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Cytogenetics and Genome Size Evolution in *Illicium* L.

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Abstract. Illicium **is an ancient genus and member of the earliest diverging angiosperms known as the Amborellales, Nymphaeales, and Austrobaileyales (ANA) grade. These adaptable, broadleaf evergreen shrubs, including ≈40 species distributed throughout Asia and North America, are valued for diverse culinary, medicinal, and ornamental applications. The study of cytogenetics of *Illicium* can clarify various discrepancies and further elucidate chromosome numbers, ploidy, and chromosome and genome size evolution in this basal angiosperm lineage and provide basic information to guide plant breeding and improvement programs. The objectives of this study were to use flow cytometry and traditional cytology to determine chromosome numbers, ploidy levels, and relative genome sizes of cultivated *Illicium*. Of the 29 taxa sampled, including ≈11 species and one hybrid, 2C DNA contents ranged from 24.5 pg for *Illicium lanceolatum* to 27.9 pg for *Illicium* aff. *majus*. The genome sizes of *Illicium* species are considerably higher than other ANA grade lineages indicating that *Illicium* went through considerable genome expansion compared with sister lineages. The New World sect. *Cymbostemon* had a slightly lower mean 2C genome size of 25.1 pg compared with the Old World sect. *Illicium* at 25.9 pg, providing further support for recognizing these taxonomic sections. All taxa appeared to be diploid and $2n = 2x = 28$, except for *Illicium floridanum* and *Illicium mexicanum* which were found to be $2n = 2x = 26$, most likely resulting from dysploid reduction after divergence into North America. The base chromosome number of $x = 14$ for most *Illicium* species suggests that *Illicium* are ancient paleotetraploids that underwent a whole genome duplication derived from an ancestral base of $x = 7$. Information on cytogenetics, coupled with phylogenetic analyses, identifies some limitations, but also considerable potential for the development of plant breeding and improvement programs with this genus.**

Illicium, previously considered as the sole genus in Illiiaceae, has more recently been placed in the Schisandraceae within the order Austrobaileyales (The Angiosperm Phylogeny Group, 2016). The Austrobaileyales, along with the orders Nymphaeales and Amborellales, form the most basal branches of the angiosperm phylogeny, cumulatively referred to as the ANA grade (Vialette-Guiraud et al., 2011), and have origins dating back to the Late Jurassic to Early Cretaceous ≈160–130 million years ago (Soltis et al., 2008). The ancient origin of ANA grade angiosperms (including *Illicium*), morphological similarity with early fossils, and limited molecular divergence suggests that these lineages may provide features and insights into the foundational traits of early angiosperms (Morris et al., 2007; Soltis et al., 2009).

Chromosome numbers and nuclear genome sizes vary widely among angiosperms. The original base chromosome number of the angiosperm linage was most likely somewhere between $x = 6$ and 9 (Ehrendorfer et al., 1968; Raven, 1975; Stebbins, 1971) and increased over time with repeated cycles of whole genome duplication events (Soltis et al., 2003). Genomes can further expand through amplification of noncoding, repetitive DNA including retrotransposons (Leitch and Leitch, 2013). However, this ''one-way ticket to genomic obesity'' (Bennetzen and Kellogg, 1997) is often tempered by genome downsizing that can occur through recombination-based processes, such as unequal recombination and illegitimate recombination (Grover and Wendel, 2010; Soltis et al., 2015). There have only been limited reports on chromosome numbers and relative genome sizes for species and cultivars of *Illicium*. A base chromosome number of $x = 14$ and diploidy has been reported for *Illicium anisatum*, *Illicium parviflorum*, *Illicium ternstroemioides*, and *Illicium verum* (Baolian, 1990; Lepper, 1982; Lin, 1989; Stone and Freeman, 1968; Whitaker, 1933). However, conflicting chromosome counts for *I. floridanum* exist, with different sources reporting a base chromosome number of either $x = 13$ (Stone, 1965; Stone and Freeman, 1968) or $x = 14$ (Whitaker, 1933). Reports of genome sizes for *Illicium* are also limited and variable. Nagl et al. (1977) reported a 2C genome size of 6.72 pg (determined with scanning densitometry of Feulgen-stained nuclei) whereas Pellicer et al. (2013, Supplemental Table 2) reported genome sizes for *Illicium henryi* and *Illicium simonsii* to be 29.3 and 29.2 pg, respectively (determined with flow cytometry). Additional study of cytogenetics of *Illicium* can clarify various discrepancies and further elucidate chromosome and genome size evolution in this basal angiosperm lineage.

