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One-step, two-step: Whole-genome-duplication pathways in *Artemisia tridentata* and potential consequences for genome evolution

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Abstract

Premise: The presence of more than two full chromosome sets, polyploidy, is an important component in plant evolution. However, knowledge of the frequency of polyploidization and which pathways contribute to polyploid formation and gene flow among cytotypes remains limited.

Methods: Flow cytometry and chromosome counts were used to determine genome size of *Artemisia tridentata* parents and offspring. Reduced-representation sequencing was used on the parents, offspring, and conspecific taxa to confirm genetic relationships among parents and offspring.

Results: A polyploidization rate of 0.22% was determined from seedlings of diploid parents producing one triploid and one tetraploid. Genome-size estimates coupled with chromosome counts provide evidence for both one-step and two-step pathways in *A. tridentata*. A wild triploid produced viable offspring with varied aneuploidy ($2n = 18 + 1$ to $4n = 36 - 4$) and genome sizes ranging from 11 pg to 18.2 pg.

Conclusions: Our results indicate that one- and two-step pathways to WGD can occur in *A. tridentata*. The two-step pathway could provide a mechanism for bidirectional gene flow via triploid bridge. These pathways could generate substantial genomic variation within the *A. tridentata* polyploid complex. Given the fecundity and WGD rates in this species, the generation of polyploid offspring from diploid progenitors can be relevant to environmental adaptation at generational timescales.

KEYWORDS

aneuploidy, chromosome, flow cytometry, genome size, polyploidy, triploid bridge

Whole-genome duplication (WGD) is widely recognized as important in plants and may be a stimulus for adaptation and diversification (Soltis and Soltis, 2016; Van de Peer et al., 2017). Despite its broad importance, relatively little is known about the pathways involved in generating polyploids (Kolář et al., 2017; Bartolić et al., 2024). Pathways to polyploidy include three types of unreduced gamete fusion: one-step, two-step, and polyspermy (Harlan and deWet, 1975; Ramsey and Schemske, 1998), the latter is not discussed here (Nakel et al., 2017). One-step (bilateral) WGD involves the fusion of two unreduced (diploid) gametes. Two-step (unilateral) WGD involves the fusion of an unreduced (diploid) and a reduced (haploid) gamete producing triploid intermediate (i.e., triploid bridge), followed by fertilization by a reduced gamete in a subsequent generation (Ramsey and Schemske, 1998; Comai, 2005; Kreiner et al., 2017a). Each pathway can have important consequences for gene flow across ploidy levels. The one-step pathway may indicate a triploid block, where chromosomally unbalanced gametes between 2n and 4n plants may lead to sterility, thereby preventing interploidy gene flow back to the diploid progenitors. In contrast, fertile triploids or other odd-numbered cytotypes in a two-step pathway could provide a conduit for bidirectional interploidy gene flow (Brown et al., 2024; Ramsey and Schemske, 1998; Kreiner et al., 2017a; Sutherland and Galloway, 2017).

At present, the frequency of polyploid formation remains largely unknown, especially in wild populations. An understanding of the polyploidy formation pathway(s) and WGD rates may also have practical implications for ecological restoration efforts, particularly with respect to seed increase and transfer guidelines.

Most assessments of polyploidization rates are inferred from the rates of unreduced gamete formation (i.e., pollen), rather than from viable seeds, and therefore the actual frequencies of stable WGD events are mostly unknown (Ramsey and Schemske, 1998; Weiss-Schneeweiss et al., 2013). The average rate of 2*n* gamete production for various species has been empirically estimated to range from <0.1% to 2.50%, although higher outlier rates have also been reported (Bretagnolle, 2001; Ramsey, 2007; Kreiner et al., 2017a; DeVries et al., 2024). Rates of unreduced gamete formation may not directly correlate with frequencies of WGD events because fertilization from unreduced gametes at different ploidy levels may lead to the formation of mainly nonviable progeny (Ramsey, 2007; Köhler et al., 2010). More promising in this respect are surveys of polyploid progeny from diploid parents using flow cytometry or chromosome counts but given that unreduced gametes are a small fraction compared to reduced gametes, ca. 0.1% to 2.5% (Bretagnolle, 2001; Ramsey 2007; Kreiner et al., 2017a; DeVries et al., 2024), this approach requires sample sizes of hundreds or thousands to detect polyploid seedlings.

The *Artemisia tridentata* Nutt. (big sagebrush, Asteraceae) subspecies complex provides an excellent study system to infer mechanisms of WGD and its ecological and evolutionary consequences. This species is a perennial, wind-pollinated, outcrossing shrub, in which subspecies occupy distinct ecological niches throughout the intermountain region in the western United States (Shultz, 2009). This species consists of three widespread subspecies: *Artemisia tridentata* subsp. *tridentata* Beetle (hereinafter subsp. *tridentata*, basin big sagebrush), occurring on deep, well-drained soils; *A. tridentata* subsp. *wyomingensis* Beetle and Young (Wyoming big sagebrush) grows in similar climates as subsp. *tridentata* but is defined by slower growth (Richardson et al., 2021) and dominates landscapes with shallow soils; *A. tridentata* subsp. *vaseyana* (Rydb.) Beetle (mountain big sagebrush) occupies cooler and moderately moist habitats at higher elevations (Mahalovich and McArthur, 2004; Shultz, 2009). Subspecies *tridentata* and *vaseyana* are predominantly diploid ($2n = 2x = 18$). Subspecies *wyomingensis* is strictly tetraploid ($2n = 4x = 36$; McArthur and Sanderson, 1999) and is ecologically important because it occupies a preponderance of the arid part of the *A. tridentata* range and has greater environmental tolerance than both diploid progenitors (Kolb and Sperry, 1999; Brabec et al., 2017).

