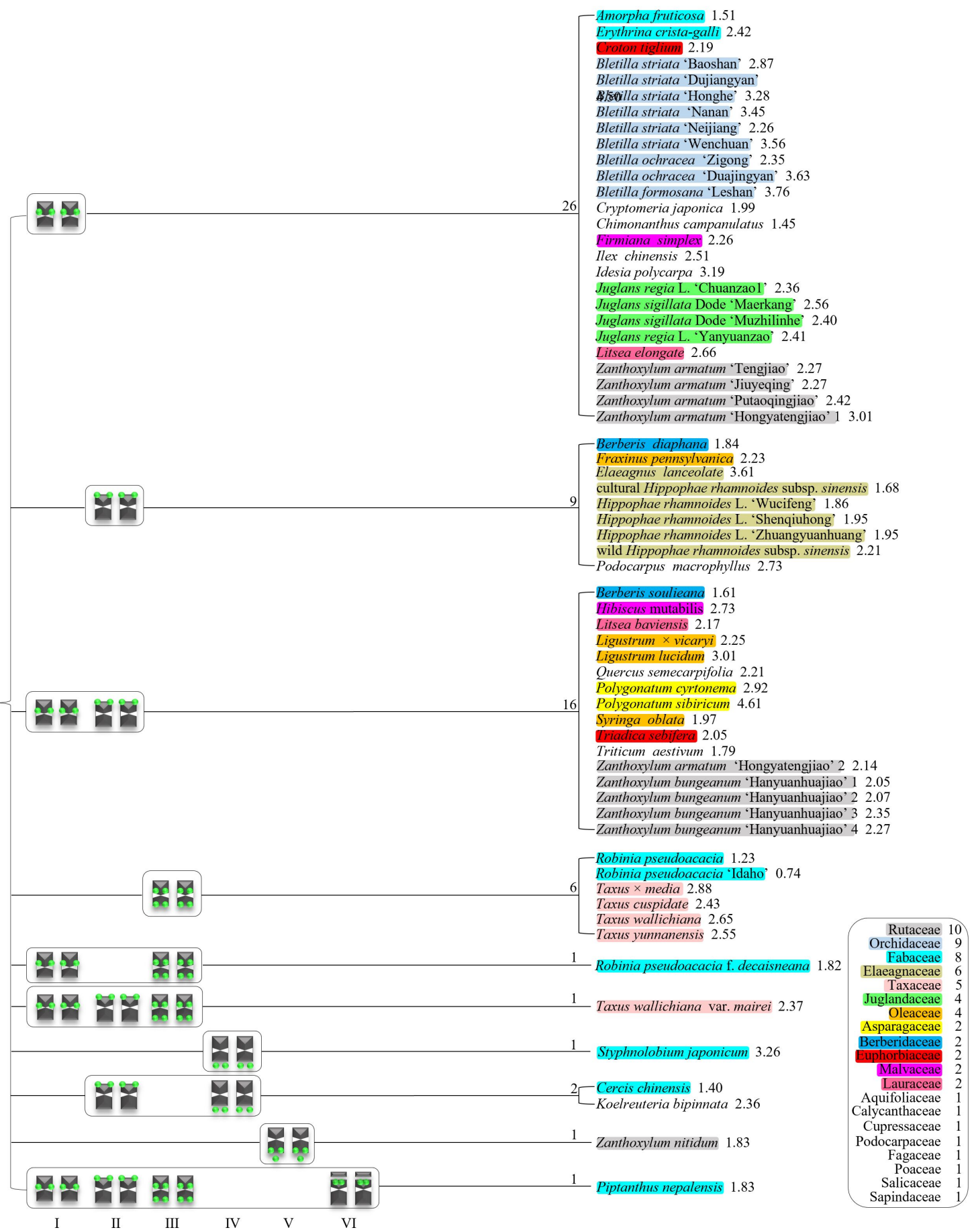




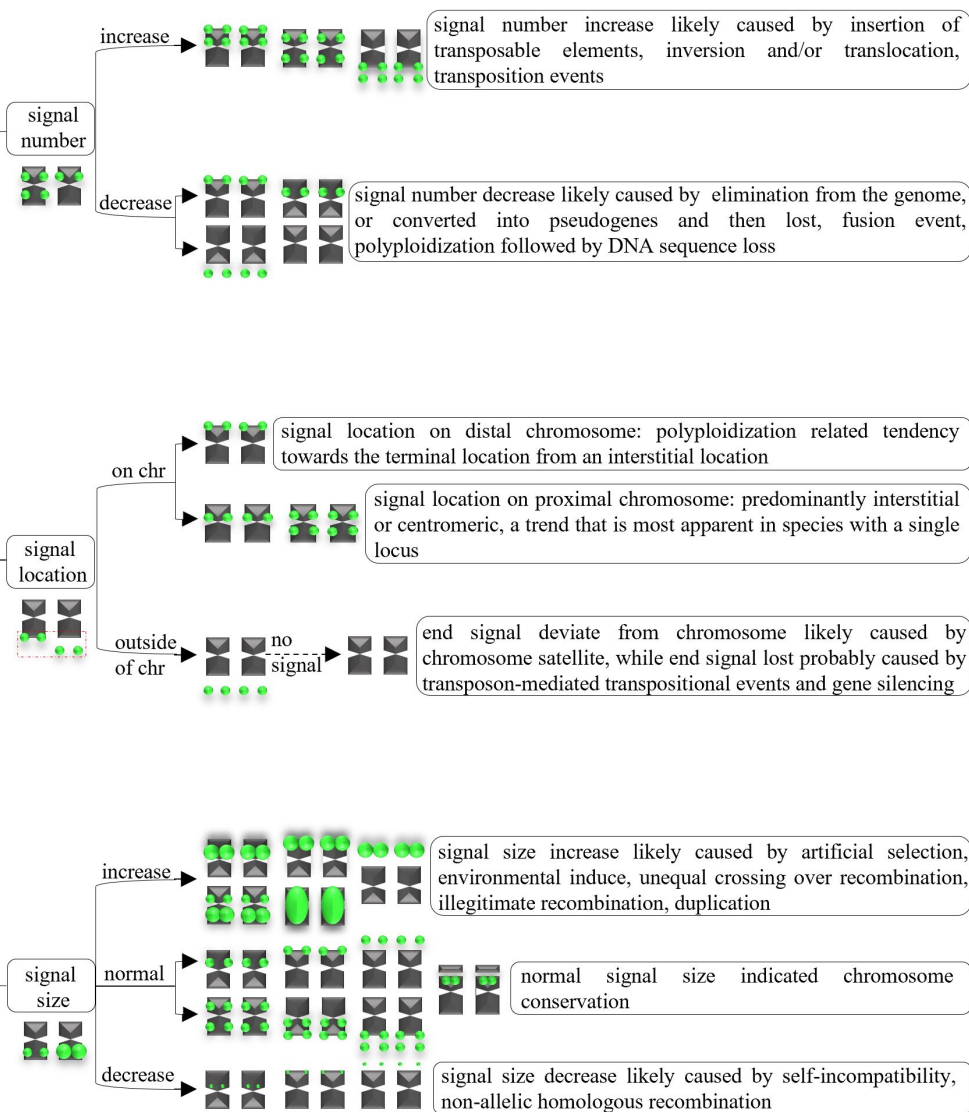




Signal pattern of 5S rDNA in 64 plants



# Proposed Origin of 5S rDNA Signal Diversity



