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Investigating sexual and asexual modes of reproduction in Palmer amaranth (*Amaranthus palmeri*)

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Research Article

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Abstract

Palmer amaranth (*Amaranthus palmeri* S. Watson), a dioecious wind-pollinated plant, is one of the most troublesome crop weeds in the United States and is spreading northward. The prodigious production of seed contributes to establishment of populations and spread across the landscape. Sexual reproduction via outcrossing is likely the primary mode of seed production for this dioecious plant. However, *A. palmeri* may also be capable of autonomous asexual seed production (apomixis), which could be beneficial during colonization. We conducted two studies of female isolation from pollen to investigate the propensity for autonomous seed production in 19 populations across eastern North America. In the first, we observed low-frequency seed production on many isolated females. Using flow cytometry of seed samples (FCSS) we primarily found patterns of ploidy consistent with sexual reproduction; no significant differences in ploidy between seeds produced on isolated females (putative apomicts) and non-isolated females (putatively sexual) were detected. We also investigated patterns of DNA content and found no evidence in 153 samples for polyploidy, which is often observed in apomictic species. The second female isolation trial utilized sex-specific molecular markers to identify and remove males before flowering, and we observed zero seed production. Overall, we did not detect evidence in support of apomixis in these populations of *A. palmeri*, suggesting that apomixis is unlikely to have played a role in the northward advance of this species in eastern North America. We also investigated whether there is variation between females and males in size and secondary reproductive traits. We found evidence for sexual dimorphism in three of six traits investigated: females are taller at senescence and produce longer secondary branches and more axillary flowers than males. Differences in cost of reproduction and strategies for pollen release versus pollen capture are likely factors shaping the evolution of sexual dimorphism in this wind-pollinated dioecious plant.

Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson), one of the most troublesome agricultural weeds in the United States (Van Wychen 2016, 2017, 2022), has rapidly expanded its geographic range within the last century. Originating in northwestern Mexico and the southwestern United States, this species gradually spread eastward throughout the 1900s (Sauer 1957). Following the widespread adoption of broad-spectrum herbicides in the 1990s, *A. palmeri* rapidly expanded its range northward, establishing in the midwestern states North Dakota (Crawford et al. 2018) and Wyoming (Kniss 2018). It is now recorded in at least 27 of the 48 contiguous U.S. states (USDA-APHIS 2020) and has been recently reported but eradicated in Manitoba, Canada (RealAgriculture Agronomy Team 2021). This rapid northern range expansion also coincides with increasing incidence of herbicide resistance (Chahal et al. 2015) to 10 herbicide modes of action, with multiple resistance to up to 6 modes of action (Chahal et al. 2015; Heap 2024). Although herbicide resistance clearly contributes to the local demographic success of *A. palmeri* in agricultural populations, questions remain regarding what factors contribute to the geographic spread of both the species and resistance and the demographic success of this weed across a wide range of latitudes. Mode(s) of reproduction for this dioecious (separate female and male plants) weed have been suggested to contribute to its demographic success (Franssen et al. 2001; Gaines et al. 2010; Ribeiro et al. 2014; Trucco et al. 2007; Ward et al. 2013). Here we focus on understanding the roles of sexual and asexual reproductive modes, and whether a putative mixed reproductive system could contribute to *A. palmeri*'s geographic spread.

Introduced weeds are predicted to have a greater probability of being hermaphroditic (male and female organs within the same flower) or monoecious (separate sex flowers on the same plant) than introduced non-weeds, as they are often capable of self-fertilization in mate-limited

populations, which allows the establishment of a new population from a single propagule (Van Etten et al. 2017). Across U.S. weeds, hermaphroditism is the most common reproductive strategy, and monoecy is the second most common reproductive strategy (Van Etten et al. 2017). This is consistent with "Baker's law," which posits that species capable of uniparental reproduction are more likely to colonize new populations than those that require mates (Baker 1955). The strict separation of sex function among individuals in dioecious species prevents self-fertilization. *Amaranthus palmeri* is a wind-pollinated dioecious species, expected to be exclusively outcrossing. This sexual and outcrossing mode of reproduction is not expected to be favored during colonization, raising the question of how *A. palmeri*, a dioecious weed, is so successful at rapid colonization.

Another reproductive hypothesis is that *A. palmeri* possesses the ability to produce seeds asexually, via autonomous apomixis (i.e., seed production in the absence of pollen) (Ribeiro et al. 2014; Ward et al. 2013). The possibility of apomictic seed production in *Amaranthus* crop weeds, to our knowledge, first appeared in the early 2000s (Franssen et al. 2001), stemming from reports of apomixis in agricultural crops (Poehlman and Sleper 1995). Observations of maternally biased inheritance patterns in crossing experiments, first in waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (female *A. tuberculatus* × male *A. palmeri*; Franssen et al. 2001), and then in *A. palmeri* (Georgia: Gaines et al. 2010; Illinois: Trucco et al. 2007) indirectly suggested (autonomous) apomixis as an explanation for observed deviations from expected sex ratios or expected hybrid progeny. To test the apomixis hypothesis more directly, Ribeiro et al. (2014) grew *A. palmeri* females (Mississippi) in a greenhouse isolated from males and observed seed production by 117 of 118 females. Some individuals produced up to 6,000 seeds, but the majority (60% to 100%, depending on the population) produced a small number of seeds (1 to 1,000 seeds per plant; Ribeiro et al. 2014), and genetic confirmation of apomictic origin is pending. Many plants combine sexual reproduction with asexual or clonal reproduction (Barrett 2015), but overall apomixis is rare, occurring in <1% of flowering plants (Whitton et al. 2008). Autonomous apomixis provides reproductive assurance during pollen scarcity and maintains favorable genotypes due to lack of recombination (Antonovics 1968; Baker 1955; Niklas and Cobb 2017). These benefits may contribute to a disproportionately high representation of apomixis in disturbed and recently colonized areas (Bierzychudek 1985; Richards 2003). A mixed reproductive mode capable of exploiting disturbed habitats and maintaining genotypes that maximize herbicide resistance without compromising the long-term potential for adaptation could be advantageous for crop weeds. If asexual reproduction plays a role in promoting the spread of *A. palmeri*, we might observe populations in more recently colonized regions (i.e., northern) exhibiting higher rates of apomixis.

Apomixis and dioecy are both relatively rare reproductive conditions in angiosperms (Renner 2014; Whitton et al. 2008), and there are few reports of these two reproductive modes co-occurring: For example, in Asian spicebush [*Lindera glauca* (Siebold & Zucc.) Blume], both females and males occur in China, but female-only populations have established in Japan and, based on isolation experiments (Dupont 2002) and observed genetic uniformity (Nakamura et al. 2021; Zhu et al. 2020), are thought to reproduce through apomixis. Autonomous apomixis has also been proposed to occur in females and some hermaphrodites of the subdioecious shrub smallleaf fuchsia (*Fuchsia microphylla* Kunth) (Cuevas et al. 2014) and the cryptic dioecious species *Geniostoma borbonicum* (Lam.) Spreng. (Aliyu et al. 2010; Rojek et al. 2018). In contrast, apomixis is commonly associated with polyploidy (Hojsgaard and Hörandl 2019; Whitton et al. 2008). Autonomous apomixis is the production of genetically identical seeds without meiosis or pollen fertilization (Bicknell and Koltunow 2004). In contrast to sexually produced seed, which is composed of a 2C embryo (from two parents), and a 3C endosperm (2 polar nuclei + 1 sperm), an autonomous apomictic seed consists of a diploid parthenogenic embryo (2C) and a tetraploid endosperm (4C) containing two unreduced female gametes (Matzk et al. 2000). For polyploid individuals, a diploid embryo and tetraploid endosperm can arise from mitotic division; however, for diploid individuals, meiosis must be altered to produce unreduced gametes (Hojsgaard and Hörandl 2019). This complexity is one hypothesis of many (Whitton et al. 2008) to explain why apomixis is typically associated with ploidy variation (Pegoraro et al. 2020), and diploid apomicts are rare (but see Aliyu et al. 2010; Rojek et al. 2018). To date, all assessments of ploidy in *A. palmeri* are consistent with diploid status (Molin et al. 2017; Montgomery et al. 2020; Rayburn et al. 2005). Finally, to our knowledge, aside from the preliminary reports in *A. tuberculatus* and *A. palmeri*, there are no other reports of autonomous apomixis in Amaranthaceae. Most reports of autonomous apomixis come from the Melastomataceae, Orchidaceae (Hojsgaard and Hörandl 2019), and Asteraceae (Whitton et al. 2008), families known to exhibit weak endosperm formation. Therefore, overall, the phylogenetic background and dioecious and diploid status of *A. palmeri* suggest the probability of apomixis occurring may be low, but not impossible.

