Relative fecundity and ploidy of 34 *Buddleja* cultivars[1]

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Abstract

Buddleja davidii (butterfly bush) is a popular landscape plant that is also an invasive species in Oregon and other temperate locations. In Oregon, the plants are classified as a class B noxious weed (which enacts a ban on the species within state lines), with exemptions to the ban based on fertility data or interspecific pedigree alone. To date, there is no evidence that suggests all interspecific hybrids of butterfly bush exhibit reduced fecundity that would lower invasive potential. This study investigates what correlation, if any, there is between interspecific hybridization and lowered fecundity in butterfly bush as well as the cytogenetic effects of interspecific hybridization in the genus. Relative fecundity of 34 *Buddleja* cultivars of mixed pedigree was analyzed with three populations: the cultivars in a study field, a greenhouse population for controlled crossing, and an open pollinated population generated from cultivars. Interspecific cultivars were consistently both within the highest range of fecundity as well as the lowest range, with greenhouse crossing generally supporting field findings. Flow cytometry conducted on cultivars revealed lower than expected variation in genome size. This study shows interspecific hybridization does not appear to guarantee reduction in fecundity.

Species used in this study: butterfly bush *Buddleja davidii* L.

Index words: interspecific hybrid, invasive species, butterfly bush, flow cytometry.

Significance to the Horticulture Industry

There are several ways regulatory bodies are addressing the spread of invasive ornamental plants, including banning species from commerce. This poses a threat to the nursery industry, as many of these species remain popular landscape plants among consumers. Advances in plant breeding now offer cultivars of invasive species that present little or no ecological threat, but the evaluation process and regulation of these exceptions remains unclear. The current regulation of *Buddleja* L. in Oregon attempts to balance the needs of growers with protecting wild areas from invasion but has thus far de-regulated interspecific hybrids. This study showed that testing all cultivars of an invasive species is necessary, regardless of its hybrid status. The pedigree exemption is a loophole in regulation that was a well-intentioned attempt to strike a balance but has the potential to negatively impact the nursery industry by endangering future exceptions to species bans. Our work documented reliably low fecundity cultivars and provides clarity regarding which cultivars among those evaluated may be deemed "ecologically safe". This provides an example of what data regulators should consider and will help strike the balance of commerce and protecting Oregonian ecosystems. The methods in our study could also inform testing of purportedly low fecundity cultivars of other ornamental species with high invasive potential.

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Introduction

Buddleja davidii (butterfly bush) is native to central to southwestern Himalayan China and can be invasive to disturbed natural areas in many temperate to Mediterranean environments (USDA Zone 5 to 10; Tallent-Halsell and Watt 2009). Their current non-native range includes the United Kingdom, Western Europe, South Africa, Zambia and Zimbabwe, both seaboards of North America, South and Central America, Australia and New Zealand (Tallent-Halsell and Watt 2009). Based on the current invasive range, the plants seem to be restricted by extreme cold (below −28 C) (−18 F) and drought stress (Kriticos et al. 2010). Disturbance of the ecosystem is an important requirement for invasion (Tallent-Halsell and Watt 2009). It is registered as a class B noxious weed in Oregon (Oregon Noxious Weed Control Program 2016).

Buddleja davidii is primarily a threat to ecosystems in Oregon that are experiencing disturbance in some way and are thus in early successional stages or disrupted late successional stages. Riparian ecosystems (which remain in early successional stages due to flood-drought cycles), reforestation projects (disturbed through clear cutting), and roadsides are primarily where naturalized populations of *B. davidii* crop up in Oregon (Ream 2006). Human disturbance of ecosystems, wind, and water help distribute seed.