**Table 1** Details of all 64 plants used in this work.

| Family       | No. | Species  | Collection Location   |
|--------------|-----|--|-----------------------|
| Rutaceae     | 1   | <i>Zanthoxylum nitidum</i> (Roxb.) DC.   | Hanyuan, Sichuan      |
|              | 2   | <i>Zanthoxylum bungeanum</i> Maxim. 'Hanyuanhuajiao'                               | 1 Hanyuan, Sichuan    |
|              | 3   | <i>Zanthoxylum bungeanum</i> Maxim. 'Hanyuanhuajiao'                               | 2 Hanyuan, Sichuan    |
|              | 4   | <i>Zanthoxylum bungeanum</i> Maxim. 'Hanyuanhuajiao'                               | 3 Hanyuan, Sichuan    |
|              | 5   | <i>Zanthoxylum bungeanum</i> Maxim. 'Hanyuanhuajiao'                               | 4 Hanyuan, Sichuan    |
|              | 6   | <i>Zanthoxylum armatum</i> DC. 'Hongyatengjiao'                                    | 1 Hongya, Sichuan     |
|              | 7   | <i>Zanthoxylum armatum</i> DC. 'Hongyatengjiao'                                    | 2 Hongya, Sichuan     |
|              | 8   | <i>Zanthoxylum armatum</i> DC. 'Jiuyeqing'   | Jiangjin, Chongqing   |
|              | 9   | <i>Zanthoxylum armatum</i> DC. 'Putaoqingjiao'                                     | Hanyuan, Sichuan      |
|              | 10  | <i>Zanthoxylum armatum</i> DC. 'Tengjiao'  | Hanyuan, Sichuan      |
| Orchidaceae  | 11  | <i>Bletilla striata</i> (Thunb. ex Murray) Rchb. f. 'Dujiangyan'                   | Dujiangyan, Sichuan   |
|              | 12  | <i>Bletilla striata</i> (Thunb. ex Murray) Rchb. f. 'Neijiang'                     | Neijiang, Sichuan     |
|              | 13  | <i>Bletilla striata</i> (Thunb. ex Murray) Rchb. f. 'Wenchuan'                     | Wenchuan, Sichuan     |
|              | 14  | <i>Bletilla striata</i> (Thunb. ex Murray) Rchb. f. 'Baoshan'                      | Baoshan, Yunnan       |
|              | 15  | <i>Bletilla striata</i> (Thunb. ex Murray) Rchb. f. 'Honghe'                       | Honghe, Yunnan        |
|              | 16  | <i>Bletilla striata</i> (Thunb. ex Murray) Rchb. f. 'Nanan'                        | Nanan, Chongqing      |
|              | 17  | <i>Bletilla ochracea</i> Schltr. 'Dujiangyan'                                      | Dujiangyan, Sichuan   |
|              | 18  | <i>Bletilla ochracea</i> Schltr. 'Zigong'  | Zigong, Sichuan       |
|              | 19  | <i>Bletilla formosana</i> (Hayata) Schltr. 'Leshan'                                | Leshan, Sichuan       |
| Fabaceae     | 20  | <i>Cercis chinensis</i> Bunge  | Wenjiang, Sichuan     |
|              | 21  | <i>Piptanthus nepalensis</i> (Hook.) D. Don  | Qingbaijiang, Sichuan |
|              | 22  | <i>Robinia pseudoacacia</i> L.   | Wenjiang, Sichuan     |
|              | 23  | <i>Robinia pseudoacacia</i> f. <i>decaisneana</i> (Carr.) Voss                     | Suqian, Jiangsu       |
|              | 24  | <i>Robinia pseudoacacia</i> 'Idaho'  | Suqian, Jiangsu       |
|              | 25  | <i>Styphnolobium japonicum</i> (L.) Schott   | Suqian, Jiangsu       |
|              | 26  | <i>Amorpha fruticosa</i> L.  | Suqian, Jiangsu       |
|              | 27  | <i>Erythrina crista-galli</i> L.   | Wenjiang, Sichuan     |
| Elaeagnaceae | 28  | <i>Hippophae rhamnoides</i> L. 'ShenqiuHong'                                       | Huai' an, Hebei       |
|              | 29  | <i>Hippophae rhamnoides</i> L. 'Wucifeng'  | Huai' an, Hebei       |
|              | 30  | wild <i>Hippophae rhamnoides</i> subsp. <i>sinensis</i> Rousi                      | Huai' an, Hebei       |