If not apomictic, how does *A. palmeri* achieve such reproductive success, given the reproductive challenges experienced by strictly dioecious plants? Female *A. palmeri* exhibit prodigious seed production in many contexts, putatively through sexual reproduction, with individual females producing 20,000 to 600,000 seeds per plant when growing with cotton (*Gossypium hirsutum* L.), peanut (*Arachis hypogaea* L.), and soybean [*Glycine max* (L.) Merr.] (Ward et al. 2013), although in competition with corn (*Zea mays* L.), estimates were much lower at ~200 seeds per plant (Mahoney et al. 2021). Dioecious plants are obligately outcrossing and thus do not experience reproductive assurance from self-fertilization. Outcrossing promotes opportunity for gene flow from other populations, and this has the potential to either increase or decrease adaptive evolution (Ellstrand 2014). Dioecious plant populations also have the opportunity for natural and sexual selection to result in differences in traits between females and males. This is an opportunity to alleviate sexual conflict that interferes with either sex function in the hermaphroditic state (Barrett and Hough 2013; Dorken and Van Drunen 2018; Geber et al. 1998) and to optimize fitness of both sexes through sexual dimorphism of secondary sex or life-history traits (Geber et al. 1998). One classic prediction by Bateman (1948) suggested that males are most often limited by mate availability, and females by resource acquisition. In addition, males may be taller to enhance pollen distribution, whereas females may be smaller to preserve resources for seed maturation (Barrett and Hough 2013; Lloyd and Webb 1977; Nakahara et al. 2018; Niklas 1985). Investigating whether morphological traits exhibit sexual dimorphism, and whether dimorphism varies among populations, could be key to understanding the sexual reproductive success of the dioecious weed *A. palmeri*.

Reproduction, both sexual and asexual, plays a substantial role in determining the genetic variation passed on to each generation.

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Sexual reproduction is associated with the generation of diverse genetic combinations, whereas asexual reproduction preserves and replicates a single genetic combination. In this study of the range-expanding crop weed *A. palmeri*, we investigate the following reproductive biology questions: (1) Do female *A. palmeri* plants from eastern North America (Georgia, North Carolina, and Illinois) exhibit autonomous apomixis (i.e., the ability to produce seed in the absence of pollen)? To answer this question, we conducted two female isolation trials, with an emphasis on assessing the quality of female isolation. (2) If females do produce seeds in isolation, is there evidence based on patterns of seed embryo and endosperm ploidy that seeds have been produced through apomixis rather than sexual reproduction? (3) Using leaf tissue to estimate nuclear DNA content, is there any evidence of polyploidy, a condition that frequently co-occurs with apomixis? (4) Is there variation among populations in the propensity of autonomous apomixis? And further, is there geographic structure of the propensity of apomixis; specifically—do more northern populations, which have likely been more recently colonized, exhibit a higher propensity for apomixis? (5) For 22 populations of *A. palmeri*, is there a pattern of sexual dimorphism for plant height, stem diameter, number of axillary flower buds, number of inflorescences, total inflorescence length, and number and length of secondary stems?

Material and Methods

Female Isolation (i) to Estimate Autonomous Apomixis

Amaranthus palmeri seeds were collected from 22 populations across eastern North America (Teitel 2021): 13 populations from North Carolina (NC), five populations from Illinois (IL), and four populations from Georgia (GA) (Supplementary Table S1; figure 1 in Yakimowski et al. 2021).

To estimate the propensity for autonomous apomixis we conducted this initial female isolation trial (see section on female isolation (ii) employing genetic markers). Plants for female isolation (i) were grown from October 2017 to April 2018. Seed families (collected from a single maternal plant) sampled from the abovementioned 22 populations (mean = 8.8 families per population) were used. Five to 10 (mean = 6.6 seeds) seeds per family were planted 1-cm deep in 15-cm round pots with commercial soil (Sun Gro®, Horticulture, Agawam, MA, USA, Professional Growing Mix #1) and grown at 22 to 26 C, 30% to 40% humidity with a 13-h day photoperiod in the Queen's University Phytotron. At the 2- to 4-leaf stage, individual seedlings ($n = 539$) were transplanted to 15-cm pots and randomly spaced across four tables in the greenhouse at a density of ~25 plants m^{-2}. Plants were watered two times per day and fertilized (Master Plant-Prod Inc., Brampton, ON, Canada, 20N-20P-20K Part ID # 70440) three times, at ~6-wk intervals.

As flower buds developed and flowering commenced, plants were surveyed daily for first visible anthers or stigmas and identified as female or male. Note that no hermaphroditic plants were observed in our studies, consistent with the detailed microscopic investigation of *A. palmeri* buds and flowers (Wu et al. 2023). As females were identified, they were first distanced from males (~5 to 10 m) within the same greenhouse zone for 12 h ($n = 144$ females). This distance was to reduce the amount of pollen landing on females from flowering males before full isolation. The length of this distancing was to increase the probability that pollen that had landed on a female plant from neighboring flowering males was inviable; pollen is estimated to remain viable for only 4 h (Sosnoskie et al. 2007).

Following this distancing, females were moved to an isolated greenhouse zone under the same conditions, where up to three pollination bags per plant were secured to branches with unopened inflorescences (total bags: $n = 333$, mean = 2.03 bags per female). The estimated range of pollen diameter for *A. palmeri* is 21 to 38 µm; we used the following two materials that, based on their mesh size, are expected to effectively exclude pollen. We custom-made bags using Tyvek® Homewrap® (Dupont Canada) that was sewn or sealed (Uline Canada, Milton, ON, Canada, plastic seal) in sizes of 12 by 45 cm, 12 by 22 cm, and 6 by 22cm (reported to exclude pollen of 21 to 30 µm; see Smith and Mehlenbacher 1994). We also used PBS International (Scarborough, UK) 3D.55 (duraweb®, pore size: 15 µm) with dimensions of 55 by 15.8 by 15.8 cm. Bag sizes were chosen based on the size of the inflorescence and enclosed by plastic zip ties. The large 3D bags were placed over the top of some plants, encompassing up to 92 inflorescences in addition to the primary inflorescence. Any flowers that were already open before complete isolation (i.e., stigmas visible) were marked with paint and/or zip ties, and any seeds produced in these positions were not counted as seed produced in isolation. Pollination bags remained on plants for a minimum of 7 wk.

