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Authors: Pelosi, Jessie A., Zumwalde, Bethany A., Hornych, Ondřej, Wheatley, Kayla, Kim, Emily H., et al.

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Lygodium japonicum (Lygodiaceae) Is Represented by a Tetraploid Cytotype in Florida

Jessie A. Pelosi*
Department of Biology, University of Florida, Gainesville, FL, USA 32611
Email: jessiepelosi@ufl.edu

Bethany A. Zumwalde
Department of Biology, University of Florida, Gainesville, FL, USA 32611
Email: bzumwalde@ufl.edu

Ondřej Hornych
Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ 37005
Email: hornych.ondrej@gmail.com

Kayla Wheatley
Department of Biology, University of Florida, Gainesville, FL, USA 32611
Institute of Economic Botany, New York Botanical Garden, Bronx, NY USA 10458
Email: kwheatley@nybg.org

Emily H. Kim
Department of Microbiology & Cell Sciences, University of Florida, Gainesville, FL USA 32611
Email: kim.emily@ufl.edu

Emily B. Sessa
Department of Biology, University of Florida, Gainesville, FL 32611
William and Lynda Steere Herbarium, New York Botanical Garden, Bronx, NY USA 10458
Email: esessa@nybg.org

Abstract.—Invasive species are one of the largest threats to vulnerable ecological communities and biodiversity today and are economic burdens across the globe. It is therefore crucial that we understand the origins and the driving forces that promote the establishment, persistence, and spread of these taxa. Polyploidy, or whole genome duplication, has been suggested as a possible factor facilitating the success of invasive taxa, yet is an understudied aspect in invasion biology. Although ferns are often neglected in invasive species inventories, several fern families are over-represented as naturalized and invasive taxa including the vining ferns in the family Lygodiaceae. The Japanese climbing fern, *Lygodium japonicum*, is native to eastern Asia, and since its introduction in the early 1900s through the ornamental plant trade, it has rapidly spread throughout the southeastern United States, creating dense thickets that smother native plants and disrupt agricultural pine logging. While previous chromosome counts of *L. japonicum* suggest that both diploid and tetraploid cytotypes occur in its native range, there are no data for populations in the invaded range to date. Using chromosome counts, flow cytometry, and spore size measurements, we assessed the ploidy of invasive populations of *L. japonicum* in the state of Florida. We found that *L. japonicum* is represented by a tetraploid cytotype throughout Florida. Our study is the first to examine the ploidy of invasive *L. japonicum* populations, although additional work will be needed to determine if this species is tetraploid throughout its invaded range.

Key Words: polyploidy, invasive species, cytology, fern, *Lygodium*, chromosomes

*Corresponding author

Polyploidy has long been considered a possible driver of invasive species' success (Baker and Stebbins, 1965). Multiple genomes may mask deleterious alleles and increase genetic diversity in the form of fixed heterozygosity, and compared to their diploid progenitors, polyploids may show altered morphology, increased ecological tolerance, and/or niche differentiation (reviewed by te Beest *et al.*, 2012). Variations in ploidal levels between native and invaded ranges have been observed in multiple angiosperms including *Senecio inaequidens* DC. (Asteraceae; Lafuma *et al.,* 2003), *Solidago gigantea* Aiton (Asteraceae; Schlaepfer *et al.,* 2008), and in Cactaceae (Lopes *et al.,* 2021). However, similar cytological patterns have yet to be explored in invasive ferns despite the prevalence of polyploidy (Wood *et al.,* 2009; Pelosi *et al.,* 2022) and invasive species (Jones *et al.,* 2019) in the clade.

Lygodium japonicum (Thunb.) Sw. is a highly invasive vining fern that has colonized much of the southeastern United States. Native to eastern Asia, records of *L. japonicum* in North America suggest an introduction occurred as early as the 1880s (Pemberton and Ferriter, 1998). In its native range, chromosome counts of *L. japonicum* have found both diploid ($2n$=58; Roy and Manton, 1964; Nakato, 1990) and tetraploid cytotypes (n=58; Manton and Sledge, 1954; Mitui,1965; Mitui, 1968). The specimen examined by Roy and Manton (1964) was from southern China, while the specimens analyzed by Mitui (1965), Mitui 1968, and Nakato (1990) were from Japan, and the Manton and Sledge (1954) sample was from an unidentified location. Compared to the genome size of *L. microphyllum* (Cav.) R.Br., a known diploid (1C=5.56pg, Kuo and Li, 2019), the only published 1C genome size estimate of 11.66pg for *L. japonicum* would make it a tetraploid (Hanson and Leitch, 2002). However, the specimen measured by Hanson and Leitch (2002) does not have an associated location, and the ploidal level of this species in its invaded range is therefore unknown. Here we used a combination of flow cytometry, spore size measurements, and chromosome counts to determine whether *L. japonicum* is represented by single or multiple cytotypes in the invaded range in Florida.