|              | 31  | cultural <i>Hippophae rhamnoides</i> subsp. <i>sinensis</i> Rousi                  | Tieling, Liaoning     |
|              | 32  | <i>Hippophae rhamnoides</i> L. 'Zhuangyuanhuang'                                   | Wenchuan, Sichuan     |
|              | 33  | <i>Elaeagnus lanceolate</i> Warb. apud Diels                                       | Wenchuan, Sichuan     |
| Taxaceae     | 34  | <i>Taxus cuspidata</i> Siebold & Zucc.   | Suqian, Jiangsu       |
|              | 35  | <i>Taxus</i> × <i>media</i> Rehder   | Ya' an, Sichuan       |
|              | 36  | <i>Taxus wallichiana</i> var. <i>mairei</i> (Lemee & H. Léveillé) L. K. Fu & N. Li | Ya' an, Sichuan       |
|              | 37  | <i>Taxus wallichiana</i> Zucc.   | Xichuang, Sichuan     |
|              | 38  | <i>Taxus yunnanensis</i> W.C. Cheng & L.K. Fu                                      | Ya' an, Sichuan       |
| Juglandaceae | 39  | <i>Juglans regia</i> L. 'Chuanzao1'  | Qingbaijiang, Sichuan |
|              | 40  | <i>Juglans sigillata</i> Dode 'Maerkang'   | Maerkang, Sichuan     |
|              | 41  | <i>Juglans sigillata</i> Dode 'Muzhilinhe'   | Gulin, Sichuan        |

|                |    |   |                     |
|----------------|----|---|---------------------|
|                | 42 | <i>Juglans regia</i> L. 'Yanyuanzao'                      | Yanyuan, Sichuan    |
|                | 43 | <i>Fraxinus pennsylvanica</i> Marsh.                      | Wenjiang, Sichuan   |
| Oleaceae       | 44 | <i>Syringa oblata</i> Lindl.                              | Wenjiang, Sichuan   |
|                | 45 | <i>Ligustrum</i> × <i>vicaryi</i> Rehder                  | Wenjiang, Sichuan   |
|                | 46 | <i>Ligustrum lucidum</i> Ait.                             | Wenjiang, Sichuan   |
| Asparagaceae   | 47 | <i>Polygonatum cyrtoneura</i> Hua                         | Wenchuan, Sichuan   |
|                | 48 | <i>Polygonatum sibiricum</i> Delar. ex Redoute            | Dujiangyan, Sichuan |
| Berberidaceae  | 49 | <i>Berberis diaphana</i> Maxim.                           | Wenchuan, Sichuan   |
|                | 50 | <i>Berberis soulieana</i> Schneid.                        | Wenchuan, Sichuan   |
| Lauraceae      | 51 | <i>Litsea baviensis</i> Lec.                              | Jinniu, Sichuan     |
|                | 52 | <i>Litsea elongate</i> (Wall. ex Nees) Benth. et Hook. f. | Jinniu, Sichuan     |
| Malvaceae      | 53 | <i>Firmiana simplex</i> (L.) W. Wight                     | Wenjiang, Sichuan   |
|                | 54 | <i>Hibiscus mutabilis</i> L.                              | Jinniu, Sichuan     |
| Euphorbiaceae  | 55 | <i>Croton tiglium</i> L.                                  | Ya' an, Sichuan     |
|                | 56 | <i>Triadica sebifera</i> (Linnaeus) Small                 | Jiangyou, Sichuan   |
| Cupressaceae   | 57 | <i>Cryptomeria japonica</i> (L.f.) D. Don                 | Wenjiang, Sichuan   |
| Aquifoliaceae  | 58 | <i>Ilex chinensis</i> Sims                                | Dujiangyan, Sichuan |
| Calycanthaceae | 59 | <i>Chimonanthus campanulatus</i> R.H. Chang & C.S. Ding   | Jinniu, Sichuan     |
| Fagaceae       | 60 | <i>Quercus semecarpifolia</i> Smith                       | Wenchuan, Sichuan   |
| Poaceae        | 61 | <i>Triticum aestivum</i> L.                               | Wenjiang, Sichuan   |
| Podocarpaceae  | 62 | <i>Podocarpus macrophyllus</i> (Thunb.) Sweet             | Wenjiang, Sichuan   |
| Salicaceae     | 63 | <i>Idesia polycarpa</i> Maxim.                            | Wenjiang, Sichuan   |
| Sapindaceae    | 64 | <i>Koelreuteria bipinnata</i> Franch.                     | Wenjiang, Sichuan   |