After 7 wk, plants exhibiting signs of senescence, including stem yellowing, which we observed to be the most reliable indicator of senescence, were harvested. Height from the base of the soil to the base of each bag was recorded to the nearest millimeter, and the pollination bags were removed. Isolated (bagged) and non-isolated (unbagged) inflorescences were counted, and length was measured (to the nearest millimeter). Isolated inflorescences were hand-threshed, and the number of filled seeds were counted. The proportion of total inflorescences bagged was calculated to determine the extent to which bagging affected inflorescence production. Seeds produced by axillary inflorescences were not considered a part of this exclusion trial, because they were observed to commonly be the first inflorescences to become receptive, and thus it was difficult to ensure their isolation from pollen sources. However, see below (Results Autonomous Apomictic Seed Production and Figure 4) our quantification of axillary inflorescences and consideration of ploidy variation.

To account for variation in the opportunity for apomixis, in addition to total seed count, we calculated the number of seeds produced by isolated females per total length (cm) of inflorescence produced within bags. To investigate whether any of the variation in seed production from female isolation (i) is geographically structured, we investigated Pearson correlations between latitude and total seed production, as well as seed production per centimeter of inflorescence.

To investigate variation in seed production by isolated females, in addition to total seed production within an isolation bag, individuals were scored for the following four nested criteria: (1) seed production by more than one isolated inflorescence; (2) a minimum of one isolated inflorescence produced more than one seed; (3) low probability of pollen contamination: seed producing inflorescences were at least 1 cm from painted or tied inflorescences; and (4) seed-producing inflorescences produced more than three seeds each. Individuals that scored 4 also met criteria 1, 2, and 3, and so on. Seeds from isolated inflorescences from individuals meeting criteria 4 were considered most likely to have exhibited spontaneous apomictic seed production (i.e., least likely that seeds produced were due to pollen contamination) and were selected for flow cytometric analysis.

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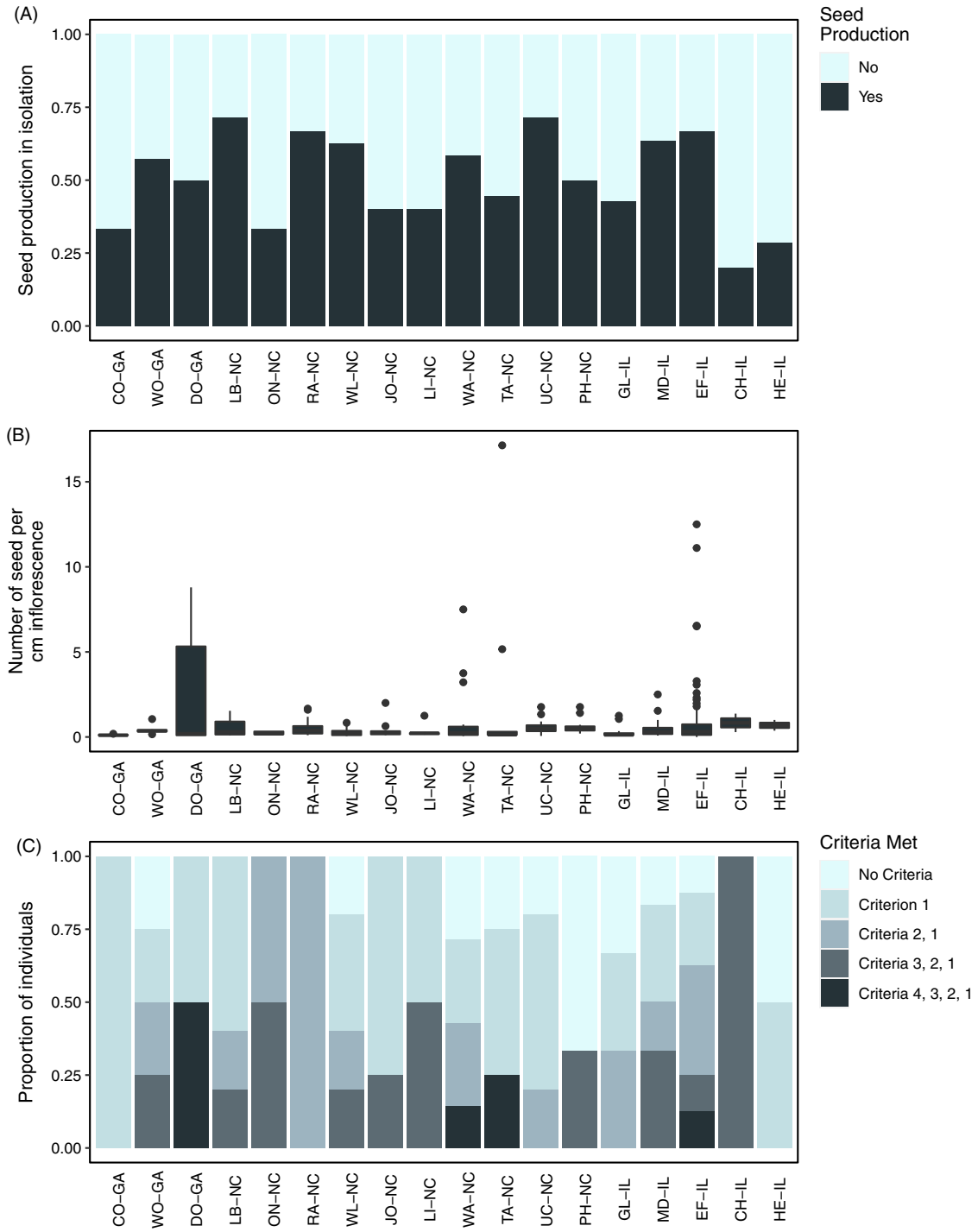

Figure 1. Seed production by *Amaranthus palmeri* females from 23 populations collected from Georgia, North Carolina, and Illinois, grown in isolation from males (female isolation (i)). (A) Proportion of isolated female individuals that produced seed (dark bar) or did not produce seed (light blue) for each population. (B) Number of seeds produced by each seed-producing inflorescence in isolation for each population. (C) Proportion of individuals in isolation that met each of the following nested criteria: (1) more than one inflorescence produced seed; (2) at least one inflorescence produced more than one seed; (3) no inflorescences were painted or tied (i.e., cross was isolated before female flowers opening); (4) inflorescences produced more than three seeds each. Individuals that meet criterion 4 (and therefore also criteria 1–3) are least likely to exhibit seed production due to contamination by male pollen.

Flow Cytometric Analysis: DNA Content Estimation from Leaf Tissue and Flow Cytometric Seed Screen (FCSS)

Plants for flow cytometric analysis of DNA content were grown from March to April 2019. We germinated one seed per family from each of 10 to 11 families per population. The 10 to 11 seeds were split between two 15.24-cm pots per population (i.e., 5 to 6 seeds per pot) at the Queen's University Phytotron. At the 2- to 4-leaf stage, one or two fresh leaves were harvested from seedlings ($n = 153$) and refrigerated for up to 3 d. Common vervain (*Verbena officinalis* L.) individuals were grown in April 2019, and

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leaf tissue was used as a diploid reference (Temsch et al. 2022) for flow cytometric analysis.

Flow cytometric analysis (Flow Cytometry Services, Husband Lab, University of Guelph) was performed on *A. palmeri* leaf tissue to estimate DNA content by picogram and on *A. palmeri* seeds to investigate patterns of seed embryo and endosperm ploidy as they relate to mode of reproduction (Bicknell and Koltunow 2004; Matzk et al. 2000). Fresh *A. palmeri* leaf tissue was prepared for nuclei isolation to estimate DNA content (pg). Samples of *A. palmeri* and *V. officinalis* leaf tissue (7-mm^2) were finely chopped with a razor into approximately 1-mm^2 pieces in separate petri dishes. Next 0.7 ml of ice-cold LB01 buffer (Dpoležel et al. 1989) containing 100 µg/ml propidium iodide was added to the chopped leaf tissue in a petri dish for 20 min. This solution was then poured through a 30-µm Celltrics® filter (Sysmex, Lincolnshire, IL, USA) and collected in a 5-ml Falcon™ (Corning, Glendale, AZ, USA) round-bottom polystyrene tube (12-mm diameter, 75-mm length).