Anthropogenic dispersal of butterfly bush is therefore non-trivial and introductions of fully fecund plants to readily invasible environments should be prevented. The nursery industry is a large part of anthropogenic dispersal of *B. davidii*, and accounts for dispersal of the species across the globe (Ream 2006). The nursery industry contributes to introductions of invasive species currently and historically (Van Kleunen et al. 2018). Though breeding for increased resilience in landscape plants is important in combatting climate change and increasing urban tolerance, it can lead to the perfect storm of factors in an age with increased habitat fragmentation (disturbance), unregulated e-commerce, and potentially faulty invasive potential predictions (Contreras

2022, Humair et al 2015, Kriticos et al. 2010). Additionally, marketing patterns in the nursery industry, including low price point, the number of operations that grow the plant, and the number of years the plant is on the market, are correlated to potential for the introduction to become invasive (Dehnen-Schmutz et al. 2007). Given how close the nursery industry is to this issue, it behooves the green industry to pursue and follow environmentally protective legislation.

In response to the invasive threat posed by *B. davidii* to Oregonian ecosystems, the Oregon Department of Agriculture (ODA) imposed a plant ban on *B. davidii* in 2004 (Oregon Noxious Weed Control Program 2016). However, *B. davidii* remained an economically lucrative selection, accounting for approximately 2% of deciduous shrub sales, amounting to about $13 million in 2019 (U.S. Department of Agriculture, National Agricultural Statistics Service 2019). In response, the legislation was amended in 2009 to allow sale of cultivars for which the apparent ecological risk was low. The ODA defined reduced ecological risk in the amendment as a *B. davidii* cultivar exhibiting a 98% reduction in viable seed compared to fully fertile cultivars or an interspecific hybrid of any pedigree. The second qualification for reduced ecological risk may not be stringent enough to protect Oregonian ecosystems, as there is no proof that all interspecific hybrids are sterile and there is wide variety in the pedigree of interspecific hybrids available in the market today.

Interspecific hybridization can be used as a method to induce sterility, especially when the interspecific cross is also an interploid cross (Vining et al. 2012). There are several potential *Buddleja* species that would result in an interploid cross where *B. davidii* is used as a parent, as the ploidy level variation in *Buddleja* encompasses everything between 2*x* to 24*x* cytotypes (Chen et al. 2007). Current breeding efforts utilize species across different ploidy levels and geographic distributions, including Sino- Himalayan tetraploid species *B. davidii* and *B. fallowiana* Balf.f. & W.W.Sm., to Sino Himalayan diploid species *B. alterinfolia* Maxim., *B. asiatica* Lour., and *B. lindleyana* Fortune as well as South American diploid *B. globosa* Hope (Scott Trees 2015, Werner 2016a, 2016b, 2017, Werner and Snelling 2013a, 2013b, 2013c, 2014). Some interspecific hybrids are obvious interploid crosses, such as 'Asian Moon' which is a cross between *B. davidii* 'Moonshadow' (female) and *B. asiatica* (male) (Renfro et al. 2007). Other pedigrees are not as clear or are simply more complex than is easily predictable, such as CranRazz™ which includes *B. davidii*, *B. fallowiana*, and *B. globosa* in its pedigree (Scott Trees 2015). Furthermore, interploid crosses can occur when unreduced gametes from diploid species such as *B. globosa* and *B. lindleyana* hybridize with the tetraploid *B. davidii* (Van Laere et al. 2009), which can prevent easy confirmation of hybrids through flow cytometry. This suggests that there is probably significant variation in fertility amongst interspecific hybrids of *Buddleja* rather than a blanket reduction in fertility across all hybrids.

Interspecific hybridization can also increase variation and potentially environmental plasticity (Ellstrand and Schierenbeck 2000). This may take the form of complementary hybridization (a cross in which the shortcomings of one parent are compensated for by the strengths of the other) where parents are closely related, or introgression of certain alleles into one parent's genetic background that increases environmental tolerance (Anderson and Stebbins Jr. 1954). When the hybrid is exposed to novel environments, such as urban or disturbed ecosystems, populations of the hybrid can increase quickly. Observations of interspecific hybridization increasing fitness in ornamental plants are documented. A study documenting fecundity of Japanese barberry (*Berberis thunbergii* DC.) and related interspecific hybrids found that interspecific hybrids (though not interploidy hybrids) of *Berberis koreana* Palib. and *Berberis thunbergii* were more fecund than cultivars of *Berberis thunbergii* (Brand 2012, Lehrer et al. 2012).