Materials and Methods

A total of 43 *L. japonicum* individuals was collected from nine populations throughout its range in Florida (Fig. 1A). Leaf material was stored at 4°C prior to preparation for flow cytometry or used fresh. For sporophytes, the flow cytometry procedure followed the simplified two-step protocol published by Doležel, Greilhuber, and Suda (2007). The sample was mixed with leaf tissue of an internal standard (*Vicia faba* 'Inovec', 2C=26.9pg [Doležel, Sgorbati, and Lucretti, 1992] for DAPI and *Chlorophytum comosum*, 2C=24.1pg [Hornych *et al.,* 2019] for PI) and stained with either DAPI or PI. The stained sporophyte samples were then analyzed using Partec PA II (Sysmex, Münster, Germany) and Sysmex CyFlow Space (Sysmex, Münster, Germany) flow cytometers, for DAPI and PI-stained samples, respectively. A total of 17 sporophyte samples were analyzed via DAPI staining to test whether samples differ in their ploidy level and to estimate AT base percentage. Absolute genome size was calculated

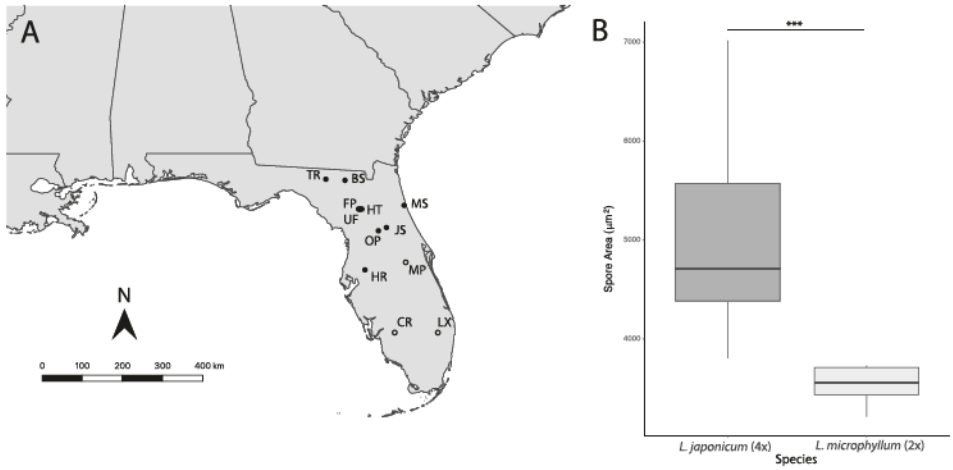

FIG. 1. A) Populations of *Lygodium japonicum* (black) and *L. microphyllum* (light gray) analyzed in this study. Population abbreviations for *L. japonicum* are as follows: BS – Big Shoals State Forest, FP – Forest Park, HR – Hillsborough River State Park, HT – Hawthorne Trail, JS – Juniper Springs, MS – Matanzas State Forest, OP – Ocklawaha Prairie Restoration Area, TR – Twin Rivers State Forest, UF – University of Florida Campus. Population abbreviations for *L. microphyllum* are as follows: CR – CREW Bird Rookery, LX – Loxahatchee National Wildlife Refuge, MP – Moss Park. B) Average spore area of *Lygodium japonicum* (4x, dark gray) and *L. microphyllum* (2x, light gray). *** significance level denotes $p < 0.001$.

for three sporophyte samples analyzed using the PI stain. The ratio of mean sample and standard fluorescence was calculated for all analyses. Consequently, the percentage of AT was estimated using the formula of Šmarda *et al.* (2008).

Spores were collected from fertile *L. japonicum* samples (21 of 43) and from *L. microphyllum* from three populations in Florida for comparison. Several spores from each specimen were sown on Bold's media (Bold, 1957) with Nitsch's micronutrients (Nitsch, 1951) and grown in a Percival growth chamber at 25°C on a 12:12 light:dark schedule. Up to three gametophytes from a specimen were pooled and used for genome size estimation with flow cytometry following a modified protocol from Roberts, Gladis, and Brumme (2009) and Loureiro *et al.* (2007); see Appendix 1 for a full protocol. Gametophyte samples were mixed with leaf tissue from an internal size standard (*Pisum sativum,* 2C=9.09pg [Doležel *et al.,* 1998]) and stained with PI. Stained samples were then analyzed using a BD Accuri C6 flow cytometer (Franklin Lakes, New Jersey, U.S.) at the University of Florida ICBR Cytometry Core Facility, RRID:SCR_019119. Although spores have been used previously to estimate genome size (*e.g.*, Kuo *et al.,* 2017), the number of spores isolated from each specimen was insufficient for flow cytometry. Furthermore, given that *Lygodium* gametophytes grow rapidly from spores (Lott *et al.,* 2003) and are mostly composed of a single cell layer (Takahashi *et al.,* 2015), these tissues are ideal for isolating nuclei for flow cytometry.