For seed ploidy analysis, individual *A. palmeri* seeds were chopped with a razor in 0.7 ml of ice-cold LB01 buffer. One 7-mm^2 sample of *V. officinalis* leaf tissue was chopped into ~1-mm^2 pieces and added to the LB01 buffer to provide a DNA standard. We conducted this analysis on 40 individual seeds produced by each of the four *A. palmeri* females ($n = 10$ seeds per female) ranked as most likely to have produced seed in the absence of pollen based on the amount of seed produced and the quality of the isolation (see Materials and Methods for Female Isolation (i) to Estimate Autonomous Apomixis). We also analyzed 40 seeds, individually, produced by inflorescences of the same females that were not isolated or bagged, and are thus putatively sexually produced. We also analyzed: (1) 5 seeds individually, produced in axillary inflorescences; and (2) 10 seeds in bulk to determine whether other variation in ploidy would be apparent with a larger volume of seed tissue; and (3) 15 F_1 seeds from controlled crosses individually.

Flow cytometry data were acquired using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) operated with CellQuest Pro software (v. 6.0, 2007; BD Biosciences). Relative nuclei fluorescence was measured using the FL2 (485-nm) detector, with fluorescence area (FL-A, integrated fluorescence) as the parameter of interest. Sample gating was done by testing *A. palmeri* leaf tissue nuclei peaks, with *V. officinalis* leaf tissue nuclei peaks as standard. After testing, significant debris was observed in FL2-A/FL3-H and FL2-A/SSC-H scatter plots; debris was excluded by gating on a fluorescence area (FL2-A, 485 nm) by fluorescence height (FL3-H, 670 nm) scatter plot to eliminate particles with distinct fluorescence properties relative to nuclei. Additional gating was done on a side-scatter fluorescence area (SSCFL2A, 485 nm) and side-scatter height (SSC-H) scatter plot to ensure all ploidy-level variation of *A. palmeri* leaf tissue was captured. Data from individual leaf samples were collected until a minimum of 1,000 nuclei were acquired for both *A. palmeri* 2C and *V. officinalis* 2C peaks. Fewer nuclei (400 to 1,600 counted at peaks) were acquired for individual seed samples due to their small size.

Raw flow cytometric data were analyzed using the R package FLOWPLOIDY (Smith et al. 2018) to estimate peak means, coefficients of variation, and nuclei number. We used single-cut (SC) debris models, and peaks were manually measured in the FLOWPLOIDY package. Peaks of relative nuclei fluorescence for *A. palmeri* and *V. officinalis* leaf tissue were measured manually. The peak of *V. officinalis* leaf tissue was divided by the *A. palmeri* leaf tissue peak to calculate a peaks ratio, which was then multiplied by the known size of *V. officinalis* DNA content (Temsch et al. 2022). Two ploidy profile types were detected for seed data, and we used a chi-square test to determine whether the occurrence of these two types depended on whether the seed was from a putatively sexual seed or produced by an isolated female.

Female Isolation (ii)

We conducted a second female isolation trial, applying sex-specific markers to increase our ability to identify and remove pollen-producing plants before female receptivity. To further minimize the potential for contamination of female plants isolated from pollen, we applied *Amaranthus palmeri* male-specific Y-chromosomal region markers (Montgomery et al. 2021) to determine the sex of juvenile plants before the formation of inflorescences. Plants for female isolation (ii) were grown from August 2022 to March 2023. Seeds from 10 different seed families (Supplementary Tables S2 and S3) were germinated in 3.81-cm plug trays with clear plastic lids using commercial potting soil (Sun Gro® Professional Growing Mix #1) and transplanted into 15-cm-diameter pots 3 to 5 d post-germination in Queen's University Phytotron. The greenhouse condition was set to 15:9-h photoperiod, and the temperature ranged from 22 to 30 C. Transplanted seedlings were fertilized using 20-20-20 NPK fertilizer (50 ppm) at 1 wk posttransplant and after primary inflorescences emerged.

We sampled one leaf per plant at the 3- to 4-leaf stage, ~2 wk post-germination, and flash-froze tissue samples using liquid nitrogen in 1.5-ml microcentrifuge tubes. We used a modified CTAB (hexadecyltrimethylammonium bromide) DNA extraction protocol (adapted from Yakimowski et al. 2021). We repeated the cold CTAB wash buffer step two times and incubated samples in hot CTAB extraction buffer for 120 to 180 min, mixing and releasing pressure every 15 min. Following 100% isopropanol precipitation, we used 500 µl of 70% molecular-grade ethanol to wash the DNA pellet twice. Ethanol was removed, and the resulting DNA pellets were dried completely and resuspended in 30 µl of nuclease-free water.

Each DNA sample ($n = 113$; mean = 434.44 ng μl^{-1}; range: 101.54 to 17,365.9 ng μl^{-1}) was diluted to 100 ng μl^{-1}, and any samples ($n = 18$; mean = 60.95 ng μl^{-1}; range: 3.73 to 99.44 ng μl^{-1}) that yielded less than 100 ng μl^{-1} were processed as is. We used the JM940 primer design from (Montgomery et al. 2021): forward primer (5′-3′)–AAAGCATGTGTGGGTCT; reverse primer (5′-3′)–CAGCCTTCTCGCACT. PCR Master Mix (25 µl per sample) included the following: 1X Taq Frogga Mix (12.5 µl per sample; FroggaBio, Concord, ON, Canada); 1.25 µl of 10 µM forward primer; 1.25 µl of 10 µM reverse primer; 3 µl of template DNA; and 7 µl of nuclease-free water. We processed samples in a thermocycler with the following conditions: 95 C for 1 min; 95 C for 15 s, 52 C for 15 s, 72 C for 30 s, repeating 35 times; 72 C for 5 min; and 4 C for 5 min with a ramp rate of 2.0 C s^{-1}. We visualized PCR products through gel electrophoresis using 2% agarose gel at (84 V) for 60 to 75 min with a 100-bp DNA ladder (Bio-Helix Co. Ltd., New Taipei City, Taiwan).

Samples that exhibited a male-identifying band ~100-bp were putatively male (Supplementary Figure S2); any samples with inconclusive gel visualizations were also assumed to be male. We discarded all presumed male *A. palmeri* plants ($n = 85$) before any inflorescences formed, and isolated presumed female plants ($n = 46$), that is, those lacking a male-identifying band, in a greenhouse zone housing no other *A. palmeri* plants. Each presumed female plant was bagged with custom pollination bag (PBS International, Scarborough, UK, type 3D) to further prevent

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potential pollen contamination. Two presumed female plants (APO.TANC33_4 and APO.WANC13_9) were left unbagged due to their small size. Plants were monitored closely as inflorescences emerged and flowers opened; any plants incorrectly classified as "presumed female" ($n = 4$) (Supplementary Table S2) were discarded as soon as identified as male based on inflorescence phenotype, yielding a final number of 42 female plants. Once inflorescences were visibly mature, we removed pollination bags and measured: stem height (from the base of the plant to the tip of the primary inflorescence), number of axillary buds, number of inflorescences, length of the primary inflorescence, and total and average lengths of inflorescence(s). Any seeds in the axillary buds or in inflorescences were counted (Supplementary Table S3).