Knight et al. (2011) also question the fertility of progeny of reduced fertility cultivars of invasive plants. They posit that progeny may pose an invasive threat if significant reversion to fully fertile phenotypes occurs between generations. This would mean that cultivars with reduced fertility may simply delay invasion by a generation rather than slowing or halting it. As each cultivar may include genetic information from previously isolated gene pools in the species types, interbreeding between cultivars in production and feral populations may also pose a risk of heightening invasive potential (Culley and Hardiman 2009).

The objectives of the study were 1) to measure relative fecundity of *Buddleja* hybrids and *B. davidii* cultivars and determine if interspecific hybridization reduced fertility, 2) to assess the fecundity of seedlings derived from low fecundity cultivars and their potential for reversion to full fertility and 3) to measure the genome size of interspecific hybrids, *B. davidii* cultivars, and select *Buddleja* species to document if genome size in hybrids matches expectations.

Materials and Methods

Overview of experimental design. Fecundity was evaluated with three different populations, described in detail in the following sections. Fecundity was evaluated in extant cultivars in a field setting by harvesting seeds from the field population in 2020 and 2021. A single inflorescence from each plant was sown and the number of seedlings was counted. This was multiplied by the number of inflorescences each plant generated over the study season to estimate the total number of seedlings each cultivar could produce under ideal conditions. Seedlings were saved from that original assay and grown out as an open pollinated (OP) population. The OP population was used to evaluate potential for reversion to invasive biotypes in 2021 and 2022. Fecundity was evaluated in male and female roles specifically through greenhouse crossing in 2020 and 2021.

Establishing study population. The study plot was located at Lewis Brown Farm (OSU, Corvallis OR, USDA Zone 8b). A 34 m wide by 53.3 m long (112 ft by 175 ft) field was prepared for planting in the fall of 2019. Twelve 2.4 m wide × 47.5 m long (8 ft by 155 ft) rows were plowed and worked with a power harrow prior to marking and planting. Soil type at the planting site was a Malabon silty clay loam. Plants were spaced 2.4 m in rows. A

Table 1. Taxa and sources of *Buddleja*.

Taxon	Original source
B. alternifolia	Arrowhead Alpines
B. 'Asian Moon'	Garland Nursery
B. Blaze Pink™ USPP25,731	Ball Horticultural Company
B. 'Blue Chip' USPP19,991	Spring Meadow
B. 'Blue Chip Jr.' USPP26,581	Spring Meadow
B. 'Boscranz' USPP25,730 CranRazz™	Ball Horticultural Company
B. CP 17WO1363	Ball Horticultural Company
Dapper® Blue	Ball Horticultural Company
B. CP17WO2051	Ball Horticultural Company
B. CP17WO2083	Ball Horticultural Company
B. CP17WO2092	Ball Horticultural Company
B. 'Ice Chip' USPP24,015	Spring Meadow
B. 'Lilac Chip' USPP24,016	Spring Meadow
B. 'Miss Molly' USPP23,425	Spring Meadow
B. 'Miss Violet' USPP28,448	Spring Meadow
B. 'PIIBD-II' USPP26,627 Funky Fuchsia™	Bailey Nursery
B. 'PIIBD-III' USPP26,306 Psychedelic Sky™	Bailey Nursery
B. 'Pink Microchip' USPP26,547	Spring Meadow
B. 'Podaras#13' USPP22,177 Flutterby Petite® Tutti Fruitti	Ball Horticultural Company
B. 'Podaras#8' USPP22,069 Flutterby Petite® Blue Heaven	Ball Horticultural Company
B. 'Purple Haze' USPP24,514	Spring Meadow
B. davidii 'Attraction'	Ball Horticultural Company
B. davidii 'Black Knight'	Bailey Nursery
B. davidii 'Buddaplav' USPP33,625 Dapper® Lavender	Ball Horticultural Company
B. davidii 'Dapconwhi' USPP33,566 Dapper® White	Ball Horticultural Company
B. davidii 'Grand Cascade' USPP30,868	Ball Horticultural Company
B. davidii 'Harlequin'	Ball Horticultural Company
B. davidii 'Nanho Blue'	Bailey Nursery
B. davidii 'Pink Delight'	Bailey Nursery
B. davidii 'PIIBD-I' USPP26,305 Groovy Grape™	Bailey Nursery
B. davidii 'Podaras#9' USPP22,065 Flutterby® Pink	Ball Horticultural Company
B. davidii Royal Red	Ball Horticultural Company
B. davidii 'Tobud0615' USPP23,461 BUZZ™ Sky Blue	Ball Horticultural Company
B. davidii 'Tobud0703' USPP23,462 BUZZ™ Velvet	Ball Horticultural Company
B. globosa	Dancing Oaks
B. japonica	Arrowhead Alpines
B. lindelyana	Dancing Oaks
B. ×*weyeriana* 'Honeycomb'	Oregon State University campus