All three subspecies are ecologically, cytologically, chemically, and genomically distinct (McArthur and Sanderson, 1999; Richardson et al., 2012; Jaeger et al., 2016; Grossfurthner et al., 2023). Previous studies suggest that *A. tridentata* polyploids may be the result of (recurrent) autopolyploidy (McArthur et al., 1981; Richardson et al., 2012), but genetic similarity within tetraploids was higher inter se than to their different putative diploid progenitors (Faske, 2023; Grossfurthner et al., 2023). Co-occurrence of and hybridization between distinct diploid subspecies in basin-mountain ecotones (McArthur et al., 1988; McArthur and Sanderson, 1999) may give rise to allopolyploidy, whereas production of unreduced gametes within subspecies may lead to autotetraploidy. In both cases, one-step and two-step reproduction are possible pathways to tetraploidy. Based on the genetically distinct subspecies (Richardson et al., 2012; Grossfurthner et al., 2023), we refer to polyploids that have an admixed genetic signature between diploid subsp. *tridentata* and subsp. *vaseyana* as putative segmental allopolyploids (i.e., polyploids occurring between subspecific taxa with similar chromosome sets and hereafter referred to as allopolyploids). Polyploids with high genetic similarity to their parental group are referred to as autopolyploids.

To identify the frequency of one-step and two-step pathways to polyploidy in *A. tridentata*, we used two experiments. For the one-step experiment, we grew 900 seeds from 12 diploid subsp. *tridentata* open-pollinated mothers. These mothers were growing in two environments (i.e., common gardens) with six mothers in each garden. Tetraploid offspring would indicate a one-step pathway, while triploid offspring would indicate a two-step pathway. Cultivated seedlings were screened for WGD using flow cytometry. In the two-step experiment, seeds from a wild, open-pollinated triploid subsp. *vaseyana* were collected and used to estimate the ploidy of its offspring. Viable offspring from the triploid would indicate the potential for a triploid bridge.

We used flow cytometry to estimate genome size, coupled with chromosome counts to estimate ploidy, and double-digested restriction-site-associated DNA (ddRAD) sequencing of the cultivated individuals and their close and distant relatives to assess the frequency of auto- and allopolyploidy origins of neopolyploids. Specifically, we (1) assessed the frequency and ploidy of neopolyploids from diploid progenitors in the common garden plants and (2) evaluated aneuploidy of progeny from a wild triploid. We further (3) tested whether interspecific hybridization with related *Artemisia* species or subspecies may have given rise to allopolyploids or whether autopolyploidy occurred within an *A. tridentata* subspecies.

MATERIALS AND METHODS

One-step experiment

Seeds were collected from a total of 12 diploid *A. tridentata* subsp. *tridentata* mothers (Appendices S1, S2), growing in two common gardens, Orchard, Idaho ($N = 6$) and Majors Flat, Utah ($N = 6$). Common gardens were chosen as seed sources because the gardens are located in different climate regimes (Chaney et al., 2017), subspecies of the source populations were previously genetically identified (Richardson et al., 2012), and extensive trait data were gathered since establishment of the gardens (Chaney et al., 2017; Richardson et al., 2021). Even though common gardens contained plants from a wide

geographic range, the plants were observed to largely retain the natural phenology (Richardson et al., 2017) among environmentally different source populations (Richardson and Chaney, 2018), mitigating pollination by plants non-native to the respective garden climates. Seeds from common garden parents were collected in mesh bags (see Richardson et al., 2021) in November 2013 cleaned and stored at –20°C until January 2018. Seeds were then directly sown into potting trays containing a soil mix of 1:1 v:v peat to sand. Seeds from each parent were placed in rows with ca. 3.5 cm separating the seeds. Seeds were germinated in the greenhouse with regular misting to ensure the soil remained moist. Seedlings were grown for 8 weeks, before harvesting leaves for flow cytometry.

Two-step experiment

Seeds from a triploid from a natural site in Oregon (Appendices S1, S2) were collected in mesh bags following the methods described above. Seeds were then cleaned to remove chaff and stratified for at least 2 weeks at –20°C. A total of 45 seeds were placed on a moist filter paper (Whatman Filter; Thermo Fisher Scientific, Waltham, MA, USA) in a petri dish at 4°C until radicles were detectable and subsequently transferred into pots and grown in the greenhouse.

Ploidy and genome size estimates

About 1 cm^2 of fresh leaf tissue was chopped in 330 µL Nuclei Extraction Buffer (Sysmex, Kobe, Japan) with internal standards. Due to different sampling periods and availability of internal standards, we used fresh leaf tissue of *Atriplex canescens* (Amaranthaceae; 1.653 pg/1 C) for screening common garden progeny; dried leaf tissue of *Acer negundo* (Sapindaceae; 0.535 pg/1 C; Loureiro et al., 2007) for the population screening at the natural site, and dried *Acer rubrum* 'Autumn Spire' (Sapindaceae; 2.045 pg/1 C; Contreras and Shearer, 2018) for genome size estimates of the progeny cultivated in the greenhouse. Genome sizes of internal *Acer* standards were confirmed with fresh *Pisum sativum* 'Ctirad' (Fabaceae; 4.38 pg/1 C; Contreras and Shearer, 2018), to ensure suitability and comparability of dried standards for genome size estimates. Cell wall remnants were removed by filtering the isolate through a 30-µm filter (CellTrics, Sysmex, Kobe, Japan). For field sample screening and genome size estimates, a glycerol treatment (Kolář et al., 2012) was performed to remove UV-fluorescing coumarin compounds. In short, 0.5 mL of 60% glycerol solution was added to the isolates; samples were then centrifuged at 3200 rpm for 3 min, and the supernatant was discarded. The nuclei were then re-suspended with 0.5 mL Nuclei Extraction Buffer (Sysmex, Kobe, Japan) and prepared for flow cytometry. All measurements were performed on a Partec Cyflow Space (Sysmex), equipped with a green laser (532 nm, 30 mW) and a UV-LED (365 nm, 60 mW).