Estimating Sexual Dimorphism of Morphological Traits

Plants for the sexual dimorphism dataset were grown at the same time, in the same greenhouse zone, and using the same growth methods as those for female isolation (i) from germination until initiation of flowering. Females ($n = 144$) that were isolated and bagged (female isolation (i), above) were moved to the adjacent greenhouse zone with similar growth conditions. A subset of females ($n = 114$) remained in the original greenhouse zone with male plants, unbagged, and thus were pollinated and set seed. At senescence, the following traits were measured for all plants: height from base of stem to the intersection of the main stem and the primary inflorescence to the nearest millimeter ($n_{FemaleBagged} = 144$, $n_{FemaleUnbagged} = 114$, $n_{Male} = 281$), base stem diameter (~1 cm from soil surface) was measured with digital calipers to the nearest 0.01 mm ($n_{FemaleBagged} = 144$, $n_{FemaleUnbagged} = 94$, $n_{Male} = 267$), number of axillary flower bud clusters ($n_{FemaleBagged} = 131$, $n_{FemaleUnbagged} = 36$, $n_{Male} = 176$), number of inflorescences ($n_{FemaleBagged} = 144$, $n_{FemaleUnbagged} = 94$, $n_{Male} = 267$), total inflorescence length to the nearest millimeter ($n_{FemaleBagged} = 144$, $n_{FemaleUnbagged} = 94$, $n_{Male} = 267$), and the number and length of secondary stems >20 cm to the nearest millimeter ($n_{FemaleBagged} = 144$, $n_{FemaleUnbagged} = 94$, $n_{Male} = 267$). Plants exhibiting more and longer secondary branches are wider, that is, more "bushy," which could be relevant to the dynamics of dispersing and capturing pollen.

To investigate whether there are differences in reproductive and morphological features between sexes, mixed-effects linear models and generalized linear mixed-effects models were used with sex type, bagging, and latitude as fixed effects; population and population by sex type were random effects. Because the bagging treatment was only applied to females and not males, sex type and bagging were combined as a single "sex type/bagging" variable with three levels (females bagged, females unbagged, and males). Linear mixed models (LMM) of height, stem diameter, axillary inflorescence number, inflorescence number, and total inflorescence length were analyzed using the *lmer()* function from the LME4 (Bates 2015) package in R (v. 3.5.2; R Core Team 2018). Secondary branch lengths >20 cm included a large number of zeros, preventing transformations for normality. Separate models were analyzed for whether or not secondary branches were produced (binary zero [0] vs. nonzero [1] data; *glmer()*) and continuous nonzero lengths of secondary branches produced using *lmer()*. To determine the best models for all variables, a full model of Response variable ~ Sex type/Bagging × Latitude + (1 | Population) + (1 | Sex:Population), was tested using the *step()* function from the LMERTEST (Kuznetsova et al. 2017) package, and then confirmed by a likelihood ratio test between pairs of simplified models using an ANOVA. For all variables, latitude did not have a significant effect. Test assumptions for models were plotted using the *plot_model()* function from the SJPLOT (Lüdecke 2024) package. For variables analyzed with LMMs, no deviations from assumptions were observed in homoscedasticity, normality of residuals, and multicollinearity.

This dataset includes females that were grown with and without pollen-exclusion bags; therefore, we used contrasts between levels of "sex type/bagging" for morphological traits to test whether female morphology differed between bagged and unbagged females. In cases in which no difference was detected, differences between both type of female versus males was considered consistent with a sexually dimorphic trait. For traits that did exhibit a significant difference between bagged and unbagged females, a significant difference between a bagged female and male was not considered sufficient for sexual dimorphism; a significant contrast between unbagged females and males was required for evidence of sexual dimorphism.

Results and Discussion

Autonomous Apomictic Seed Production in Amaranthus palmeri across Eastern North America?

In female isolation trial (i), seed production in isolation was observed in 20% to 71% of individual females from each population (Figure 1A). However, 47% of these females produced three or fewer seeds. Overall, seed production per inflorescence in isolation ranged from 0 to 233 (mean = 16.4). Accounting for the variation in total length of inflorescence isolated, seed production per centimeter isolated ranged from 0 to 17.1 (mean = 0.8) (Figure 1B). Latitude of the collected germplasm did not have a significant effect on whether inflorescences produced seed ($t = -0.4$, $P = 0.7$), total seed production ($t = 0.3$, $P = 0.7$) or seed production per centimeter of inflorescence ($t = 0.007$, $P = 1$).

Overall, seed production in isolation was observed in 50% of females ($n = 68$) in female isolation trial (i). Criterion 1 (seed production by more than one inflorescence) was met by 19.1% of isolated females ($n = 26$). Criteria 2 (at least one of these inflorescences produced more than one seed) and 1 were met by 11.0% of females ($n = 15$). Criteria 3 (low probability of pollen contamination), 2, and 1 were met by 8.1% of individuals ($n = 11$). Only four individuals (2.9%) met all four criteria. Therefore, we considered these four individuals (Figure 1C; Supplementary Table S1) most worthy candidates for further investigation of whether seed was produced by autonomous apomixis using flow cytometric analysis.

A total of 40 seeds from isolated females and 39 seeds from non-isolated females (open pollinated) were individually analyzed for variation in ploidy. Across non-isolated and isolated seeds, the peak mean values detected using FLOWPLOIDY were consistent with two patterns: (1) 2C, [3C], 4C, and 6C (the detection of [3C] peak is discussed later); or (2) 2C and 4C. Patterns observed in all other samples (axillary seeds, bulked seeds, seeds produced via controlled crossing) were consistent with these patterns; thus, we focus here on interpreting these two patterns.

Plant cells, and especially cells associated with reproductive function, commonly exhibit endopolyploidy—elevated ploidy within cells that is not observed at the organismal level (Barow and Jovtchev 2007). Thus, it is likely that the observed 4C peak arises from doubling of the 2C embryonic peak, the 6C peak from doubling of the 3C endosperm (Figure 2A and 2B). Note that 3C was always low in magnitude and not detected by peak calling;

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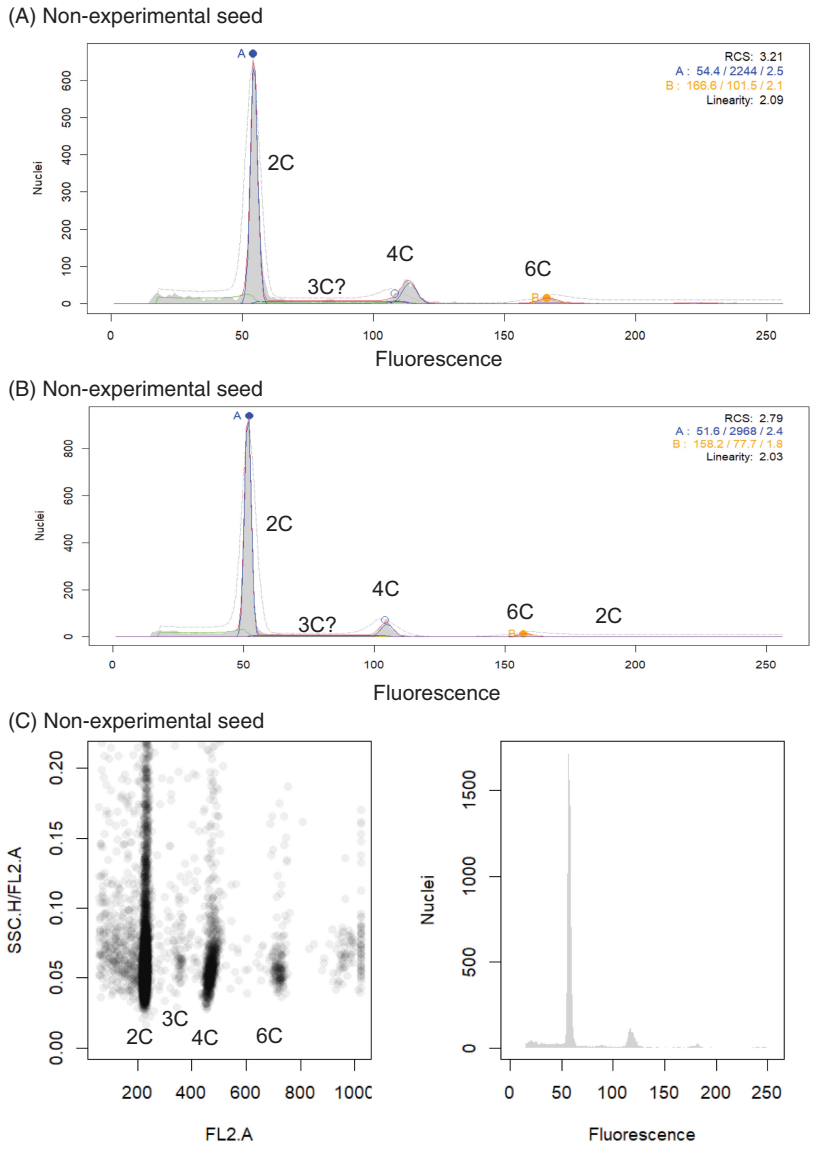