randomized complete block design was used with six blocks. Thirty-four *Buddleja* cultivars were selected to include a variety of putative and reported fertility levels (Table 1). Each block contained one replicate of the 34-cultivar collection, along with three extra replicates of full fertility check cultivars in the study to ensure adequate pollen flow. As such, the study included 6 complete replicates of the study cultivars along with 18 extra spaces for full fertility pollen donors. The block was established in Fall 2019 and study years were 2020 and 2021.

Each year following establishment of the block, plants were irrigated with overhead impact sprinklers. No schedule was established for irrigation, but plants were watered as needed based on plant demand, soil, and environmental conditions. This generally resulted in 1-2, 2-hour irrigations weekly between June and October. Weeds were controlled manually via side tilling as needed, with a pre-emergent herbicide application conducted during Spring and Fall each year. Nitrogen was applied 3 times yearly through spring to early summer, using band applications of ureasul [(33-0-0) 10% ammoniacal nitrogen, 23% urea nitrogen, 12% sulfur derived from ammonium sulfate; Marion Ag Service, Aurora, OR] at 22.4 kg per hectare (20 lb N per acre).

Field Measurements. Fecundity of field grown plants was assessed in 2020 and 2021. A single inflorescence was harvested from each field plant of visually average size and seed set. Inflorescences were marked and left to ripen on the plant until capsules began shifting color from green to brown and suture lines began to form. In cases where capsules did not form, the inflorescence was harvested after all flowers had dehisced. Inflorescences were stored at room temperature until sowing for fecundity estimates.

All uncollected inflorescences were deadheaded to prevent spread of a potentially invasive species outside of the test area. Deadheading was completed periodically in the complete block as labor allowed. Span of deadheading season ran from the end of July both years according to the senescence of the first flush of flowers until after frost in the Fall to account for all subsequent flushes of flowers. At time of deadheading in field season 2020, each separate inflorescence was counted for all plants. A separate inflorescence included an isolated panicle of flowers. Branched panicles were counted as a single inflorescence and branches with panicles separated by vegetative nodes were counted separately. For study year 2021, plants had grown such that there were too many separate inflorescences to