The common garden progeny and field sample population were screened using DAPI (4′,6-diamidino-2-phenylindole; CyStain UV Precise P Kit; Sysmex) and the manufacturer's protocol. DAPI is suitable for rapid ploidy screening but lacks accuracy for DNA content and genome size estimates due to the preferential binding of the fluorochrome to A-T rich regions (Doležel et al., 1992).

Two outlier samples from the common garden and two of their diploid half siblings and progeny from the field site were additionally analyzed with propidium iodide (PI; CyStain PI Absolute P Kit, Sysmex) because the DNA intercalating fluorochrome does not have any base preference and allows increased accuracy of DNA content and genome size estimates (Doležel et al., 1992). Measurements were taken three times to assess consistency of measurements within a sample and calculated as 2C-values (Sample 2 C [pg] = Standard G1 mean/Sample G1 mean × Standard 2 C [pg]; Doležel and Bartoš, 2005).

To test for the presence of triploids, we analyzed sample pools containing samples with intermediate genome size and confirmed diploid and tetraploid samples.

Chromosome counts

Actively growing root tips, about 1 cm long, were excised from the putative tetraploid, triploid, and offspring of the putative triploid plants growing in potting soil in the greenhouse using fine-pointed forceps. The root tips were immediately pretreated with 2.5 mM 8-hydroxyquinoline (HQ) for 4 h in the dark, then rinsed briefly with double-distilled water (ddH_2O) twice for 2 min each, with two to three gentle swirls each to remove any residual HQ. The root tips were then fixed in a 3:1 95% v/v ethanol to glacial acetic acid in small glass vials. The fixative was replaced with fresh fixative after 5 min and was stored at room temperature (RT) until enzymatic processing for chromosome spreads.

Root tips were enzymatically digested as described by Jewell and Islam-Faridi (1994). Briefly, fixed root tips were rinsed with ddH_2O for 30 min to remove the fixative, then treated with 0.2 N HCl at 60°C for 15 min, then at RT for 10 min. The root tips were rinsed twice with ddH_2O for 10 min each, then treated with cold 0.01 M citrate buffer (pH 4.5) at RT. The meristematic portion of the root tips (appearing milky white) was excised and transferred into 0.5-mL centrifuge tubes containing approximately 50 µL of an enzyme mixture (2.5% w/v cellulase RS, 1% w/v macerozyme R10 (Yakult Pharmaceutical, Tokyo, Japan), 1.5% w/v pectolyase Y23 (Kyowa Chemical Products, Kagawa, Japan), and 30% v/v cellulase (C2730), 30% (v/v) pectinase (P2611) (both from Sigma-Aldrich, St. Louis, MO, USA), and 40% 0.01 M citrate buffer pH 4.5). One to three or four root tips of similar sizes (based on tip's thickness) can be processed together in slightly higher volume of enzyme mixture. The digestion time varied from 18 to 50 min depending on the thickness of the root tips, then the solution was removed without disturbing the tips. Samples were washed three times with fresh buffer,

then used to prepare chromosome spreads as described by Jewell and Islam-Faridi (1994).

Chromosome spreads were stained with azure-B (A4043, Sigma) and permanently mounted in Permount (SP15-100, Thermo Fisher Scientific). The stained chromosome spreads were observed using bright-field optics with a green filter, and a 63× plan-apochromatic oil-immersion objective on the AxioImager M2 microscope (Zeiss, Göttingen, Germany). Images were recorded with a charge-coupled device (CCD) camera (Cool Cube 1, MetaSystems Group, Boston, MA, USA), then processed first using ISIS v5.1 (MetaSystems Group, Altlussheim, Germany) and further refined with Photoshop 2025 (Adobe Systems, San Jose, CA, USA).

DNA extractions

Leaves from the common garden plants and 11 progeny from plants at the natural site were freeze dried (Freezone 6, Labconco, Kansas City, KS, USA), a ball mill (Retsch MM400, Retsch, Haan, Germany) was used to grind leaf tissue. The ground tissue was treated with a sorbitol wash buffer, then DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol (Inglis et al., 2018) as detailed by Grossfurthner et al. (2023). DNA was quantified using Qubit dsDNA HS and BR Assay kits (Thermo Fisher Scientific).

ddRAD library preparation

Two double-digest (dd)RAD libraries were prepared for sequencing using the method of Grossfurthner et al. (2023). The first library included four progeny from the common garden, the mother of the field samples, and five randomly selected individuals per subspecies from an earlier study for which we sampled *A. tridentata* and four outgroups across several elevational transects (Grossfurthner et al., 2023). The second library included the mother and 11 progenies from the field site (O1). In short, samples for the first library were prepared using EcoR1 and Mse1 restriction enzymes (New England Biolabs, Ipswitch, MA, USA), following the protocol of Parchman et al. (2012), then shipped to the Genomics and Cell Characterization Core Facility (GC3F) at the University of Oregon (https://gc3f.uoregon.edu/) for fragment size selection (Blue Pippin Prep, 400 bp to 550 bp; Sage Sciences, Beverly, MA, USA) and sequencing using 150-bp paired-end reads (Illumina NovaSeq S4 lane; Illumina, San Diego, CA, USA).

The second library was prepared at the genome technology laboratory (https://microcollaborative.atlassian.net/wiki/spaces/MICLAB/overview) using the same ddRAD method, except using 20 PCR cycles instead of 30 as in previous studies (Parchman et al., 2012; Grossfurthner et al., 2023). Samples were subsequently sequenced at Admera Health (South Plainfield, NJ, USA; NovaSeq X Plus lane, Illumina) as 150-bp paired-end reads.