Figure 2. Examples of flow cytometric seed screen (FCSS) of individual *Amaranthus palmeri* seeds. Peak analysis is shown for (A) a seed produced on a non-isolated female (i.e., not bagged, produced via sexual reproduction) from population EF-IL and (B) a seed produced on an isolated female, within an isolation bag from population EF-IL. (C) Non-isolated seed, from population DO-GA, for which the 3C peak was again not detected by peak analysis, but for which a 3C cluster is visible in the side scatter plot, albeit small (left panel).

however, a clear cluster of 3C nuclei was commonly observed in the size distribution of nuclei (Figure 2C). No predicted ploidy patterns for apomixis involve a 6C peak (Hojsgaard and Hörandl 2019) consistent with the 6C arising from the 3C signature of sexual reproduction. The small magnitude of the 3C in our study may be due to the small size of the endosperm; seeds used in this study were 1 to 2 yr old and endosperm tissue was likely already used to keep the embryo viable for germination. We also explored whether the observed 4C or 6C peaks could occur due to technical error involving "doublets" or "triplets" (i.e., two or three nuclei measured together): it is exceedingly improbable that the 6C is explained by 3C doublets, because the 3C peak consistently exhibits lower event count. The 2C triplets are an unlikely explanation, because the relative size of 4C and 6C peaks is not consistent with the dramatically lower rate at which triplets form relative to doublets. Finally, we conducted doublet discrimination (Wersto et al. 2001) for several samples and found no evidence of significant doublets.

Moreover, whether a seed was observed to exhibit the 2C, [3C], 4C, 6C versus 2C, 4C patterns did not depend on whether a seed was from an isolated or non-isolated female (chi-square: $\chi^2 = 3.14$, $P = 0.08$). Therefore, the expected apomictic 2C, 4C pattern was not associated with female isolation. Overall, the analysis of ploidy variation in seeds is most consistent with sexual reproduction and does not provide evidence that seeds produced within isolation bags are of apomictic origin, but rather suggests that despite pollen-isolation efforts, the seeds produced in female isolation (i) were likely due to pollen contamination (Liedl and Anderson 1993) due to the presence of males as plants became reproductive and/or due to movement of small amounts of pollen between the greenhouse containing bagged females and the neighboring greenhouse containing unbagged males.

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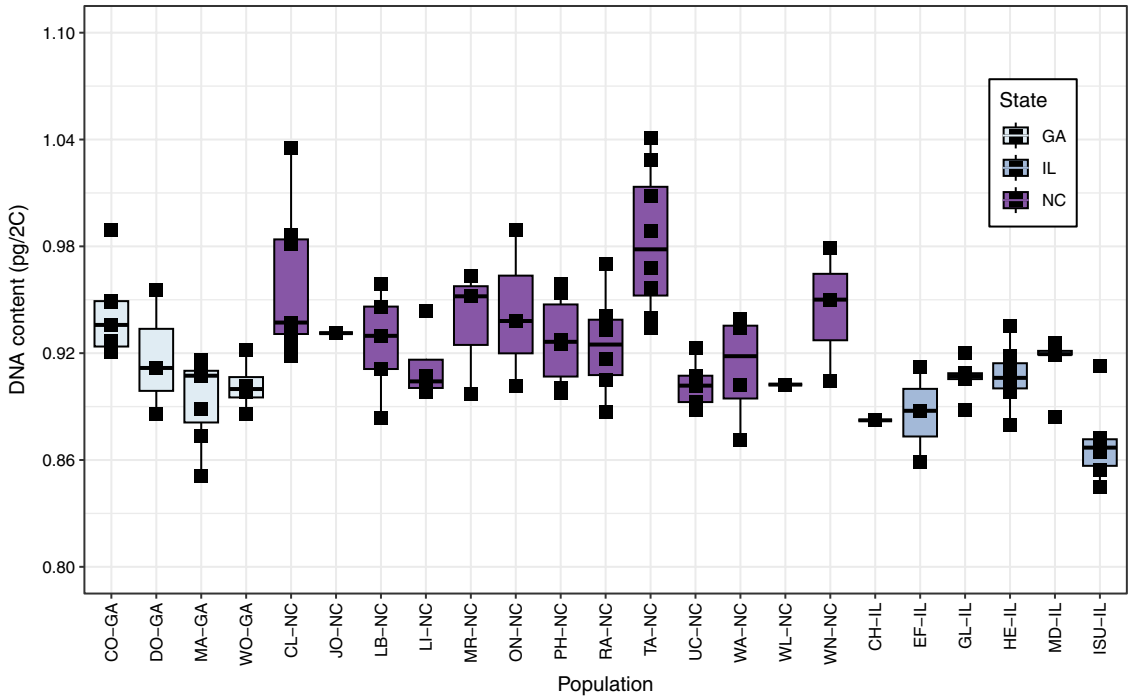

Figure 3. DNA content estimated by flow cytometry from *Amaranthus palmeri* leaf tissue for individuals from populations from eastern North America (Georgia, North Carolina, and Illinois).

Although polyploidy is not a strict requirement for apomixis, it is a common affiliate (Hojsgaard and Hörandl 2019), and thus it is relevant to note that across several studies of DNA content so far, there is no evidence for polyploid individuals in *A. palmeri*. Our investigation of whether any ploidy variation exists in the species more generally involved 104 samples of *A. palmeri* leaf tissue, with *V. officinalis* leaf tissue as a standard. The average DNA content across all samples was 0.922 pg per 2C. By state location within the United States, the mean DNA content for samples from Georgia was 0.915 pg per 2C, for North Carolina, 0.937 pg per 2C, and for Illinois, 0.897 pg per 2C (Figure 3). We tested whether this observed individual variation in DNA content was correlated with previously estimated population mean *EPSPS* copy number (Yakimowski et al. 2021). We found a positive correlation, but the correlation was not statistically significant ($r = 0.29$, $P = 0.22$); similar nonsignificant positive correlations were observed within each state (Supplementary Figure S1). Overall, these results are consistent with previous estimates of DNA content (0.82 to 0.96) and the previously reported diploid status of *A. palmeri* (Molin et al. 2017; Rayburn et al. 2005).