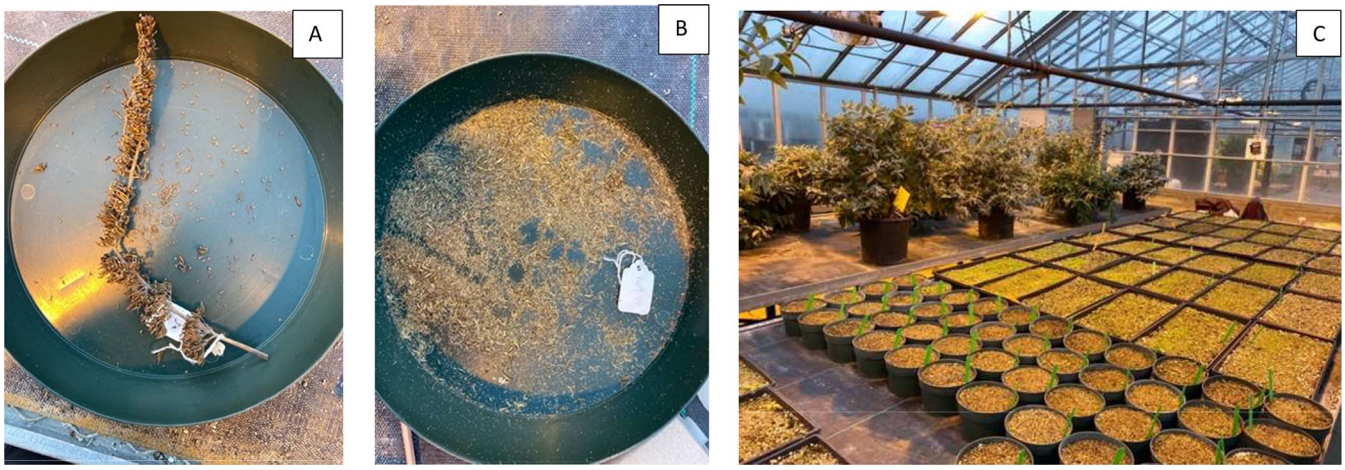

Fig. 1. Progression of whole field inflorescence sowing of butterfly bush. Dried whole inflorescence before crushing (A), chaff and seeds after crushing (B), sown seeds in 1020 flats or containers filled with soilless substrate, topped with vermiculite (C).

count with our labor constraints. Therefore, 3 of 6 blocks were counted.

OP seedling establishment. During summer 2020 and summer 2021, seeds were collected from the 34 field-grown cultivars to establish an open pollinated (OP) study population. The seeds for the OP population were sown as described as for the seedling count protocol outlined below. Seedlings from each parent cultivar were selected and pricked out of community flats before establishing a developed network of roots. These seedlings were planted in 50 cell trays filled with Sunshine Mix (Sungro, Agawam, MA), and covered with a humidity dome for 48-72 hours following transplanting. They were then grown in glasshouse conditions until roots were well established and held together the integrity of the soil ball when removed from cell trays. In 2021, the 2020 collected seedlings were then transplanted into 10 cm (4 in) square pots containing 2 parts Metromix (Sungro):1 part fine perlite amended with 250 grams of 18-6-12 plus micronutrients 5-6 month controlled release fertilizer granules (18N-2.62P-9.96K; Harrells LLC, Florida, United States) and allowed to establish for 2 months. They were then transplanted into 11.4 L (3 gal) nursery cans in fresh douglas-fir bark media and then maintained on a container pad with timed overhead irrigation. The 2021 collected OP population was transplanted to 2.8 L (3 qt) containers with fresh douglas-fir bark media from 50 cell trays and kept on the container pad. The container pad site was monitored to ensure pollinator presence and thus cross pollination in the OP population.

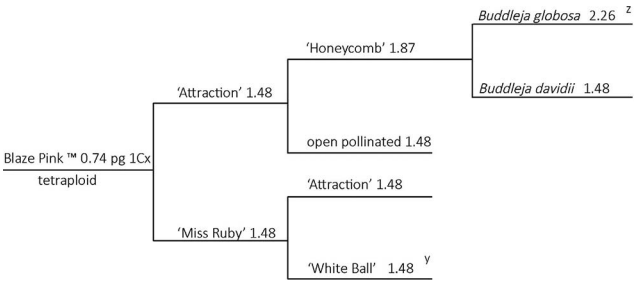

Fig. 2. Reconstructed pedigree calculations used to infer ploidy based on holoploid DNA content (2C) of butterfly bush cultivars. Example shown is for Blaze Pink™. Each branch of pedigree shows crossing partner next to haploid genome size (1Cx) that indicates its DNA content contributed to progeny. [z]White Ball- interspecific hybrid containing both *Buddleja davidii* and *Buddleja fallowiana*, however *B. fallowiana* has not been measured. Counted as *Buddleja davidii*. [y]Assumes unreduced gametes on part of the *B. globosa* crossing partner.