To compare spore sizes (a common proxy for ploidy in ferns, *e.g.*, Barrington, Paris, and Ranker, 1986; Schuettplez, Pryer, and Windham, 2015) spores were fixed on slides and photographed at 400x magnification with an AmScope T340B-LED microscope and an AmScope MU1000-HS camera. Composite images were generated from multiple focus depths. At most ten spores per sporophyte were photographed. Spore length and width were measured following Barrington, Patel, and Southgate (2020). We further measured the area of the spore using the free-form polygon function in ImageJ (Schneider, Rasband, and Eliceiri, 2012). We used nested ANOVAs with populations nested within species to compare average spore length, width, and area between *L. japonicum* and *L. microphyllum* in R ver. 4.1.3 (R Core Team, 2022).

Sporophytes derived from sporophytic selfing (*sensu* Haufler *et al.*, 2016) were used for chromosome squashes. Actively dividing root tips were placed in 2mM 8-hydroxyquinoline for 4-6 hours at 4°C, fixed in Carnoy's solution (3:1 ethanol:glacial acetic acid) for 18-24 hours, and stored at room temperature in 70% ethanol until squashed. Tissues were transferred to 1M HCl at 60°C for 45-60 minutes depending on the size of the root, the root cap was removed to release the underlying cells which were macerated for about 30 seconds with a razor blade, and then squashed on a glass microscope slide with 1% aceto-orcein dye and 45% acetic acid. Squashes viewed with an AmScope Phase Contrast Kit-B/T 400/490/590/600 series lens and photographed at 1000x with the same scope and camera as above.

Results and Discussion

We determined that *L. japonicum* is tetraploid in Florida, with an average 1C genome size of 14.38 ± 0.67pg (mean ± 1SD) and 48.0 ± 2.69% GC base content. The GC content of *L. japonicum* is notably high but ferns seem to have higher GC content compared to angiosperms (Šmarda *et al.*, 2019). We estimated the absolute genome size of 13 total samples from six populations: three sporophyte samples from three populations and ten gametophyte samples from three populations. The average genome size from sporophyte samples (13.28 ± 0.1pg) was slightly lower than from gametophyte samples (14.72 ± 0.26pg), which may be attributable to the use of different size standards and flow cytometers.

Compared to Hanson and Leitch (2002), our genome size estimates of *L. japonicum* are about 1.23 times larger than their reported size estimate of 1C=11.66pg, which could be explained by regional or methodological/technological differences. In both studies, the same method (flow cytometry) was used but with a different internal standard (Hanson and Leitch used *Allium cepa*, 2C=33.5pg). To infer the ploidy level of *L. japonicum,* we combined these flow cytometric data with chromosome counts. Compared to *L. microphyllum*, which is diploid with $2n$=60 (Fig. 2), the chromosome count of *L. japonicum* in this study is tetraploid with $2n$=116 (Fig. 2). Given that the 1C