After many millions of years of divergence, there are now ≈40 extant species of shrubs and small trees within the genus *Illicium* (Morris et al., 2007), including six found in the New World and the remaining species distributed throughout Asia (Shu, 2008; Vincent, 1997). Parsing the taxonomy and systematics of *Illicium* has been challenging because of the somewhat surprising morphological similarities between species despite the age and broad distribution of the genus (Morris et al., 2007). Molecular phylogeny studies have helped to clarify some species relationships (Hao et al., 2000; Morris et al., 2007; Oh et al., 2003). Morris et al. (2007), in agreement with Hao et al. (2000), provided a revised sectional classification of the genus with two sections: sect. *Illicium* (including the Old World species) and sect. *Cymbostemon* (including the New World species). Within sect. *Cymbostemon* there was also strong support for separation between the *I. floridanum* + *I. mexicanum* clade and the *I. parviflorum* + *Illicium hottense* + *Illicium cubense* + *Illicium ekmanii* clade.

Illicium are also of interest because of their unique and diverse plant metabolites that have both medicinal and culinary uses. Certain species of *Illicium* have been used in traditional medicine for treating pain, rheumatism, and skin inflammation (Liu et al., 2009). Most plant organs of *Illicium* are noticeably pungent with a strong odor of anise/terpenes. Extensive studies have been conducted to identify these compounds that include prenylated C_6–C_3 compounds, neolignans, and secoprezizaane-type sesquiterpenes that are found exclusively in *Illicium* (Liu et al., 2009). Many of these compounds and/or crude extracts from *Illicium* are

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biologically active and demonstrate antibacterial, anticancer, anti-inflammatory, antioxidant, antiviral (antiHIV), insecticidal, neurotoxic, neurotrophic, and phytotoxic activities (Liu et al., 2009). *Illicium verum* is of importance as a crop and the seedpods are well known in kitchens as the spice star anise (not to be confused with other species, which have a similar appearance and can be toxic). However, most *I. verum* grown as a crop is used as a source of shikimic acid and is the primary ingredient in the antiflu medication oseltamivir phosphate, sold as Tamiflu® (Wang et al., 2011).

Illicium have a suite of desirable ornamental qualities that make them valuable as nursery and landscape crops. Many species have attractive, tropical-looking evergreen leaves and distinctive showy flowers in whites, pinks, and reds in spring and summer/fall. They are also broadly adaptable with some taxa being cold hardy to USDA zone 6, tolerant of shady and wet sites (Griffin et al., 2004), and resistant to many diseases and pests, most notably deer. Nonetheless, *Illicium* are relatively uncommon in American landscapes and few cultivars exist for taxa beyond the three North American native species, *I. floridanum*, *I. mexicanum*, and *I. parviflorum*, and the Asian *I. anisatum*. Fewer interspecific hybrids exist and only *I. floridanum* × *I. mexicanum* selections are currently available in the horticultural trade. Additional data on cytogenetics, including chromosome numbers and ploidy, of taxa in this genus would provide basic information to better enable plant breeding and improvement programs.

The objectives of this study were to determine chromosome numbers, ploidy levels, and relative genome sizes of cultivated *Illicium* taxa to gain further insights into the evolution, systematics, and the potential for interspecific hybridization.