**Table 2** Chromosome number and length of the 64 plants used in this work.

| Accession | Species   | Chromosome Number | Chromosome Length | Karyotype Asymmetry |
|-----------|---|-------------------|-------------------|---------------------|
| A1        | <i>Cercis chinensis</i>                                     | 2n=14             | 1.58-2.21 μm      | 1.40                |
| A2        | <i>Piptanthus nepalensis</i>                                | 2n=18             | 1.65-3.02 μm      | 1.83                |
| A3        | <i>Robinia pseudoacacia</i>                                 | 2n=22             | 1.37-1.68 μm      | 1.23                |
| A4        | <i>Robinia pseudoacacia</i> f. <i>decaisneana</i>           | 2n=22             | 0.77-1.40 μm      | 1.82                |
| A5        | <i>Robinia pseudoacacia</i> 'Idaho'                         | 2n=22             | 1.09-1.47 μm      | 0.74                |
| A6        | <i>Styphnolobium japonicum</i>                              | 2n=28             | 0.98-3.19 μm      | 3.26                |
| A7        | <i>Amorpha fruticosa</i>                                    | 2n=40             | 1.44-2.18 μm      | 1.51                |
| A8        | <i>Erythrina crista-galli</i>                               | 2n=42             | 1.37-3.32 μm      | 2.42                |
| A9        | <i>Polygonatum cyrtonema</i>                                | 2n=18             | 1.54-4.49 μm      | 2.92                |
| A10*      | <i>Polygonatum sibiricum</i>                                | 2n=18             | 1.47-6.77 μm      | 4.61                |
| A11       | <i>Croton tiglium</i>                                       | 2n=20             | 1.65-3.61 μm      | 2.19                |
| A12       | <i>Chimonanthus campanulatus</i>                            | 2n=22             | 1.33-1.93 μm      | 1.45                |
| A13       | <i>Cryptomeria japonica</i>                                 | 2n=22             | 3.09-6.14 μm      | 1.99                |
| A14       | <i>Quercus semecarpifolia</i>                               | 2n=24             | 1.47-3.10 μm      | 3.11                |
| A15       | <i>Litsea baviensis</i>                                     | 2n=24             | 1.86-4.04 μm      | 2.17                |
| A16       | <i>Litsea elongate</i>                                      | 2n=24             | 1.58-4.21 μm      | 2.66                |
| B1        | <i>Taxus yunnanensis</i>                                    | 2n=24             | 2.46-6.28 μm      | 2.55                |
| B2        | <i>Taxus</i> × <i>media</i>                                 | 2n=24             | 2.39-6.88 μm      | 2.88                |
| B3        | <i>Taxus cuspidata</i>                                      | 2n=24             | 2.60-6.32 μm      | 2.43                |
| B4        | <i>Taxus wallichiana</i>                                    | 2n=24             | 2.42-6.42 μm      | 2.65                |
| B5        | <i>Taxus wallichiana</i> var. <i>mairei</i>                 | 2n=24             | 2.81-6.67 μm      | 2.37                |
| B6        | <i>Hippophae rhamnoides</i> 'Wucifeng'                      | 2n=24             | 1.40-2.60 μm      | 1.86                |
| B7        | <i>Hippophae rhamnoides</i> 'Shenqiu hong'                  | 2n=24             | 1.26-2.46 μm      | 1.95                |
| B8        | <i>Hippophae rhamnoides</i> 'Zhuangyuanhuang'               | 2n=24             | 1.44-2.81 μm      | 1.95                |
| B9        | cultural <i>Hippophae rhamnoides</i> subsp. <i>sinensis</i> | 2n=24             | 1.30-2.18 μm      | 1.68                |
| B10       | wild <i>Hippophae rhamnoides</i> subsp. <i>sinensis</i>     | 2n=24             | 1.05-2.32 μm      | 2.21                |
| B11       | <i>Elaeagnus lanceolate</i>                                 | 2n=28             | 1.02-3.68 μm      | 3.61                |
| B12       | <i>Berberis diaphana</i>                                    | 2n=28             | 1.54-2.84 μm      | 1.84                |
| B13       | <i>Berberis soulieana</i>                                   | 2n=28             | 2.18-3.51 μm      | 1.61                |