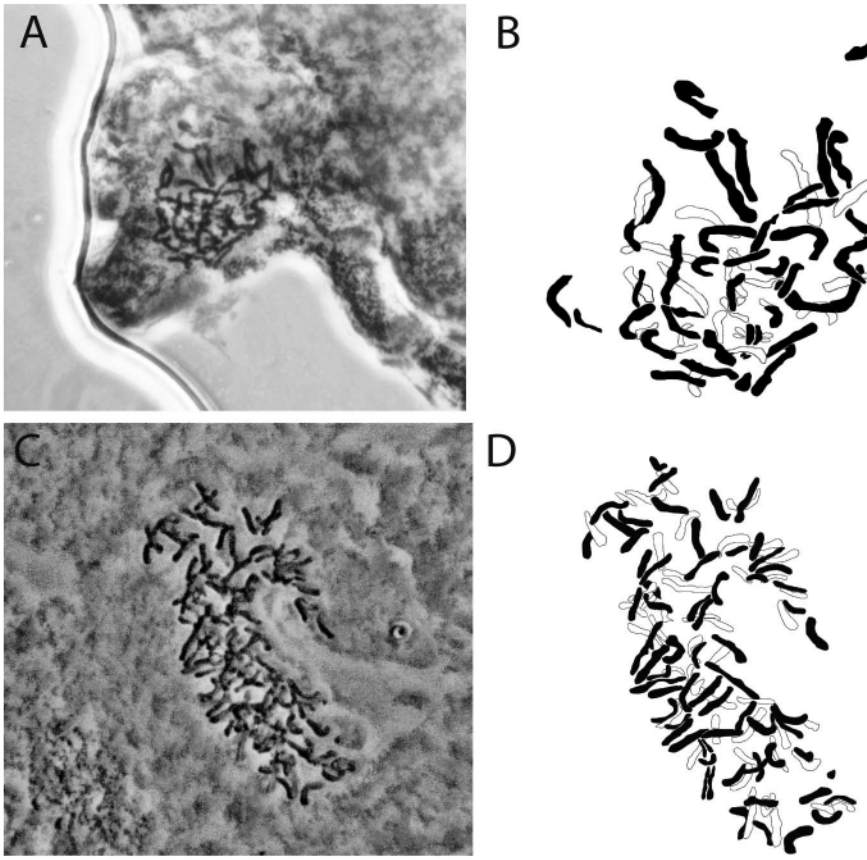

FIG. 2. Mitotic chromosome squashes from root tips of *Lygodium microphyllum* (A, B) and *L. japonicum* (C, D). Explanatory diagrams are provided in B and D, with white outlined chromosomes underlying black chromosomes, to enhance clarity.

genome size of diploid *L. microphyllum* is 6.20pg, the genome size estimates of *L. japonicum* of 1C = 14.38pg correspond to a tetraploid cytotype.

Although chromosome counts are the most definitive way to assess ploidy, there are drawbacks and difficulties with generating these data. Flow cytometry has therefore been an important supplement for inferring ploidy levels in plants (Doležel, Greilhuber, and Suda, 2007). Chromosome counts are time- and labor-intensive and require actively dividing material, such as root tips for mitotic counts or spore mother cells for meiotic counts. In contrast, several samples can be run in a few hours on a flow cytometer and a variety of tissues can be stored for up to months for use in genome size estimation. Particular difficulties with generating chromosome counts in *Lygodium* included hardiness of the root cap, "sticky" chromosomes (*e.g.*, Walker, 1966), the relatively high number of chromosomes resulting in a high degree of overlap, and initial overstaining of the cytoplasm. By integrating chromosome

TABLE 1. Chromosome numbers, ploidy, genome size, and average spore measurements of two invasive *Lygodium* species in Florida. *Genome size from Clark *et al.* (2016); [+]Genome size from Kuo and Li (2019).

Species	Approximate 2*n* Chromosome Number	Ploidy	Genome Size (1C, pg) Prior Study / This Study	Spore Length (μm)	Spore Width (μm)	Spore Area (μm^2)
L. japonicum	116	4x	11.66* / 14.38	70.7	78.3	4993
L. microphyllum	60	2x	5.56[+] / 6.20	58.2	64.0	3538

counts and flow cytometry, we were able to expand our ability to infer cytotypes of samples across its range in Florida.

The average spore lengths of *L. japonicum* (70.7μm) and *L. microphyllum* (58.2μm, Table 1) are similar to measurements reported by Hanks (1998) based on Scanning Electron Micrographs of herbarium material, with ranges of 70-79μm and 62-73μm for *L. japonicum* and *L. microphyllum*, respectively. There was a larger variation in spore measurements in *L. japonicum* (ranges in length: 62.0-85.2μm, width: 68.8-95.4μm, area: 3805-7014μm^2) compared to *L. microphyllum* (ranges in length: 56.6-60.6μm, width: 61.3-65.8μm, area: 3212-3729μm^2, Fig. 1B). This difference in variation may be due to larger sample sizes for *L. japonicum,* which was the focus of our study. There was a significant difference in spore area between species ($F_{1,19} = 16.297$, $p < 0.001$, Fig. 1B), but not between populations within species ($F_{7,19} = 0.369$, $p > 0.05$). We also found a significant difference in spore length ($F_{1,19} = 25.985$, $p < 0.001$) and spore width ($F_{1,19} = 22.814$, $p < 0.001$) between species, but not within populations within species ($F_{7,19} = 0.629$, $p > 0.05$; $F_{7,19} = 0.603$, $p > 0.05$ for length and width, respectively). Interestingly, Hanks (1998) did not find a correlation between spore size and ploidal level in *Lygodium*, although this correlation is supported in several fern genera with both monolete and trilete spores (*e.g.*, *Adiantum* and *Polystichum*; Barrington, Patel, and Southgate, 2020) and in the present study. Unlike Barrington, Patel, and Southgate (2020) and this study, Hanks (1998) took measurements along the longest axis of each tetrahedral spore, which may account for differences in spore sizes. Spores of *Lygodium* spp. also vary in ornamentation, which may impact these measurements. Furthermore, spore measurements by Hanks (1998) were not made on the same samples used for ploidal estimates, rather, ploidy was determined based on literature searches. Future phylogenetic and cytological studies across the genus will be necessary to verify if spore size is a viable proxy for ploidal level in *Lygodium*.