Materials and Methods

Cytology. Chromosome counts were determined for six species representing major phylogenetic clades and sub-clades (Morris et al., 2007; Oh et al., 2003) to verify chromosome numbers and to calibrate genomes size measurements with ploidy levels. Actively growing root tips were excised and placed in a mixture of 4 mM 8-hydroxyquinoline and 0.249 mM cycloheximide for 3 h at room temperature and then three to five additional hours in the dark at 4 °C. Roots were placed into 3 mL of Carnoy's solution (six 95% ethanol: three chloroform: one glacial acetic acid by volume) until the following morning, when the roots were stored in 70% ethanol. Fixed roots were hydrolyzed in a 3:1 95% ethanol: 12 M HCl solution for ≈6–15 min. *Illicium anisatum* Crowder #2 (2007-117) and *I. floridanum* 'Swamp Hobbit' (2009-148) were then stained with a modified carbol fuchsin stain for at least 5 min (Kao, 1975). Because of poor staining with carbol fuchsin, *I. henryi* (1996-039), *I. parviflorum* 'Forest Green' (1998-329), *I. mexicanum* 'Aztec Fire' (1996-041), and *I. verum* (2007-063) were stained with 1% acetocarmine stain for at least 5 min after which the root tips were sectioned onto a slide with a drop of stain, overlaid with a cover slip, and heated warm to the touch (Singh, 2003). Root tips were squashed underneath a cover slip on a microscope slide, and chromosomes were counted under oil immersion at ×1000 magnification.

Flow cytometry. Relative 2C genome sizes were determined using a flow cytometer (Partec PA-II; Partec, Münster, Germany). Leaf tissue from 29 *Illicium* taxa was obtained from the JC Raulston Arboretum, Raleigh, NC; Mountain Crop Improvement Laboratory, Mills River, NC; Dan Hinkley, Indianola, WA; and Ken Cox and Steve Hootman, Federal Way, WA (Table 1). Young leaf tissue of *Illicium* samples and an internal standard [*Pisum sativum* 'Ctirad' 2C DNA = 8.76 pg (Greilhuber et al., 2007)] were finely chopped together using a razor blade in a petri dish containing 400 µL of nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer; Sysmex Partec, Görlitz, Germany). The chopped sample and internal standard were then filtered through a 50-µm nylon mesh filter into a test tube and stained with 1600 µL of 4′, 6-diamidino-2-phenylindole (DAPI) staining buffer (Cystain ultraviolet Precise P Staining Buffer; Sysmex Partec) immediately before analysis. The flow cytometer was used to process the stained nuclei, with at least 3000 counts per subsample, two subsamples per taxon, and a CV less than 5% where possible.

Table 1. Relative 2C genome sizes for *Illicium* taxa.

Source[z]	Accession	Taxon	Relative 2C genome size ±SE (pg)[y]
sect. *Cymbostemon*			25.13 ± 0.15 a
MCIL	2015-128	*Illicium floridanum* 'Breezy Hill'	25.22 ± 0.50
MCIL	1998-595	*I. floridanum* 'Halley's Comet'	24.74 ± 0.00
JCRA	001113	*I. floridanum* 'Jo's Variegated'	24.54 ± 0.13
JCRA	950630	*I. floridanum* 'Semmes'	25.00 ± 0.07
MCIL	1998-596	*I. floridanum* 'Semmes'	24.65 ± 0.11
MCIL	2009-148	*I. floridanum* 'Swamp Hobbit'	24.87 ± 0.25
MCIL	2007-224	*I. floridanum* 'Thayer's Choice'	25.01 ± 0.57
MCIL	2015-022	*I. floridanum* 'Zodiac'	25.01 ± 0.60
JCRA	xx0685	*I. floridanum* f. *album*	25.96 ± 0.49
MCIL	1996-041	*Illicium mexicanum* 'Aztec Fire'	25.48 ± 0.06
MCIL	1998-600	*I. mexicanum* × *floridanum* 'Woodland Ruby'	24.08 ± 0.51
JCRA	130079	*Illicium parviflorum* 'Florida Sunshine'	25.66 ± 0.50
MCIL	1998-329	*I. parviflorum* 'Forest Green'	24.84 ± 0.43
JCRA	970678	*I. parviflorum* 'Forest Green'	26.05 ± 0.29
JCRA	970796	*I. parviflorum* small leaf	25.82 ± 0.06
sect. *Illicium*			25.85 ± 0.24 b
JCRA	070640	*Illicium anisatum* 'Murasaki-no-sato'	26.10 ± 0.06
JCRA	011786	*I. anisatum* 'Pink Stars'	26.40 ± 0.07
JCRA	140326	*I. anisatum* White Margined	26.47 ± 0.27
MCIL	1996-039	*Illicium henryi*	25.39 ± 0.03
JCRA	110017	*Illicium jiadifengpi*	25.15 ± 0.03
MCIL	1998-597	*Illicium lanceolatum*	24.46 ± 0.10
JCRA	150385	*Illicium* aff. *majus*	27.87 ± 0.40
DH	DJHV 8032	*Illicium merrillianum* (wild collected from North Vietnam)	26.16 ± 0.29
MCIL	H2007-146-001	*Illicium simonsii*	25.92 ± 0.49
MCIL	H2007-147-004	*I. simonsii*	26.08 ± 0.17
MCIL	2007-063	*Illicium verum*	24.89 ± 0.02
MCIL	2001-134	*Illicium wardii*	24.91 ± 0.15
KCSH	KCSH#0374	*Illicium griffithii* (wild collected from Arunachal Pradesh, India)	25.39 ± 0.41
DH	DJHM 13141	*Illicium* sp. (wild collected from North Myanmar)	26.68 ± 0.16