Variant calling

Raw sequenced reads were demultiplexed as single-end reads due to incompatibilities between index and sequencing primers (process_radtags function of STACKS 2.60; Catchen et al., 2013) based on inline barcodes and filtering for uncalled bases (-c) and low quality (-q). To include additional samples and outgroups, we included raw demultiplexed reads originating from the same sequencing protocol (Grossfurthner et al., 2023). The demultiplexed files were then filtered for polyG tails (-g) and adapter content (-a, fastp; Chen et al., 2018; available at https://github.com/OpenGene/fastp).

Each filtered read file was mapped against the *A. tridentata* reference genome (Melton et al., 2022) using the maximum exact match (MEM) algorithm (BWA 0.7.17; Li and Durbin, 2010). The resulting SAM files were sorted by reference coordinates and annotated with read groups (picard tools 2.9.2; available from https://broadinstitute.github.io/picard/). Due to the highly repetitive genome of *A. tridentata*, only high quality (mapQ ≥ 30) uniquely mapped reads were selected (SAMtools 1.15; Li et al., 2009) for downstream analyses.

Two sets of genotypes were called with STACKS ref_map.pl (Catchen et al., 2013), requiring at least 80% of the samples to share a locus (-R). The first data set included four outgroups (*Artemisia arbuscula*, *Artemisia nova*, *Artemisia rigida*, and *Artemisia tripartita*; Asteraceae) and was filtered to retain biallelic single nucleotide polymorphisms (SNPs), a minimum coverage of five, maximum 20% missing data and a minor allele frequency of 0.03 (VCFtools v. 0.1.14; Danecek et al., 2011). Outgroups were removed from the second data set; samples were filtered for biallelic SNPs, a minimum coverage of five, maximum 15% missing data and a minor allele frequency ≤0.03 using vcftools. After demultiplexing and quality filtering, a mean of 12.1 (±12.6 SD) million high-quality reads per sample was retained, 97.5% (±3.14%) of which mapped to the reference genome. The removal of reads mapped at multiple positions or with low confidence yielded a mean (±SD) of 1.3 million (±1.1 million) reads. After filtering for 15% missing data, coverage >5, and a minor allele frequency of 0.03, the data sets with and without outgroups retained 696 and 1020 SNPs, respectively.

Evolutionary relationships of heteroploid samples

To construct a phylogeny of heteroploid *A. tridentata* subspecies in relation to the outgroups, we first converted the filtered VCF file to a nexus file (vcf2phylip; available at https://github.com/edgardomortiz/vcf2phylip). The sequence file, containing 696 concatenated SNPs from 46 individuals, was then imported to R version 4.3.1 (R Core Team, 2023) and tested for nucleotide substitution models best explaining our data (modelTest, R package phangorn; Schliep, 2011). An unrooted maximum-likelihood phylogenetic tree was constructed under the nucleotide substitution model with the lowest Bayesian information criterion (BIC) score (R package phangorn and ggtree; Yu, 2020).

TABLE 1 Genome size estimates of the parental plants and their offspring from one-step and two-step experiments.

Sample ID	Location	2C (pg)	Stain	Family origin	Experiment
COT1-1	Majors Flat	7.69	DAPI	Parent	One-step
IDT2-3	Majors Flat	7.58	DAPI	Parent	One-step
IDT2-9	Majors Flat	7.22	DAPI	Parent	One-step
UTT2-2	Majors Flat	7.78	DAPI	Parent	One-step
WAT1-4	Majors Flat	7.67	DAPI	Parent	One-step
WAT1-9	Majors Flat	7.73	DAPI	Parent	One-step
COT1-1	Orchard	9.39	DAPI	Parent	One-step
IDT2-5	Orchard	8.77	DAPI	Parent	One-step
IDT2-8	Orchard	8.52	DAPI	Parent	One-step
UTT2-5	Orchard	8.83	DAPI	Parent	One-step
WAT1-4	Orchard	7.67	DAPI	Parent	One-step
WAT1-5	Orchard	9	DAPI	Parent	One-step
IDT2-5	Greenhouse	15.3	PI	Offspring	One-step
WAT1_4	Greenhouse	17.3	PI	Offspring	One-step
O101	Lookout Mtn	12.2	PI	Parent	Two-step
O101_02	Greenhouse	18.3	PI	Offspring	Two-step
O101_03	Greenhouse	12	PI	Offspring	Two-step
O101_05	Greenhouse	11.6	PI	Offspring	Two-step
O101_06	Greenhouse	13.9	PI	Offspring	Two-step
O101_07	Greenhouse	11.6	PI	Offspring	Two-step
O101_08	Greenhouse	11.9	PI	Offspring	Two-step
O101_09	Greenhouse	11	PI	Offspring	Two-step
O101_10	Greenhouse	12.7	PI	Offspring	Two-step
O101_11	Greenhouse	11.7	PI	Offspring	Two-step
O101_12	Greenhouse	11.1	PI	Offspring	Two-step
O101_13	Greenhouse	12.8	PI	Offspring	Two-step

DAPI (4′,6-diamidino-2-phenylindole) or propidium iodide (PI) were used to calculate 2C values. Only polyploidy offspring are shown for the one-step experiment; all one-step experiment offspring are shown in Appendix S4.

To explore relationships within heteroploid *A. tridentata* subspecies, we performed a principal component analysis (PCA; R package adegenet; Jombart, 2008) and visualized the results as a scatterplot (R package ggplot). For further Bayesian clustering analyses, see Appendix S3.

RESULTS

One-step experiment

The 12 parental plants from two common gardens, Majors Flat ($N = 6$) and Orchard ($N = 6$), had a mean DAPI-based 2C-value of 8.15 pg (±0.71 SD; Table 1). Flow cytometry conducted on the offspring of these 12 plants, 900 seedlings, had a mean 2 C genome size of 7.46 pg (±0.740; Appendix S4). Two outlier seedling samples from two parents, IDT2-5 from the Orchard garden and WAT1-4 from the Majors garden, had a genome that was 1.46-fold and 2.01-fold larger than the average, respectively (Table 1, Figure 1). The two samples with the larger genomes were further analyzed using PI (Figure 2A; Appendix S5), revealing 2C-values of 15.3 pg (±0.112) and 17.3 pg (±0.178), respectively. Polyploidy was further confirmed with chromosome counts showing $2n = 3x = 27$ (Figure 2B) and $2n = 4x = 36$ (Figure 2C). In total, two of 900, 0.22% seedlings underwent polyploidization.