Our study is the first to examine the possibility of ploidal variation in *L. japonicum* in its introduced range. The difference in the ploidal level between native and invaded range can be important in determining the origin(s) of the invasion when compared to the cytotype distribution in the native range. Whether *L. japonicum* is tetraploid throughout its invaded range is unknown and further investigation is needed to determine how and if cytotype variation is present in both native and invaded ranges. Despite its ecological and

economic impacts, the mode of polyploidization (allo- or autopolyploidy) has yet to be explored for *L. japonicum*, which can impact patterns of inheritance and genetic variation (Parisod, Holdregger, and Brochmann, 2010). These and other questions remain unanswered yet are critical to understanding the evolution, tolerance, and persistence of this invasive fern.

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Literature Cited

Baker, H. G., and G. L. Stebbins (eds.). 1965. *The Genetics of Colonizing Species: Proceedings of the First International Union of Biological Sciences on General Biology*. Academic Press, New York.

Barrington, D. S., C. A. Paris, and T. A. Ranker. 1986. Systematic inference for spore and stomate size in ferns. American Fern Journal 76:149-159.

Barrington, D. S., N. R. Patel, and M. W. Southgate. 2020. Inferring the impacts of evolutionary history and ecological constraints on spore size and shape in the ferns. Applications in Plant Sciences 8(4):e11339.

Bold, H. C. 1957. *Morphology of Plants*. Harper. New York.

Clark, J., O. Hidalgo, J. Pellicer, H. Lui, J. Marquardt, Y. Robert, M. Christenhusz, S. Zhand, M. Gibby, I. J. Leitch, and H. Schneider. 2016. Genome evolution of ferns: evidence for relative stasis of genome size across the fern phylogeny. New Phygologist 210:1072-1082.

Doležel, J., J. Greilhuber and J. Suda. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nature Protocols 2:2233–2244.

Doležel, J., S. Sgorbati, and S. Lucretti. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Pysiologica Plantarum 85:625-631.

Doležel, J., J. Greilhuber, S. Lucretti, A. Meister, M. A. Lysák, L. Nardi, and R. Obermayer. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. Annals of Botany 82(Suppl.A):17-26.

Galbraith, D. W. 2009. Simultaneous flow cytometric quantification of plant nuclear DNA contents over the full range of described angiosperm 2C values. Cytometry A 75A:692-698.

Hanks, J. G. 1998. A monographic study of *Lygodium* Swartz (Pteridophyta: Lygodiaceae). PhD Dissertation, The City University of New York.

Hanson, L., and I. L. Leitch. 2002. DNA amounts for five pteridophyte species fill phylogenetic gaps in C-value data. Botanical Journal of the Linnean Society 140:169-173.

Haufler, C. H., K. M. Pryer, E. Schuettpelz, E. B. Sessa, D. R. Farrar, R. Moran, J. J. Schneller, J. E. Watkins, and M. D. Windham. 2016. Sex and the single gametophyte: revising the homosporous vascular plant life cycle in light of contemporary research. BioScience 66:928-937.

Hornych, O., L. Ekrt, F. Riedel, P. Koutecký, and J. Košnar. Asymmetric hybridization in Central European populations of the *Dryopteris carthusiana* group. American Journal of Botany 106:1477-1486.

Jones, E. J., T. Kraaij, H. Fritz, and D. Moodley. 2019. A global assessment of terrestrial alien ferns (Polypodiophyta): species' traits as drivers of naturalisation and invasion. Biological Invasions 21:861-873.

Kuo, L-Y., and F-W. Li. 2019. A roadmap for fern genome sequencing. American Fern Journal 109:212-223.

Kuo, L-Y., Y-J. Huang, J. Chang, W-L. Chiou, and Y-M. Huang. 2017. Evaluating the spore genome sizes of ferns and lycophytes: a flow cytometry approach. New Phytologist 213:1974-1983.

Lafuma, L., K. Balkwill, E. Imbert, R. Verlaque, and S. Maurice. 2003. Ploidy level and origin of the European invasive weed *Senecio inaequidens* (Asteraceae). Plant Systematics and Evolution 243:59-72.

Lopes, S., L. Mota, M. Castro, G. Nobre, A. Novoa, D.M. Richardson, J. Loureiro, and S. Castro. 2021. Genome size variation in Cactaceae and its relationship with invasiveness and seed traits. Biological Invasions 23:3047-3062.

Lott, M. S., J. C. Volin, R.W. Pemberton, and D. F. Austin. 2003. The reproductive biology of the invasive ferns *Lygodium microphyllum* and *L. japonicum* (Schizaeaceae): implications for invasive potential. American Journal of Botany 90:1114-1152.

Loureiro, J., E. Rodriguez, J. Doležel, and C. Santos. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Annals of Botany 100:875- 888.