Greenhouse controlled crossing scheme. Greenhouse crossing was conducted during summer and fall seasons of 2020 and 2021, with one representative of each cultivar housed in the West block greenhouses at OSU. The 34 plants were maintained on a 12-h photoperiod at temperature settings 20 C (68 F) days and 15 C (59 F) nights and hand watered as needed. Controlled release fertilizer (Harell's 18N-2.62P-9.13K) was topdressed to maintain vigor.

In 2020, a pooled crossing system was devised to allocate putative sterile cultivars to fertile control cultivars. The eight fertile cultivars were allocated to pairs. Each pairing shared a pool of 7 putative sterile cultivars for crossing. Each fertile cultivar was crossed to each putative sterile cultivar in its assigned pool and crossed to the fertile cultivar in its pairing. Reciprocal crosses were made of each cross to assess fertility in female and male roles. Each putative sterile cultivar was crossed to two different fertile parents, and each fertile cultivar was crossed with 7 putative sterile parents and one other fertile parent. There were 15-30 flowers pollinated per unique crossing combination. For comparing relative fecundity of greenhouse crossing in 2020, we report the top two most fertile combinations for each study cultivar.

In 2021, the crossing scheme was designed based on fertile parents identified during 2020 greenhouse crossing and field seed counting data. The top three most fecund female and male cultivars were selected to cross against all other cultivars. These cultivars were all *B. davidii*, and included 'Nanho Blue', Groovy Grape™, and 'Black Knight'. Each cultivar was crossed to each "most fecund" cultivar in

Table 2. Fecundity of 34 *Buddleja* cultivars under field conditions grown in Corvallis, OR (USDA Zone 8b).