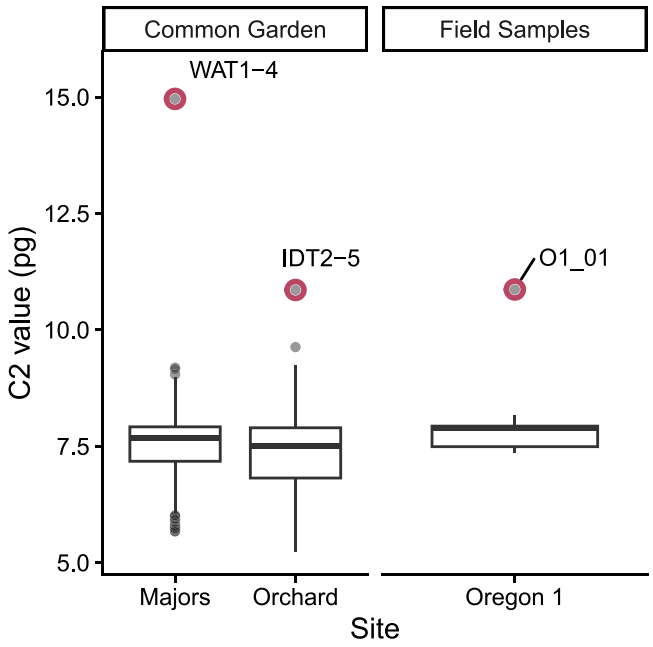

FIGURE 1 Boxplot of genome sizes of 900 cultivated seeds collected from common gardens, one-step experiment (left columns), and 11 wild-collected seeds, two-step experiment (right columns). The labelled outliers correspond to samples estimated to contain one or two additional sets of chromosomes compared to the population average. C2 values are derived from DAPI stain. Flow cytometric standards used: common garden = *Atriplex canescens*; Field samples = *Acer negundo*. See Figure 2A, B for chromosome image of IDT2-5 and WAT1-4, respectively.

Two-step experiment

At the field site, the mean DAPI-based 2C-value for the population ($N = 13$; Appendix S6) was estimated to be 7.91 pg (±0.750). One sample (O1_01) had a 1.52-fold genome size increase, 12.02 pg, compared to the population mean at this site (Figure 3A). Seeds collected from this sample (i.e., O1_01) yielded 11 offspring plants. Offspring genome sizes ranged from 11.0 pg (±0.05; O101_09) to 18.3 pg (±0.182; O101_02), with an array of intermediate genome sizes (Table 1, Figure 3A). Chromosome counts from microscopy confirm aneuploidy for the offspring of O1_01. The wide range in genome size (11–18 pg) corresponded to differences in chromosome number. For example, O101_09, 11 pg, contained one extra chromosome, $2n = 18 + 1$, O101_3, 12 pg, contained two extra chromosomes, $2n = 18 + 2$, and O101_2, 18.3 pg, contained 14 extra chromosomes, $2n = 3x = 27 + 5$ (Figure 3B–D, respectively).

Genetic relationships of heteroploid *A. tridentata*

After filtering, we generated final data sets of 696 SNPs (with outgroups) and 1020 SNPs (without outgroups) for further analysis. The phylogeny constructed under the TVM model, the nucleotide substitution model best explaining the data set including the outgroups, distinguished *A. tridentata* and its outgroups (*Artemisia arbuscula*, *Artemisia nova*, *Artemisia rigida*, and *Artemisia tripartita*; Figure 4A). Within the *A. tridentata* ingroup, subspecies were generally separated into clades, with subsp. *wyomingensis* located intermediate to its two diploid progenitors (Figure 4A). These relationships were also reflected in the PCA where outgroups were excluded. The first two eigenvectors of the PCA explained 22.5% of the variation. Principal component (PC) 1 showed a clear separation between the diploid subspecies, whereas PC 2 separates 4*x* cytotypes. In PCA space, the neotriploid common garden plant (IDT2-5) clustered within the group of subsp. *tridentata*, while the neotetraploid (WAT1-4) occupies an intermediate position between subsp. *tridentata* and subsp. *wyomingensis*. The field-collected triploid (O1_01) and its aneuploid offspring (i.e., + 1 to + 5 chromosomes) from the field site formed a subcluster, offset from other 2*x* plants in the same population and larger subsp. *vaseyana* cluster (Figure 4B). The relationships observed in the phylogenetic tree and PCA were further supported by ancestry assignments inferred using Bayesian clustering (Appendix S7).

DISCUSSION

WGD rate

In our one-step experiment, we estimated the rate of polyploidization from diploid parents using subsp. *tridentata* from two common gardens. In previous work, the frequency of stable polyploid formation has been shown to vary depending on several factors including the taxa, relatedness of the parents, mode of reproduction, and environment (Ramsey and Schemske, 1998; Kreiner et al., 2017b). Estimates of unreduced gamete production have ranged from 0.1% to 0.24% (Ramsey, 2007; DeVries et al., 2024) with reports as high as 2.5% in the Brassicaceae (Kreiner et al., 2017b). While these are indirect measures of polyploid formation based on gamete ploidy level, our direct measures from seedlings from diploid parents (0.22%) falls within the lower range of these estimates. Our results included a triploid, likely resulting from the fusion of a reduced and an unreduced gamete, and a tetraploid that could have either resulted from the fusion of two unreduced diploid gametes or an unreduced egg with a reduced tetraploid pollen donor. Nevertheless, whether the tetraploid offspring was formed from one or two unreduced gametes, our direct measure of polyploidization supports indirect estimates, suggesting that pre- or postzygotic barriers may be weak at least in the flowering plants that have been studied.