Manton, I., and W. A. Sledge. 1954. Observations on the cytology and taxonomy of the pteridophyte flora of Ceylon. Philosophical Transactions of the Royal Society of London Series B, Biological Sciences 238:127-185.

Mitui, K. 1965. Chromosome studies on Japanese ferns. Journal of Japanese Botany 40:117- 124.

Mitui, K. 1968. Chromosomes and speciation in ferns. Science Reports of the Tokyo Bunrika Daigaku Section B 13:285-333.

Nakato, N. 1990. Notes on chromosomes of Japanese pteridophytes. Journal of Japanese Botany 65:204-209.

Nitsch, J. P. 1951. Growth and development *in vitro* of excised ovaries. American Journal of Botany 38:566.

Parisod, C., R. Holdregger, and C. Brochmann. 2010. Evolutionary consequences of autopolyploidy. New Phytologist 186:5-17.

Pelosi, J. A., E. H. Kim, W. B. Barbazuk, and E. B. Sessa. 2022. Phylotranscriptomics illuminates the placement of whole genome duplications and gene retention in ferns. Frontiers in Plant Science 13:882441.

Pemberton, R. W., and A. P. Ferriter. 1998. Old World climbing fern (*Lygodium microphyllum*), a dangerous invasive weed in Florida. American Fern Journal 88:165- 175.

R Core Team. 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna Austria. www.R-project.org

Roberts, A.V., T. Gladis, and H. Brumme. 2009. DNA amounts of roses (*Rosa* L.) and their use in attributing ploidy levels. Plant Cell Reports 28:61-71.

Roy, S. K., and I. Manton. 1964. A new base number in the genus *Lygodium*. New Phytologist 64:286-292.

Schlaepfer, D.R., P. J. Edwards, J. C. Semple, and R. Billeter. 2008. Cytogeography of *Solidago gigantea* (Asteraceae) and its invasive ploidy level. Journal of Biogeography 35:2119-2127. 2008

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Imagine to ImageJ: 25 years of image analysis. Nature Methods 9:671-675.

Schuettpelz, E., K. M. Pryer, and M. D. Windham. 2015. A unified approach to taxonomic delimitation in the fern genus *Pentagramma* (Pteridaceae). Systematic Botany 40:629- 644.

Šmarda, P., P. Bureš, L. Horová, B. Foggi, and G. Rossi. 2008. Genome size and GC content evolution of *Festuca*: ancestral expansion and subsequent reduction. Annals of Botany 101:421–433.

Šmarda, P, O. Knápek, A. BŘezinová, L. Horová, V. Grulich, J. Danihelka, P. Veselý, J. Šmarda, O. Rotreklová, and P. Bureš. 2019.Genome sizes and genomic guanine+cytosine (GC) content of Czech vascular flora with new estimates for 1700 species. Preslia 91:117–142.

Takahashi, N., C. Kami, I. Ota, N. Morita, and R. Imaichi. 2015. Developmental morphology of the typical cordate gametophyte of a homosporous leptosporangiate fern, *Lygodium japonicum* (Lygodiaceae), focusing on the initial cell behavior of two distinct meristems. American Journal of Botany 102:197-207.

te Beest, M., J. J. Le Roux, D. M. Richardson, A. K. Brysting, J. Suda, M. Kubesová, and P. Pysek. 2012. The more the better? The role of polyploidy in facilitating plant invasions. Annals of Botany 109:19-45.

Walker, T. G. 1966. IX.—A Cytotaxonomic survey of the pteridpphytes of Jamaica. Transactions of the Royal Society Edenborough 66(9):170-237.1966

WOOD, T. E., N. TAKEBAYASHI, M. S. BARKER, I. MAYROSE, P. B. GREENSPOON, AND L. H. RIESEBERG. 2009. The frequency of polyploid speciation in vascular plants. Proceedings of the National Academy of Sciences. 106:13875-13879.

APPENDIX 1.

Flow cytometry protocol with modifications based on Roberts, Gladis, and Brumme (2009) and Loureiro *et al.* (2007).