Taxon	Interspecific status[z]	Seedlings (mean)[y]	Inflorescences (mean)[y]	Seedlings per plant (mean)[y]	Relative field fecundity (%)[x]
		2020 season			
'Asian Moon'	IS	0.0	548.0 a-h	0.0	0.0
'Attraction'	BD	2,096.7 ab	357.7 c-j	591,298.0 ab	41.3
'Black Knight'	BD	1,265.8 abc	433.5 a-i	515,643.5 ab	39.7
Blaze Pink™	BD	1,551.0 ab	475.2 a-h	691,701.5 ab	55.7
'Blue Chip'	IS	19.5 ghij	916.3 a, b	23,552.3 b-f	1.8
'Blue Chip Jr.'	IS	0.2 k	548.5 a-f	73.5 h, i	0.0
BUZZ™ Sky Blue	BD	328.8 a-f	266.8 f-k	87,005.7 a-e	6.5
BUZZ™ Velvet	BD	1,115.3 abc	499.2 a-g	564,511.0 ab	42.1
Dapper® Lavender	BD	261.2 a-g	349.2 c-j	87,303.0 a-e	6.5
Dapper® White	BD	71.0 d-i	312.0 e-k	21,628.3 b-f	1.6
CP17WO1363	NR	151.8 b-g	241.2 g-k	28,459.7 b-f	2.1
Dapper® Blue	NR	0.2 k	197.0 i-l	42.5 i	0.0
CP17WO2051	NR	45.3 e-i	399.0 c-i	21,499.7 b-f	1.6
CP17WO2083	NR	6.8 hijk	170.0 j-l	1,700.0 fgh	0.1
CP17WO2092	NR	302.8 a-f	341.7 c-j	115,907.8 abcd	8.6
CranRazz™	IS	1,605.2 ab	326.2 d-k	476,637.2 abc	35.5
Flutterby® Petite Blue Heaven	IS	4.7 ijk	606.3 a-e	2,641.8 e-h	0.2
Flutterby® Petite Tutti Fruitti	IS	41.0 f-i	98.2 l	4,747.2 d-g	0.4
Flutterby® Pink	BD	102.7 c-h	327.2 d-k	34,984.8 a-f	2.6
Funky Fuchsia™	IS	685.8 abcd	385.7 c-i	234,132.0 abc	17.4
'Grand Cascade'	BD	807.7 abcd	370.7 c-j	279,190.5 abc	20.8
Groovy Grape™	BD	1,440.8 abc	406.7 c-i	470,550.5 abc	39.6
'Harlequin'	BD	169.3 b-g	347.0 c-j	50,749.7 a-f	3.5
'Honeycomb'	IS	25.8 f-i	410.3 b-i	13,153.2 c-f	1.0
'Ice Chip'	IS	0.4 j-k	433.6 a-i	142.8 g-i	0.0
'Lilac Chip'	IS	174.5 b-g	414.3 b-i	87,449.0 a-e	6.5
'Miss Molly'	IS	637.5 a-e	742.7 a-c	524,846.8 ab	46.1
'Miss Violet'	IS	240.4 a-g	748.6 a-d	186,435.6 abcd	13.9
'Nanho Blue'	BD	2,575.8 a	548.7 a-f	1,253,526.0 a	100.0
'Pink Delight'	BD	148 b-g	211.2 h-l	39,106.8 a-f	5.0
'Pink Microchip'	IS	0.0	270.8 e-k	0.0	0.0
Psychedelic Sky™	IS	730.5 abcd	938.8 a	729,948.2 ab	57.3
'Purple Haze'	IS	0.0	145.7 k, l	0.0	0.
'Royal Red'	BD	967.3 abcd	330.0 d-j	284,657.8 abc	19.8
		2021 season[w]			
'Asian Moon'	IS	0.0	Not recorded	Not recorded	0.0
'Attraction'	BD	591.2 a-e	1,055.0 c-i	669,673.7 a-e	16.0
'Black Knight'	BD	1,077.5 abc	1,391.0 a-g	1,581,985.5 abc	37.9
Blaze Pink™	IS	2,252.2 a	1,809.0 a-e	4,074,169.5 a	97.5
'Blue Chip'	IS	12.2 gh	2,640.3 a	32,123.7 ef	0.8
'Blue Chip Jr.'	IS	0.0	1,165.7 b-h	0.0	0.0
BUZZ™ Sky Blue	BD	1026.0 abc	1,213.0 a-g	1,244,538.0 abc	29.8
BUZZ™ Velvet	BD	2,315.5 a	1,805.0 a-e	4,179,477.5 a	100.0
Dapper® Lavender	BD	72.7 d-g	633.3 g-j	47,836.6 d-f	1.1
Dapper® White	BD	62.8 e-g	1,190.0 a-h	74,771.7 c-f	1.8
CP17WO1363	NR	574.8 a-e	788.0 f-j	452,968.7 a-f	10.8
Dapper® Blue	NR	0.0	988.0 c-j	0.0	0.0
CP17WO2051	NR	78.7 defg	947.7 d-j	74,552.4 c-f	1.8
CP17WO2083	NR	113.8 c-g	932.3 d-j	106,126.8 b-f	2.5
CP17WO2092	NR	823.2 abc	861.3 e-j	708,993.5 abcd	17.0
CranRazz™	IS	1,710.8 a	1,029.0 c-i	1,760,447.5 ab	42.1
Flutterby® Pink	BD	677.7 abcd	792.7 f-j	537,186.7 a-e	12.9
Flutterby Petite® Blue Heaven	IS	43.3 fg	1,685.7 a-f	73,051.3 c-f	1.7
Flutterby Petite® Tutti Fruitti	IS	100.4 c-g	176.0 k	17,670.4 fg	0.4
Funky Fuchsia™	IS	121.5 c-g	949.7 d-j	115,388.6 b-f	2.8
'Grand Cascade'	BD	1,638.7 a	533.7 h-j	874,556.4 abcd	20.9
Groovy Grape™	BD	1,674.2 a	1,391.0 a-g	2,328,766 ab	55.7
'Harlequin'	BD	464.5 a-e	1,247.7 a-g	549,271.7 a-e	13.9
'Honeycomb'	IS	72.5 d-g	1,628.7 a-f	118,080.8 b-f	2.8
'Ice Chip'	IS	0.4 h	1,871.5 a-f	749.0 g, h	0.0
'Lilac Chip'	IS	150.5 b-f	1,165.3 b-h	175,377.7 a-f	4.2
'Miss Molly'	IS	144.7 c-f	1,595.0 a-f	230,743.3 a-f	5.5
'Miss Violet'	IS	609.2 a-e	2,094.7 a-d	1,276,021.4 abc	30.5
'Nanho Blue'	BD	1,015.1 abc	2,151.0 a-c	2,346,729.7 ab	56.1
'Pink Delight'	BD	1,008.5 abc	937 d-j	905,633.0 abcd	21.7