Despite reports here and from other species that polyploidization events are comparatively rare (Bretagnolle, 2001; Ramsey, 2007; Kreiner et al., 2017a, DeVries et al., 2024), it is important to place these rates in the context of reproductive output in this species. A diploid subsp. *tridentata* can produce about 1.3 million seeds annually (Richardson et al., 2021).

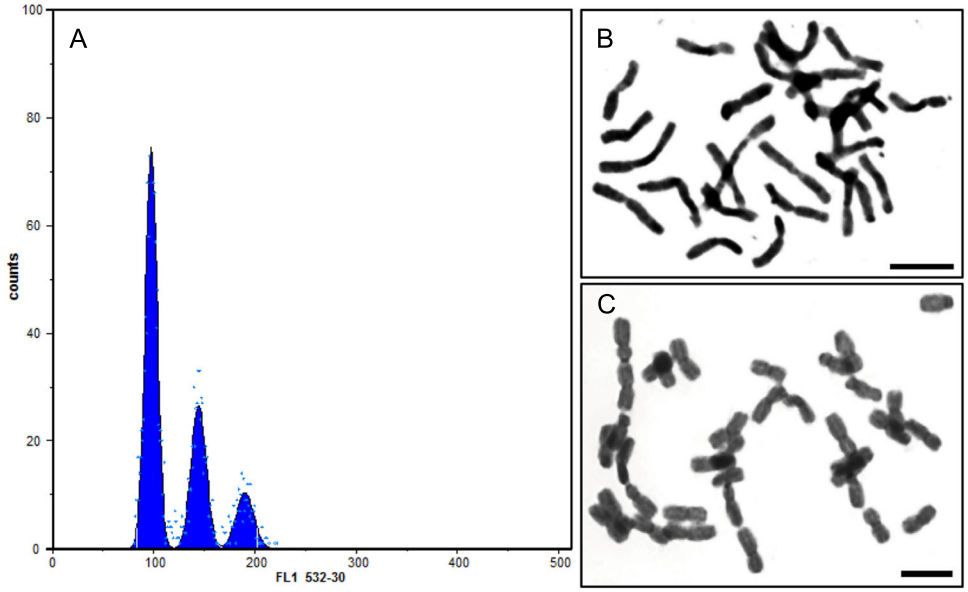

FIGURE 2 Flow cytometry and chromosome image results of the one-step experiment. (A) Flow cytometric chromatogram with peaks showing the following from left to right: diploid (IDT2-9, $2n = 8.77$ pg); triploid (IDT2-5, $3n = 15.3$ pg); and tetraploid (WAT1-4, $4n = 17.3$ pg). C2 values are derived from propidium iodide staining. The peak for the standard *Acer negundo* is not shown. See Appendix S5 for the full chromatogram. Chromosome images are shown for (B) triploid ($n = 27$) IDT2-5, and (C) tetraploid ($n = 36$) WAT1-4. Scale bar = 5 μm.

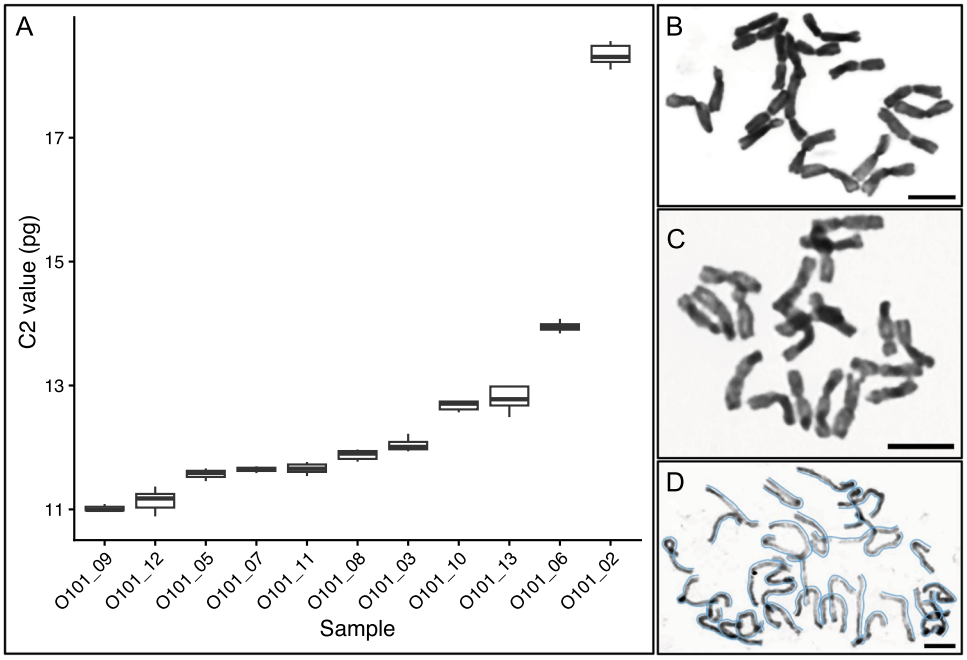

FIGURE 3 Two-step experiment genome size estimates and chromosome images for the offspring of the triploid parent. (A) The range of genome sizes from 11 pg to 18.3 pg are shown for individual offspring. Chromosome images are shown for samples (B) O101_09, $2n = 18 + 1$, (C) O101_03 $2n = 18 + 2$, and (D) O101_02, $3n = 27 + 5$, blue lines help to discern chromosomes. Scale bar = 5 μm.