| B14       | <i>Koelreuteria bipinnata</i>                               | 2n=32             | 0.77-1.82 μm      | 2.36                |
| B15       | <i>Juglans regia</i> 'Chuanzao1'                            | 2n=34             | 0.70-1.65 μm      | 2.36                |
| B16       | <i>Juglans sigillata</i> 'Maerkang'                         | 2n=34             | 0.63-1.61 μm      | 2.56                |
| C1        | <i>Juglans sigillata</i> 'Muzhilinhe'                       | 2n=34             | 0.95-2.28 μm      | 2.40                |
| C2        | <i>Juglans regia</i> 'Yanyuanzao'                           | 2n=34             | 0.91-2.19 μm      | 2.41                |
| C3        | <i>Bletilla formosana</i> 'Leshan'                          | 2n=34             | 0.98-3.68 μm      | 3.76                |
| C4        | <i>Bletilla striata</i> f. 'Dujiangyan'                     | 2n=34             | 0.88-3.96 μm      | 4.50                |
| C5        | <i>Bletilla striata</i> f. 'Wenchuan'                       | 2n=34             | 1.33-4.74 μm      | 3.56                |
| C6        | <i>Bletilla striata</i> f. 'Neijiang'                       | 2n=34             | 1.40-3.16 μm      | 2.26                |
| C7        | <i>Bletilla striata</i> f. 'Baoshan'                        | 2n=34             | 1.26-3.61 μm      | 2.87                |
| C8        | <i>Bletilla striata</i> f. 'Nanan'                          | 2n=34             | 1.12-3.86 μm      | 3.45                |



|      |   |   |        |                         |      |
|------|---|---|--------|-------------------------|------|
| C9   | <i>Bletilla striata</i> f. 'Honghe'           |   | 2n=34  | 1.23-4.04 $\mu\text{m}$ | 3.28 |
| C10  | <i>Bletilla ochracea</i> 'Zigong'             |   | 2n=34  | 1.12-2.63 $\mu\text{m}$ | 2.35 |
| C11  | <i>Bletilla ochracea</i> 'Dujiangyan'         |   | 2n=34  | 1.19-3.02 $\mu\text{m}$ | 2.63 |
| C12  | <i>Firmiana simplex</i>                       |   | 2n=36  | 0.98-2.21 $\mu\text{m}$ | 2.26 |
| C13  | <i>Hibiscus mutabilis</i>                     |   | 2n=90  | 1.09-2.98 $\mu\text{m}$ | 2.73 |
| C14  | <i>Podocarpus macrophyllus</i>                |   | 2n=36  | 1.93-5.26 $\mu\text{m}$ | 2.73 |
| C15  | <i>Idesia polycarpa</i>                       |   | 2n=38  | 0.77-2.46 $\mu\text{m}$ | 3.19 |
| C16* | <i>Ilex chinensis</i>                         |   | 2n=40  | 0.63-1.58 $\mu\text{m}$ | 2.51 |
| D1   | <i>Triticum aestivum</i>                      |   | 2n=42  | 3.85-6.88 $\mu\text{m}$ | 1.79 |
| D2   | <i>Fraxinus pennsylvanica</i>                 |   | 2n=46  | 0.88-1.96 $\mu\text{m}$ | 2.23 |
| D3   | <i>Syringa oblata</i>                         |   | 2n=46  | 0.98-1.93 $\mu\text{m}$ | 1.97 |
| D4   | <i>Ligustrum</i> $\times$ <i>vicaryi</i>      |   | 2n=46  | 0.95-2.14 $\mu\text{m}$ | 2.25 |
| D5   | <i>Ligustrum lucidum</i>                      |   | 2n=46  | 0.70-2.11 $\mu\text{m}$ | 3.01 |
| D6*  | <i>Triadica sebifera</i>                      |   | 2n=88  | 0.60-1.23 $\mu\text{m}$ | 2.05 |
| D7   | <i>Zanthoxylum nitidum</i>                    |   | 2n=66  | 1.40-2.56 $\mu\text{m}$ | 1.83 |
| D8   | <i>Zanthoxylum bungeanum</i> 'Hanyuanhuajiao' | 1 | 2n=76  | 0.84-1.72 $\mu\text{m}$ | 2.05 |
| D9   | <i>Zanthoxylum bungeanum</i> 'Hanyuanhuajiao' | 2 | 2n=134 | 0.88-1.82 $\mu\text{m}$ | 2.07 |
| D10  | <i>Zanthoxylum bungeanum</i> 'Hanyuanhuajiao' | 3 | 2n=136 | 0.91-2.14 $\mu\text{m}$ | 2.35 |
| D11  | <i>Zanthoxylum bungeanum</i> 'Hanyuanhuajiao' | 4 | 2n=160 | 0.74-1.68 $\mu\text{m}$ | 2.27 |
| D12  | <i>Zanthoxylum armatum</i> 'Hongyatengjiao'   | 1 | 2n=96  | 0.70-1.47 $\mu\text{m}$ | 3.01 |
| D13  | <i>Zanthoxylum armatum</i> 'Tengjiao'         |   | 2n=100 | 0.88-2.00 $\mu\text{m}$ | 2.27 |
| D14  | <i>Zanthoxylum armatum</i> 'Hongyatengjiao'   | 2 | 2n=102 | 0.95-2.04 $\mu\text{m}$ | 2.14 |
| D15  | <i>Zanthoxylum armatum</i> 'Jiuyeqing'        |   | 2n=104 | 0.88-2.00 $\mu\text{m}$ | 2.27 |
| D16  | <i>Zanthoxylum armatum</i> 'Putaoqingjiao'    |   | 2n=132 | 0.74-1.79 $\mu\text{m}$ | 2.42 |

Note: asterisk (\*) in Table 2 indicates chromosome number of three species are first reported