[z]DH = Dan Hinkley, Indianola, WA; JCRA = JC Raulston Arboretum, Raleigh, NC; KCSH = Ken Cox and Steve Hootman, Federal Way, WA; and MCIL = Mountain Crop Improvement Laboratory, Mills River, NC.

[y]Overall means for sect. *Cymbostemon* (25.13 pg) (a) and sect. *Illicium* (25.85 pg) (b) were significantly different at $P \leq 0.01$.

Genome size (2C) of samples was calculated as: 2C = genome size of standard × (mean fluorescence value of sample ÷ mean florescence value of standard). The experimental design was completely randomized.

Data for 2C genome sizes were subjected to analysis of variance as a function of taxonomic sections (sect. *Cymbostemon* and sect. *Illicium*) and representative species of clades within sect. *Cymbostemon* [(*I. floridanum* + *I. mexicanum*) and (*I. parviflorum*)] (Proc GLM; SAS Version 9.2; SAS Inst., Cary, NC).

Results and Discussion

Chromosome counts of accessions of *Ilicium* sect. *Illicium* taxa, including *I. anisatum*, *I. verum*, and *I. henryi* (Fig. 1A–C), were $2n = 2x = 28$. Although, chromosome counts have not been reported previously for *I. henryi*, these results are consistent with prior reports for *I. anisatum* and *I. verum* (Baolian, 1990; Whitaker, 1933). Chromosome counts for *Illicum* sect. *Cymbostemon* were more variable. *Illicium parviflorum* (Fig. 1D), was found to be $2n = 2x = 28$, substantiating reports by Stone and Freeman (1968). However, both *I. floridanum* 'Swamp Hobbit' and *I. mexicanum* 'Aztec Fire' were found to have a reduced chromosome number of $2n = 2x = 26$ (Fig. 1E–F). This reduced chromosome number is newly reported for *I. mexicanum*, but in agreement with counts by Stone and Freeman (1968) for *I. floridanum*, and further suggests that counts of $2n = 2x = 28$ for *I. floridanum* by Whitaker (1933) were in error. The reduced base chromosome number for *I. floridanum* and *I. mexicanum* relative to other *Illicium* is consistent with the phylogenetic work of Morris et al. (2007), who found these two species to form a separate clade from other *Illicium* within sect. *Cymbostemon*. Interestingly, these two species also share ligulate tepals that are unique within this section (Morris et al., 2007).