In female isolation trial (ii), with increased female isolation due to the removal of males identified by sex-specific molecular markers before plants becoming reproductive, we detected zero seed production in 40 isolated females (Supplementary Table S3). No seed production was observed in plants from the same seed families that produced the most seeds under "isolation" in female isolation (i). Note also that two females that were unbagged (due to flowering at a small size before bagging) but in the isolation greenhouse zone also did not produce seed, confirming a high level of isolation from pollen in this greenhouse. This lack of seed production is also consistent with Wu et al.'s (2023) observation that pistillate flowers (i.e., female plants) develop a fertile gynoecium with no anther primordia, limiting the risk of pollen contamination due to rare hermaphroditism in *A. palmeri*. Overall, the complete lack of seed production in female isolation trial (ii) indicates that autonomous apomictic seed production may not occur in populations of *A. palmeri* in eastern North America (specifically GA, NC, and IL) or, at the very least, that autonomous apomictic seed production is uncommon.

Geographic parthenogenesis refers to the tendency for geographic separation between sexual and asexual populations, including the tendency for asexual reproduction to be associated with larger geographic distributions (Tilquin and Kokko 2016). In this paper, we focused on estimating putative apomixis of *A. palmeri* in populations across eastern North America, with interest in whether more apomixis occurs in more northern regions of this range-expanding crop weed. Apomictic plant species tend to exhibit more northern distributions (Hörandl 2011). Many plants also combine sexual and clonal reproduction, with greater reliance on clonal reproduction toward northern range limits (Eckert 2002). However, overall, we did not detect evidence for apomixis (asexual seed production) in eastern North America. This is consistent with the finding that individuals within glyphosate-resistant *A. palmeri* populations exhibit a wide range of *EPSPS* copy number variation (Yakimowski et al. 2021). If apomixis was commonly occurring, the range of variation would be much less continuous. Yet the possibility of geographic variation in reproduction strategy remains, and we encourage further studies to confirm whether the observations in Mississippi (Ribeiro et al. 2014) or in other geographic regions are consistent with apomixis. We also encourage the use of sex-specific molecular markers to best isolate females and caution against the presence of any males or pollen-producing *A. palmeri*. In addition, we look forward to more studies that survey sex ratios when any putative apomictic seed production is observed to test the prediction of female-biased sex ratios. To date, we have observed near 1:1 sex ratios in our studies of eastern *A. palmeri* populations. However, in a study of four populations from Illinois, sex ratio variation (both female and

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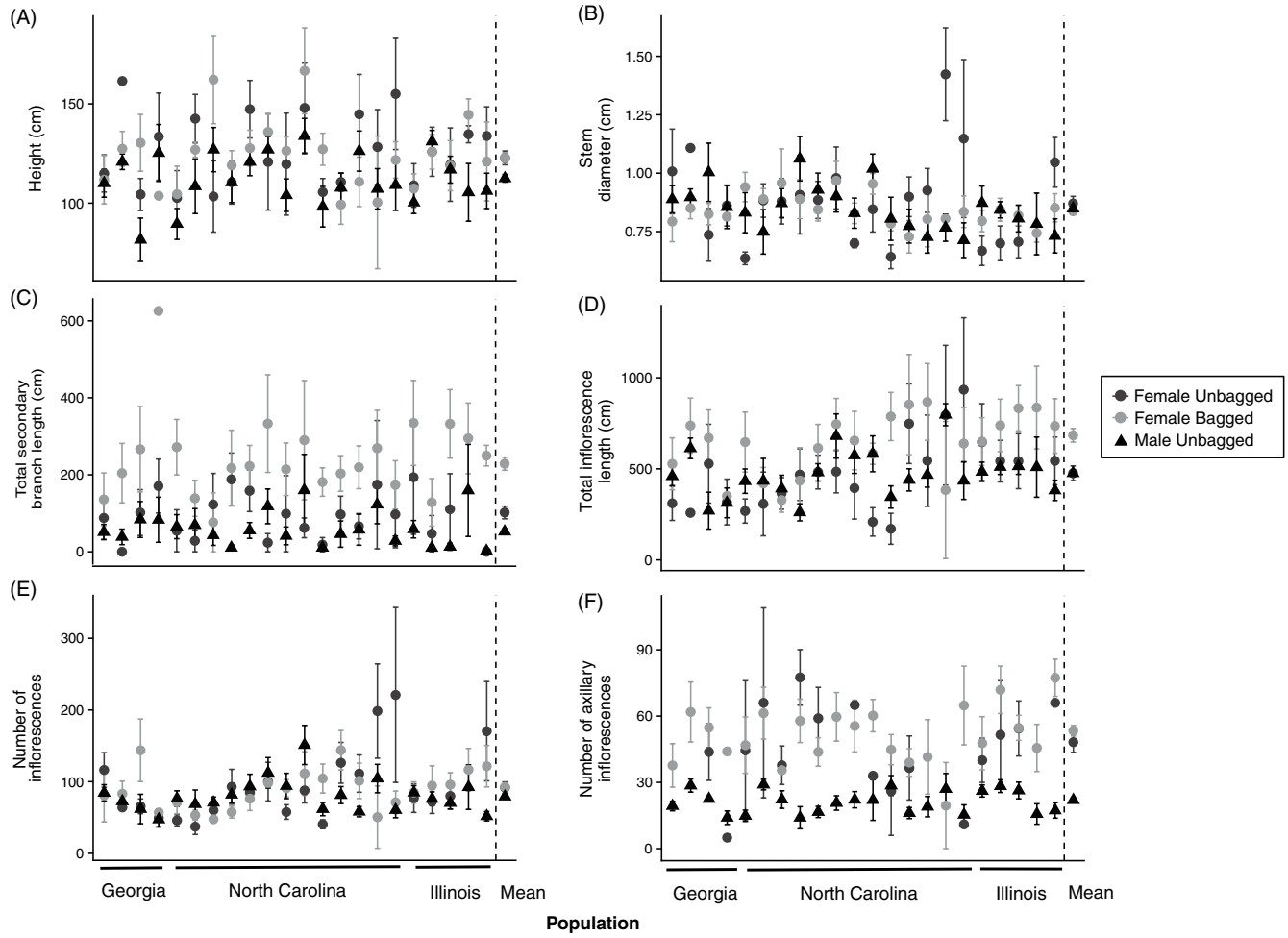

Figure 4. Average values with standard error of six phenotypic traits for female (circles) and male (triangles) *Amaranthus palmeri* from 23 populations collected from Georgia, North Carolina, and Illinois. (A) Height, in centimeters; (B) stem diameter, in centimeters; (C) total secondary branch length, in centimeters; (D) total inflorescence length, in centimeters; (E) number of inflorescences; and (F) number of axillary inflorescences. Mean sex effect with standard error is reported to the right of the dotted line. Populations occur (from left to right) in latitudinal order (Supplementary Table S1).

male biased) was detected (Rumpa et al. 2019). Further, there is some indication that the capacity for apomixis could be plastic and associated with environmental stress (Niccolò et al. 2023; Rodrigo et al. 2017). Therefore, future studies combining stressful conditions and female isolation could be worthwhile to further probe whether any capacity for apomixis exists in *A. palmeri*. Yet, at least in the eastern North American portion of the geographic range studied, sexual reproduction is primarily, or perhaps even solely, responsible for the well-known capacity of *A. palmeri* for prodigious seed production.

Sexual Dimorphism of Morphological Traits

Comparison of Plant Size between Females and Males

Of the traits characterizing plant size and architecture, two traits were not significantly affected by isolation bagging of females: height and stem diameter. Plant height was significantly different between sexes (LMM: $F = 6.53$, $P = 0.002$); both bagged and unbagged females (Supplementary Table S4A) were taller ($mean_{Bagged} = 123$, $SE = 3.4$, $mean_{Unbagged} = 124$, $SE = 3.6$) than males (mean = 113, $SE = 2.8$) at maturity (Figure 4A). There was no significant difference in stem diameter between sexes (LMM: $F = 0.4$, $P = 0.64$, $mean_{FemaleBagged} = 0.8$, $SE = 0.02$, $mean_{FemaleUnbagged} = 0.9$, $SE = 0.03$, $mean_{Male} = 0.8$, $SE = 0.02$) (Figure 4B; Supplementary Table S4B).