Assuming a conservative one-step WGD rate of 0.1%, a plant could produce ca. 1000 polyploid seeds annually. In addition, the presence of triploids, discussed below, may allow neopolyploids to overcome their minority cytotype disadvantage and aid in establishment in the presence of their diploid ancestors (Husband, 2004). The level of polyploidy production may in part explain the widespread abundance of tetraploidy in *Artemisia tridentata* (McArthur and Sanderson, 1999) and differentiation in tetraploid populations (Grossfurthner et al., 2023), likely from separate polyploidization events.

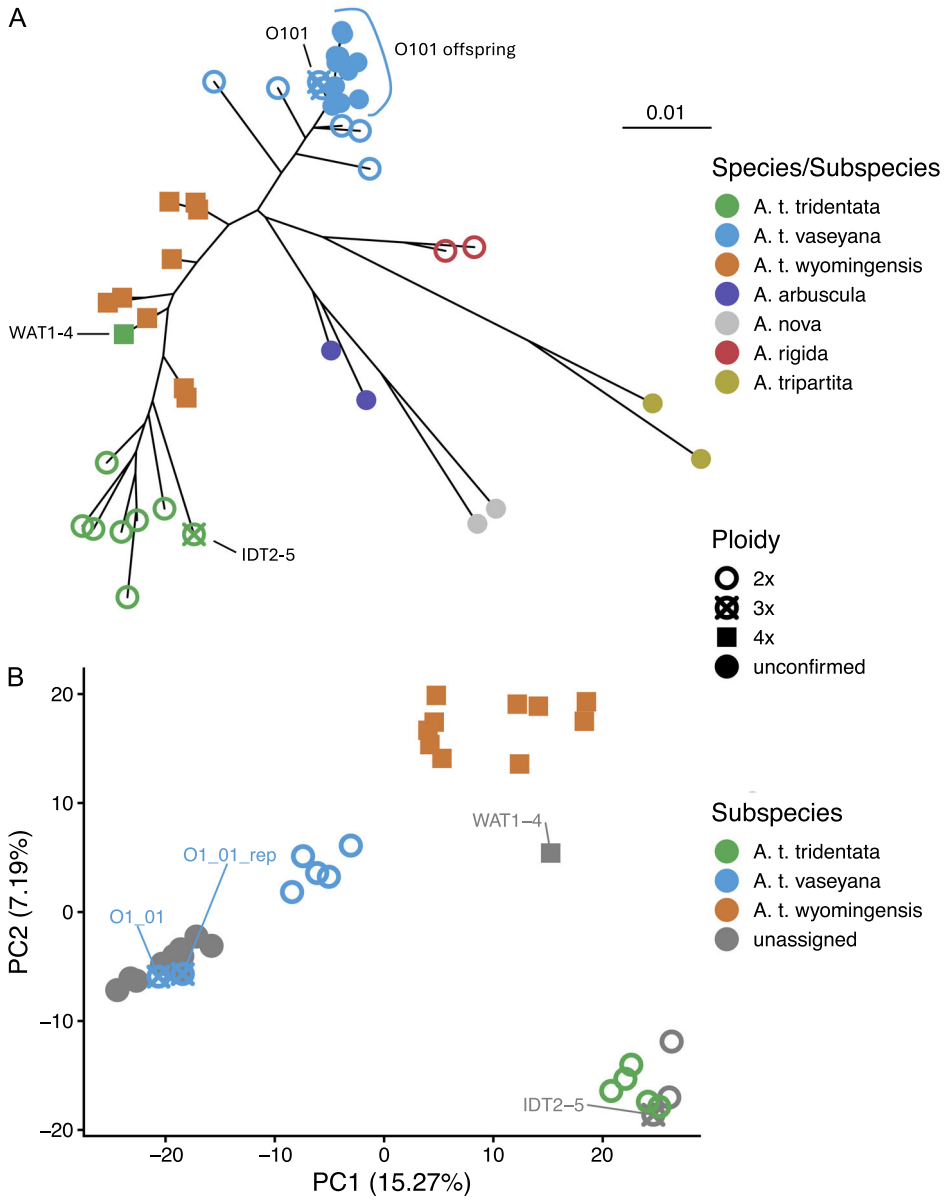

FIGURE 4 Genetic relationships among *Artemisia* taxa. (A) Phylogenetic relationships based on an unrooted maximum-likelihood phylogenetic tree of *A. tridentata* and four outgroups (*A. arbuscula*, *A. nova*, *A. rigida*, and *A. tripartita*) estimated from TVM distance based on 696 SNPs. (B) Genetic structure of *Artemisia tridentata* derived from principal components (PCs) based on 36 samples and 1020 SNPs. Colors correspond to samples previously assigned to subspecies (Grossfurthner et al., 2023), whereas gray points represent samples not previously assigned. Neopolyploid common-garden progeny and the triploid field sample are labeled. Sample ploidy labeled as unconfirmed in both A and B correspond to aneuploid individuals or those lacking cytological confirmation.

Neopolyploid pathways and ploidy variation

Our two-step experiment relied on a triploid *A. tridentata* subsp. *vaseyana* found in a natural population in a previous study (Grossfurthner et al., 2023). In contrast to our expectations of triploid infertility, 13 of the 45 wild triploid seeds germinated and survived past the seedling stage, resulting in 28.9% establishment. However, it remains unclear whether these aneuploids could reproduce. Two of the seedlings did not survive the first winter, whereas the remaining 11 potted aneuploids grew >2 years before dying, which is the typical age at which *A. tridentata* becomes reproductive (Richardson et al., 2021); notably, seven of these individuals produced inflorescences before dying. Nevertheless, these results demonstrate a wide tolerance for chromosome imbalance in *A. tridentata*, at least through prereproductive stages, and suggests that aneuploids may be intermediaries in interploidy gene flow, potentially generating neodiploids and tetraploids. These observations support previous findings where triploids generate small numbers of haploid, diploid and triploid gametes, that allow formation of (near-) diploid, triploid, and tetraploid offspring by backcrossing to diploids (Satina et al., 1938; Ramsey and Schemske, 2002; Henry et al., 2010; Schinkel

et al., 2017). The high mortality in triploid offspring supports the view that, while triploid individuals may produce viable offspring, a large proportion likely have reduced fitness and/or are infertile (Burton and Husband, 2000; Ramsey and Schemske, 2002; Morgan et al., 2021).