The base chromosome number of $x = 14$ for most *Illicium* species supports the supposition that *Illicium* are ancient paleotetraploids that underwent a whole genome duplication derived from an ancestral base of $x = 7$. Because of the similar ploidy of all modern *Illicium*, this suggests that the duplication event occurred before diversification of the New World crown group, estimated by Morris et al. (2007) to be a minimum age of 5 million years. The reduced base karyotypes of *I. floridanum* and *I. mexicanum* probably resulted more recently from dysploid reduction resulting from a reciprocal translocation (chromosomal fusion), yielding $x = 13$ (Ehrendorfer et al., 1968; Schubert and Lysak, 2011).

Implications of this research for breeding and crop improvement suggest there is wide similarity in chromosome numbers and ploidy within the genus, except for *I. floridanum* and *I. mexicanum*. The close relationship between these two species and unique chromosome numbers helps explain their ability to produce fertile hybrids (T.G. Ranney, personal observation), but most likely will limit their potential to produce viable hybrids with species with $2n = 2x = 28$. Selected crosses between species within sect. *Illicium* have been successful, including *I. anisatum* × *wardii* and *I. anisatum* × *simonsii* (T.G. Ranney, personal observation), which is not unexpected knowing that they share similar chromosome numbers and ploidy and are placed in the same phylogenetic clade/section. These results and observations provide hope that many of the sect. *Illicium* species may hybridize, allowing for breeding programs to improve these crops for medicinal, culinary, and ornamental applications. The potential for breeding between sections has yet to be determined.

Relative 2C genome sizes of 29 taxa representing ≈11 species and one hybrid of *Illicium* were surprisingly similar and ranged from 24.5 pg in *I. lanceolatum* to 27.9 pg in *I.* aff. *majus* (Table 1) and indicates a common ploidy for all *Illicium* species. The substantially higher genome size of *I.* aff. *majus* is of interest and may warrant a separate chromosome count to verify the base chromosome number in that taxon. Overall, these values were similar, but slightly lower than those values reported (Pellicer et al., 2013, Supplemental Table 2) for *I. henryi* and *I. simonsii* reported as 29.3 and 29.2 pg, respectively [determined with propidium iodide (PI stain)]. Different fluorochrome stains may give slightly different estimates of genome size, though both PI and DAPI have been found to be effective and consistent for determining and comparing ploidy levels and relative genome size among closely related taxa (Parris et al., 2010). Furthermore, DAPI typically provides more precise and repeatable results as it is specific to double-stranded DNA and is not influenced by variable chromatin structure whereas fluorescence of PI is susceptible to staining inhibitors and their antagonists (Doležel and Bartoš, 2005; Greilhuber et al., 2007). We determined the relative genome size of *I. anisatum* to be 26.2 pg, nearly four times the value of 6.72 pg reported by Nagl et al. (1977), who used scanning densitometry with Feulgen-stained nuclei. This discrepancy is most likely reflective of the more accurate and reliable measures provided by flow cytometry.