1. Place leaf material in petri dish. The amount of tissue is group specific and rather subjective. Estimate by eyeballing "the size of a pinky nail" – aim for 0.25g fresh weight tissue. ***Note:*** This may be increased to improve reads, if necessary. If desired, include standard tissue to be co-chopped. A smaller amount of standard (relative to sample) may be used to prevent the standard signal from swamping out the sample.
2. Place the petri dish with the samples on a cold brick, which will serve as a cold chopping surface, or in a dish of ice.
3. Add 1000µl cold lysis woody plant buffer (Loureiro *et al.*, 2007) to the petri dish and chop tissue with a single-edged razor blade for 60 seconds. Retain the pipette tip from this step to mix the sample later. ***Note:*** Again, the amount of chopping (fine vs. course) may be adjusted depending on the tissue. Finer chopping may result in more noise. Use a new razor blade for each sample. Do not over-chop!
4. Swirl the chopped material in the lysis buffer for approximately 20-30 seconds until a green tint appears on the liquid.
5. Remove the end of the retained pipette tip with the razor blade and use the larger opening to mix the material by pipetting up and down a couple of times.
6. Filter 800µl of suspension through Falcon® 5 mL Round Bottom Polystyrene Test Tube with 35µm nylon mesh strainer cap and keep on ice.
7. Transfer 500µl of filtrate to a new 1.5mL Eppendorf and add 2.5µl of RNase A (1-10 mg/mL). Incubate for 10 minutes.
8. Add 6µl PI stock solution for 500µl of filtrate (for an estimated final concentration of 50µg/mL) and cover tubes with foil to protect from light. Incubate on ice and analyze within 10 minutes for fresh tissue or keep on ice and protect from light; dry tissue may require incubation for 20-30 min.
9. Run on Accuri C6 flow cytometer (Galbraith, 2009).

Appendix 2. Collection and voucher information on specimens used in this study. Specimens used in this study have been deposited in the University of Florida Herbarium (FLAS). See Fig. 1 for population abbreviations.

Species	Sample ID	State	County	Population	Latitude	Longitude	Date	Voucher Specimen	Notes
Lygodium japonicum	JAP122	Florida	Alachua County	UF	29.640076	-82.3513	20 March 2021	J.A. Pelosi 122	https://www.inaturalist.org/observations/60110161
Lygodium japonicum	JAP123	Florida	Alachua County	UF	29.6441415	-82.3467	20 March 2021	J.A. Pelosi 123	https://www.inaturalist.org/observations/70577635
Lygodium japonicum	JAP124	Florida	Alachua County	UF	29.63081	-82.3702	20 March 2021	J.A. Pelosi 124	
Lygodium japonicum	JAP125	Florida	Alachua County	HT	29.636748	-82.3142	20 March 2021	J.A. Pelosi 125	
Lygodium japonicum	JAP126	Florida	Alachua County	HT	29.636748	-82.3141	20 March 2021	J.A. Pelosi 126	
Lygodium japonicum	JAP127	Florida	Alachua County	HT	29.636748	-82.3141	20 March 2021	J.A. Pelosi 127	
Lygodium japonicum	JAP128	Florida	Alachua County	FP	29.638976	-82.3938	20 March 2021	J.A. Pelosi 128	
Lygodium japonicum	JAP129	Florida	Alachua County	FP	29.638976	-82.3938	20 March 2021	J.A. Pelosi 129	
Lygodium japonicum	JAP130	Florida	Alachua County	FP	29.638976	-82.3938	20 March 2021	J.A. Pelosi 130	
Lygodium japonicum	JAP131	Florida	Alachua County	FP	29.638976	-82.3938	20 March 2021	J.A. Pelosi 131	
Lygodium japonicum	JAP132	Florida	Suwannee County	TR	30.369736	-83.1976	28 March 2021	J.A. Pelosi 132	https://www.inaturalist.org/observations/72295785
Lygodium japonicum	JAP133	Florida	Suwannee County	TR	30.369736	-83.1976	28 March 2021	J.A. Pelosi 133	https://www.inaturalist.org/observations/72295834
Lygodium japonicum	JAP134	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 134	https://www.inaturalist.org/observations/72295936
Lygodium japonicum	JAP135	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 135	https://www.inaturalist.org/observations/72295998
Lygodium japonicum	JAP136	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 136	

Appendix 2. Continued.

Species	Sample ID	State	County	Population	Latitude	Longitude	Date	Voucher Specimen	Notes
Lygodium japonicum	JAP137	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 137	https://www.inaturalist.org/observations/72296349
Lygodium japonicum	JAP138	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 138	https://www.inaturalist.org/observations/72296410
Lygodium japonicum	JAP139	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 139	
Lygodium japonicum	JAP140	Florida	Hamilton County	BS	30.34202	-82.7273	28 March 2021	J.A. Pelosi 140	
Lygodium japonicum	JAP141	Florida	St. John's County	MS	29.728336	-81.2776	28 March 2021	J.A. Pelosi 141	
Lygodium japonicum	JAP142	Florida	St. John's County	MS	29.728336	-81.2776	28 March 2021	J.A. Pelosi 142	
Lygodium japonicum	JAP143	Florida	St. John's County	MS	29.728336	-81.2776	28 March 2021	J.A. Pelosi 143	
Lygodium japonicum	JAP144	Florida	St. John's County	MS	29.728336	-81.2776	28 March 2021	J.A. Pelosi 144	
Lygodium japonicum	JAP145	Florida	Hillsborough County	HR	28.147602	-82.2373	17 April 2021	J.A. Pelosi 145	
Lygodium japonicum	JAP146	Florida	Hillsborough County	HR	28.147602	-82.2373	17 April 2021	J.A. Pelosi 146	
Lygodium japonicum	JAP147	Florida	Hillsborough County	HR	28.147602	-82.2373	17 April 2021	J.A. Pelosi 147	
Lygodium japonicum	JAP148	Florida	Hillsborough County	HR	28.147602	-82.2373	17 April 2021	J.A. Pelosi 148	
Lygodium japonicum	JAP149	Florida	Marion County	OP	29.104598	81.9077	17 April 2021	J.A. Pelosi 149	https://www.inaturalist.org/observations/52078474
Lygodium japonicum	JAP150	Florida	Marion County	OP	29.104598	81.9077	17 April 2021	J.A. Pelosi 150	
Lygodium japonicum	JAP151	Florida	Marion County	OP	29.104598	81.9077	17 April 2021	J.A. Pelosi 151	