Evolutionary relationships of heteroploid *Artemisia* species

The heteroploid *Artemisia* species in our sampling were separated into four distinct outgroups and five lineages within *A. tridentata*. The latter differ by subspecies classification, ecology, ploidy, and geography and are overall in accordance with previous findings within this subspecies complex (Richardson et al., 2012; Grossfurthner et al., 2023). The neopolyploids in this data set did not show any signs of hybridization with the four outgroup species (Figure 4A; Appendix S7), indicating that polyploidization occurred within *A. tridentata*. However, an allopolyploid origin cannot be ruled out entirely as only four of the eleven recognized species (Shultz, 2009) were included in this study, and our sample size was limited. Moreover, autopolyploidy may lessen reproductive barriers leading to tetraploid hybridization within or among species in subsequent generations. Genetic analyses support such a hypothesis, given that subspecies *wyomingensis*, exclusively tetraploid, has an intermediate position between subsp. *tridentata* and *vaseyana* in genetic analyses (Richardson et al., 2012; Faske, 2023; Grossfurthner et al., 2023).

The close genetic association between the neotriploids (IDT2-5 and O1_01) and their respective parents supports the interpretation that they formed from autopolyploidy. In contrast to our expectation, the neotetraploid originating from the common garden is located between diploid subsp. *tridentata* and tetraploid subsp. *wyomingensis* in the PCA (Figure 4B) and further shows signals of the tetraploid gene pool in Bayesian clustering derived bar plot (Appendix S7). This result supports a scenario of an unreduced diploid egg fertilized by a reduced tetraploid pollen from subsp. *wyomingensis*, indicating that backcrossing from higher ploidy levels to diploids is possible in *A. tridentata*, as has been reported in other species (Ramsey and Schemske, 1998; Brown et al., 2024).

A major goal of this study was to assess the frequency and stability of WGD events and identify pathways to polyploidy in *Artemisia*. We opted to assess the frequency of WGD events based on screening seedlings in contrast to unreduced gametes, as identification of successful WGD, which may be biologically more informative than analyzing the gametes by themselves (Sora et al., 2016; Schinkel et al., 2017; Kreiner et al., 2017b). Whole-genome duplication was rare in *Artemisia tridentata*, in the common garden and in the field, but rates were comparable to those from gamete screening (Ramsey 2007; DeVries et al., 2024). We found one neotetraploid and two neotriploids, one of which we were able to confirm (short-term) viability of the offspring, despite evidence of unbalanced chromosomes. We used ddRAD sequencing to test whether WGD occurs between or within taxa and identify taxa contributing to WGD. We found WGD in *A. tridentata* can occur via autopolyploidy, forming triploids within taxa and likely also through polyploid formation involving hybridization between distinct subspecies at different ploidies, producing tetraploids. Our results support the hypothesis of polyploid origins involving autopolypoidy within subspecies and segmental polyploidy between subspecies.

Previous studies identified evidence that link polyploid establishment to periods of environmental and ecological stress, suggesting that WGD may increase the potential for adaptation under changing environmental conditions in plants in general and in *A. tridentata* in particular (Still and Richardson, 2015; Van de Peer et al., 2017; Sharma et al., 2020; Blake-Mahmud et al., 2025). Polyploids are thought to minimize reproductive barriers between diploid progenitors and act as reservoirs for genetic variation (Schmickl and Yant, 2021). These characteristics may help facilitate expansion into clinal environments between a mosaic of diploid progenitors (Massatti et al., 2025). Considering that subsp. *wyomingensis*, a tetraploid, is the predominant subspecies found in the warm-dry clines of this species distribution (Mahalovich and McArthur, 2004; Still and Richardson, 2015), a better understanding of the frequency and modes of WGD and the consequences of polyploidy will be important to inform conservation and restoration decisions for sagebrush ecosystems. Moreover, maintaining connectivity of subspecies could be crucial to maintaining sagebrush ecosystems, especially in the most arid regions where tetraploid populations are most prevalent (Still and Richardson, 2015). These findings highlight a dynamic reproductive system in the *Artemisia tridentata* polyploid complex. The presence of both one-step and two-step polyploidization, along with the formation of diverse aneuploids, suggests a system that (1) possesses mechanisms for generating substantial genomic variation, (2) can produce polyploids over generational timescales, and (3) may provide a conduit for interploidy gene flow via a triploid bridge.

AUTHOR CONTRIBUTIONS

All authors planned and designed the experiment; L.G., N.F., and B.R. performed the experiments and analyzed the data; L.G. and B.R. wrote the manuscript; all authors revised the manuscript.

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OIA-1757324, and the USDA Forest Service Rocky Mountain Research Station. The findings and conclusions in this publication are those of the authors and should not be construed to represent any official USDA or U.S. Government determination or policy. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

DATA AVAILABILITY STATEMENT

The genome size estimates for the respective samples, the vcf and fasta files, the pipeline for reproducible data analysis, and demonstrative pictures of the plants under scrutiny can be found at https://github.com/LukeBotanist/Pathways2Polyploidy.git.

The unfiltered, demultiplexed reads can be found at NCBI (https://www.ncbi.nlm.nih.gov: accessions PRJNA942958 and PRJNA1435120).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Attributes of sampling locations.

Appendix S2. Map of sample locations from field collections and WGD experiment.

Appendix S3. Supplementary methods.

Appendix S4. Genome size estimates for 900 seedlings.

Appendix S5. Flow cytometric chromatogram of one-step experiment plants.

Appendix S6. Genome size estimates of 13 wild-collected plants.

Appendix S7. Bayesian clustering derived bar charts.

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