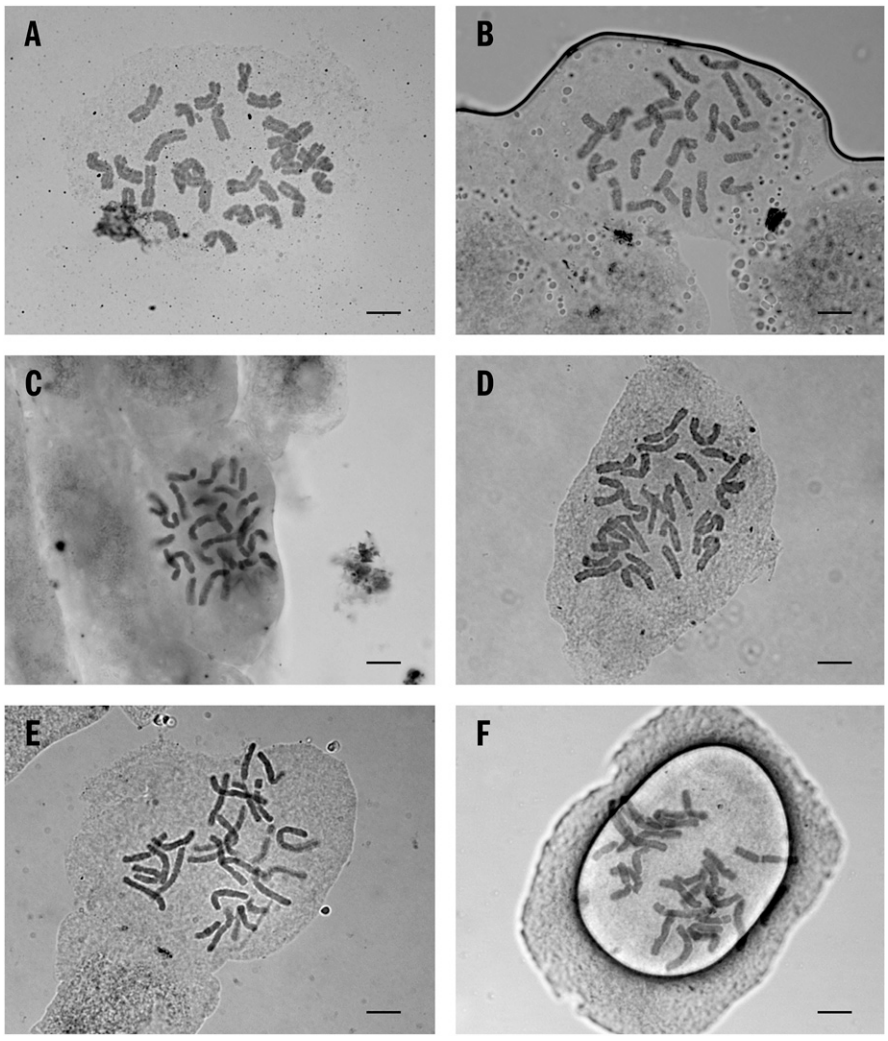

Fig. 1. Chromosomes of (**A**) *Illicium anisatum* ($2n = 28$), (**B**) *Illicium verum* ($2n = 28$), (**C**) *Illicium henryi* ($2n = 28$), (**D**) *Illicium parviflorum* ($2n = 28$), (**E**) *Illicium floridanum* ($2n = 26$), and (**F**) *Illicium mexicanum* ($2n = 26$). Bar = 15.86 µm.

Analysis of variance and comparison between sections showed that the New World sect. *Cymbostemon* had a slightly lower 2C genome size of 25.1 pg compared with the Old World sect *Illicium* at 25.9 pg, providing additional credence to these sectional designations. Despite substantial karyotypic differences and varying base chromosome numbers, there was not a significant difference in 2C genome sizes between clades (*I. floridanum* + *I. mexicanum*) and (*I. parviflorum*) within sect. *Cymbostemon*. The genome sizes of *Illicium* species are considerably higher than other ANA grade lineages. Although *Amborella*, the sole genus in Amborellales has a 2C genome size of 1.8 pg, the Nymphaeales range from 0.9 to 9.3 pg, and the Austrobaileyales range from 8.2 pg for *Trimenia* to as high as 29.3 pg for *Illicium* (Pellicer et al., 2013). Compared with other ANA grade linages, *Illicium* went through a process of considerable genome expansion, including at least one whole genome duplication, and additional within-ploidy genome increases. Despite this relatively large genome expansion, compared with the other ANA grade linages, it is somewhat surprising that genome sizes and ploidy levels of modern *Illicium* species are relatively conserved and appear somewhat static. Of course, many other angiosperm lineages have far exceeded the genome sizes of *Illicium* (e.g., *Paris japonica* with 2C = 304.5 pg, Pellicer et al., 2010).

The results of this study help to clarify and expand information on cytogenetics of *Illicium*. This information provides further insights into the evolution of chromosome numbers and genome sizes in this primitive, basal angiosperm lineage substantiating an ancestral base chromosome number of $x = 7$, ancient whole genome duplication, dysploid reduction in multiple species, substantial genome expansion compared with sister lineages, and classification of two taxonomic sections within the genus. Specific information on ploidy and chromosomes numbers, coupled with phylogenetic analyses, identifies some limitations, but also considerable potential for the development of breeding and improvement programs with this genus.

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