Appendix 2. Continued.

Species	Sample ID	State	County	Population	Latitude	Longitude	Date	Voucher Specimen	Notes
Lygodium japonicum	JAP152	Florida	Marion County	OP	29.104598	81.9077	17 April 2021	J.A. Pelosi 152	
Lygodium japonicum	JAP153	Florida	Marion County	OP	29.104598	81.9077	17 April 2021	J.A. Pelosi 153	
Lygodium japonicum	JAP155	Florida	Marion County	JS	29.185114	-81.7109	17 April 2021	J.A. Pelosi 155	
Lygodium japonicum	JAP156	Florida	Marion County	JS	29.185114	-81.7109	17 April 2021	J.A. Pelosi 156	
Lygodium japonicum	JAP157	Florida	Marion County	JS	29.185114	-81.7109	17 April 2021	J.A. Pelosi 157	
Lygodium japonicum	JAP182	Florida	Alachua County	HT	29.636748	-82.3141	5 March 2022	J.A. Pelosi 125	
Lygodium japonicum	JAP183	Florida	Alachua County	HT	29.636748	-82.3141	5 March 2022	J.A. Pelosi 125	
Lygodium japonicum	JAP184	Florida	Alachua County	HT	29.636748	-82.3141	5 March 2022	J.A. Pelosi 125	
Lygodium japonicum	JAP185	Florida	Alachua County	HT	29.636748	-82.3141	5 March 2022	J.A. Pelosi 125	
Lygodium japonicum	JAP186	Florida	Alachua County	HT	29.636748	-82.3141	5 March 2022	J.A. Pelosi 125	
Lygodium japonicum	JAP187	Florida	Alachua County	HT	29.636748	-82.3141	5 March 2022	J.A. Pelosi 125	
Lygodium japonicum	JAP188	Florida	Alachua County	UF	29.63081	-82.3702	5 March 2022	J.A. Pelosi 124	
Lygodium japonicum	JAP189	Florida	Alachua County	UF	29.63081	-82.3702	5 March 2022	J.A. Pelosi 124	
Lygodium microphyllum	JAP190	Florida	Orange County	MP	28.36655	-81.194625	5 May 2022	J.A Pelosi 193	https://www.inaturalist.org/observations/117208234
Lygodium microphyllum	OWCF1	Florida	Palm Beach County	LX	26.499859	-80.3146	26 May 2021	J.A. Pelosi 159	

Appendix 2. Continued.

Species	Sample ID	State	County	Population	Latitude	Longitude	Date	Voucher Specimen	Notes
Lygodium microphyllum	OWCF2	Florida	Palm Beach County	LX	26.499859	-80.3146	26 May 2021	J.A. Pelosi 159	
Lygodium microphyllum	OWCF4	Florida	Palm Beach County	LX	26.499859	-80.3146	26 May 2021	J.A. Pelosi 159	
Lygodium microphyllum	OWCF5	Florida	Palm Beach County	LX	26.499859	-80.3146	26 May 2021	J.A. Pelosi 159	
Lygodium microphyllum	CREW1	Florida	Collier County	CR	26.33847	-81.6198	9 March 2022		https://www.inaturalist.org/observations/108275233
Lygodium microphyllum	CREW2	Florida	Collier County	CR	26.33847	-81.6198	9 March 2022		https://www.inaturalist.org/observations/108275233
Lygodium microphyllum	CREW3	Florida	Collier County	CR	26.33847	-81.6198	9 March 2022		https://www.inaturalist.org/observations/108275233

Data: Specimens used in this study have been deposited in the University of Florida Herbarium (FLAS). Flow cytometry data, spore images and measurements, and code are available at https://github.com/jessiepelosi/LyJapCyto.