The number and length and secondary branches have the potential to increase the volume of airstream over which wind-pollinated females are capturing pollen. Both the number and total length of secondary branches were affected by isolation bagging (i.e., significant difference between bagged and unbagged females; Supplementary Table S4C and G). Although significant variation in the number of secondary branches was observed among sex type/bagging groups ($\chi^2 = 62.4$, $P = <0.0001$; Supplementary Table S4G) most of this variation is due to bagging; there is a significant difference between males and bagged females ($P < 0.0001$), but no significant difference between males and unbagged females ($P = 0.266$). Thus, the number of secondary branches did not differ between females and males in our study. We also observed significant variation among sex/bagging groups for the total length of secondary branches (LMM: $F = 17.8$, $P < 0.0001$), and for this trait the difference was observed between both males and bagged females ($mean_{Bagged} = 287$, $SE = 18.4$, nonzero $mean_{Male} = 148$, $SE = 18.9$; $P < 0.0001$), and males and unbagged females (nonzero $mean_{Unbagged} = 214$, $SE = 26.5$; $P = 0.0271$) (Figure 4C). This is consistent with sexual dimorphism in length of secondary branches, such that females produce longer

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secondary branches than males. Long secondary branches on females could be adaptive in the context of wind pollination. Increased number and length of secondary branches has the potential to increase positions available for individual female flowers, decreasing the interference for pollen reception between flowers on the same individual. However, to the best of our knowledge, secondary branching of wind-pollinated plants has not yet been studied in the context of fitness.

Male-biased sexual size dimorphism in angiosperms is common (Fairbairn 1997; Lloyd and Webb 1977; Willson 1991), likely due to females, as the seed-bearing sex, being limited in growth due to high energy (carbon) costs of reproduction (Barrett and Hough 2013). Contrary to this prediction, females in our study were taller than males and exhibited greater secondary stem length. Our observation of greater height at harvest in female *A. palmeri* than males across 22 populations from Georgia, North Carolina, and Illinois are consistent with Korres et al.'s (2017) similar finding under growth chamber conditions for an Arkansas population and Oliveira et al.'s (2022) field study using an accession from Nebraska. However, Mesgaran et al. (2021) did not find a significant difference in final height between sexes in an outdoor net house study of populations from California, Kansas, and Texas. Overall, size dimorphism is common in populations of *A. palmeri*, but can vary. This study is consistent with other studies of wind-pollinated dioecious species, in which larger female size is often reported (Conn 1981; Hesse and Pannell 2011; Pickup and Barrett 2012; Teitel et al. 2016). Larger female plant size, despite the cost of seed maturation, has generally been hypothesized to be due to a greater male nitrogen cost (Ishida et al. 2005), especially in wind-pollinated species, due to the production of large quantities of nitrogen-intensive pollen (Harris and Pannell 2008; Tonnabel et al. 2017). In addition, ongoing growth of females during seed maturation, a period during which males might senesce, can contribute to the overall larger size of females (Lloyd and Webb 1977; Pickup and Barrett 2012).

In addition to potential dimorphism of plant size between sexes, plant size can also vary by geography, with smaller height in particular toward northern latitudes (Moles et al. 2009). However, we did not detect a significant effect of latitude on plant height at senescence, and moreover, no latitude by sex interactions were detected, suggesting that in our sample of populations, sexual dimorphism generally does not vary by latitude. However, the overall lack of latitudinal decline in all estimates of plant size (and reproductive potential) for both females and males is worth noting. One hypothesis for the expectation of smaller plants occurring at higher latitudes is due to the shorter growing season. Smaller plant size at northern latitudes is sometimes accompanied by earlier flowering (Kollmann and Bañuelos 2004; Montague et al. 2008) relative to populations from more southern latitudes. Future work is needed to address whether earlier flowering in northern populations occurs, despite the lack of smaller plant size with latitude. In some studies, shifts in phenology (e.g., earlier flowering in northern populations) have been observed without plant size and reproduction declining (Griffith and Watson 2005), and this is critical for management planning as *A. palmeri* continues to expand its range northward.

Comparison of Secondary Reproductive Traits between Females and Males

Isolation bagging of females had no significant effect on the number of inflorescences or the number of axillary inflorescences (Supplementary Table S4E and F). Our model did not explain significant variation in inflorescence number (LMM: $F = 2.1$, $P = 0.1385$), and there was no statistical difference in inflorescence number between bagged females (mean = 92.9, SE = 6.0), unbagged females (mean = 92.7, SE = 6.9), and males (mean = 79.2, SE = 4.9) (Supplementary Table S4E). Our model did explain significant variation in total inflorescence length (LMM: $F = 17.2$, $P < 0.0001$), but this involves a significant effect of bagging on females ($P = 0.0001$), and although total inflorescence length was significantly greater ($P < 0.0001$) for bagged females ($mean_{Bagged} = 681$, SE = 32.8) than males (mean = 477, SE = 16.7), this difference was not observed for unbagged females ($mean_{Unbagged} = 483$, SE = 39.0) and males ($P = 0.9643$), suggesting a lack of sexual dimorphism for total inflorescence length (Figure 4D; Supplementary Table S4D).

Wind-pollinated species are known for their lack of showy traits in individual flowers, instead exhibiting much architectural variety. We observed striking divergence in the number of axillary inflorescences between male and female *A. palmeri*. Females produced significantly more axillary inflorescences than males, ranging from 0 to ~125 clusters of buds. In contrast, our model explained significant variation in the number of axillary inflorescences (LMM: $F = 77.6$, $P < 0.0001$), and this variation was not due to variation between bagged and unbagged females ($P = 0.3909$). Rather, both bagged ($mean_{Bagged} = 52.8$, SE = 2.1) and unbagged females ($mean_{Unbagged} = 47.7$, SE = 3.6) produced more axillary inflorescences than males (mean = 21.6, SE = 1.9) ($P < 0.0001$ for both contrasts; Figure 4F; Supplementary Table S4F). In wind-pollinated species, it is common for females to produce inflorescences in condensed clusters, directly adjacent to the main stem, in contrast to males, which extend inflorescences away from the main stem (Harder and Prusinkiewicz 2013; Niklas 1985; Payne 1963). The condensed nature of female inflorescences is also often combined with an increase in stiffness (Brulé et al. 2016) of female inflorescences, in contrast to softer, more flowy male inflorescences, which is observed in *A. palmeri*. This architectural specialization is predicted to arise due to differences in optimal airflow: male inflorescences benefit from movement and fast airflow for pollen removal and dispersal, whereas females benefit from stiller air for pollen deposition (Friedman and Barrett 2008; Harder and Prusinkiewicz 2013). The condensed nature of axillary inflorescences contrasts the inflorescences distributed along longer secondary branches on females; however, the stiffness of female branches may allow spatial stability of these more distally located flowers. Finally, female axillary flower buds exhibited rapid seed formation. We observed mature seeds occurring in the axillary positions within 2 to 3 wk of first flowering, while the primary inflorescence is still developing, and months before the development of mature seeds on main inflorescences. Consistent with this, Korres et al. (2023) found female *A. palmeri* exhibits higher mineral accumulation (e.g., N, P, K) in stems and inflorescences, relative to leaves, than do males. More detailed quantification of the spatial distribution of flowers on both sexes and their ultimate reproductive fate is needed to understand the nuances of the wide variety of architectural positions on which flowers appear in this species.

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