Table 2. Continued.

Taxon	Interspecific status[z]	Seedlings (mean)[y]	Inflorescences (mean)[y]	Seedlings per plant (mean)[y]	Relative field fecundity (%)[x]
'Pink Microchip'	IS	0.8 h	469.7 i, j	391.4 h	0.0
Psychedelic Sky™	IS	1,431.2 ab	2,416.3 a,b	3,630,297.3 a	86.9
'Purple Haze'	IS	1.5 h	447.0 j	670.5 g, h	0.0
'Royal Red'	BD	1,443.2 ab	1,427 a-g	1,959,099.2 ab	46.9

[z]Abbreviations for parentage are as follows: BD for *Buddleja davidii* only, IS for interspecific hybrid, no marking if information is not available. Parentage information retrieved from patent associated with cultivar or Dirr's manual of Woody landscape Plants. Parentage species (pedigree) either reported in patents or parent cultivar patent.

[y]Means followed by different letter indicates difference based on separation of means using a generalized linear model with a negative binomial distribution and the Tukey method for comparing a family using R (V 4.2.1). Means of zero were not included in mean separation.

[x]Relative% fecundity always compared to most fecund cultivar of the year using seedlings per plant.

[w]Three of six replicates were used for inflorescence counts during 2021. Seedlings per plant in 2021 were calculated by multiplying each location's seedling count by the cultivar's mean inflorescence count, not necessarily the exact value for that specific replicate.

male and female roles as well as all other reduced fecundity cultivars to assess relative fecundity when crossed to fully fertile parents. Ten to 15 flowers were pollinated per unique crossing combination. For comparing relative fecundity of greenhouse crossing in 2021, all combinations with 'Nanho Blue', Groovy Grape™ and 'Black Knight' are reported. For assessing fecundity of high fecundity controls, the most fecund crosses are reported.

Flowers used as the female in crossings were emasculated at least two days before crossing. All flowers used as seed parents were emasculated even though *Buddleja* are self-incompatible. Emasculation is simple for this genus, as anthers are fused to the side of the corolla. Thus, removal of corolla also removes anthers. Flowers near the emasculated flowers were removed. Pollen was collected ahead of time and dried at room temperature for 48-h before being stored in petri dishes over desiccant in sealed plastic dishes and placed in a refrigerator. At time of pollination, pollen dishes were removed from the refrigerator and brought to room temperature before opening to prevent moisture from entering pollen dishes. Anthers were applied to stigmas using a paintbrush, sterilized in 70% ethanol between male parents.

Seedling count protocol. Fecundity from field collected inflorescences, controlled crossing study, and OP population collected inflorescences was estimated via the following seedling count protocol. All capsules and inflorescences collected from field plants and OP plants were crushed, in their entirety, to release any seeds (Fig. 1). Capsules were either crushed by rubbing/rolling between fingertips or rolling under 10cm section of irrigation pipe for larger samples. Crushed capsules were mixed with fine grade vermiculite prior to sowing to ensure evenness of application across top of growing medium. For sowing, 1020 trays with drainage holes and 15.2 cm diameter containers (Fig. 1) were filled with Sunshine Mix (Sungro) and watered in prior to application of seeds and capsule chaff. Capsules with apparent seeds were sown into 1020 trays and inflorescences without capsules were sown into azalea pots for space savings.

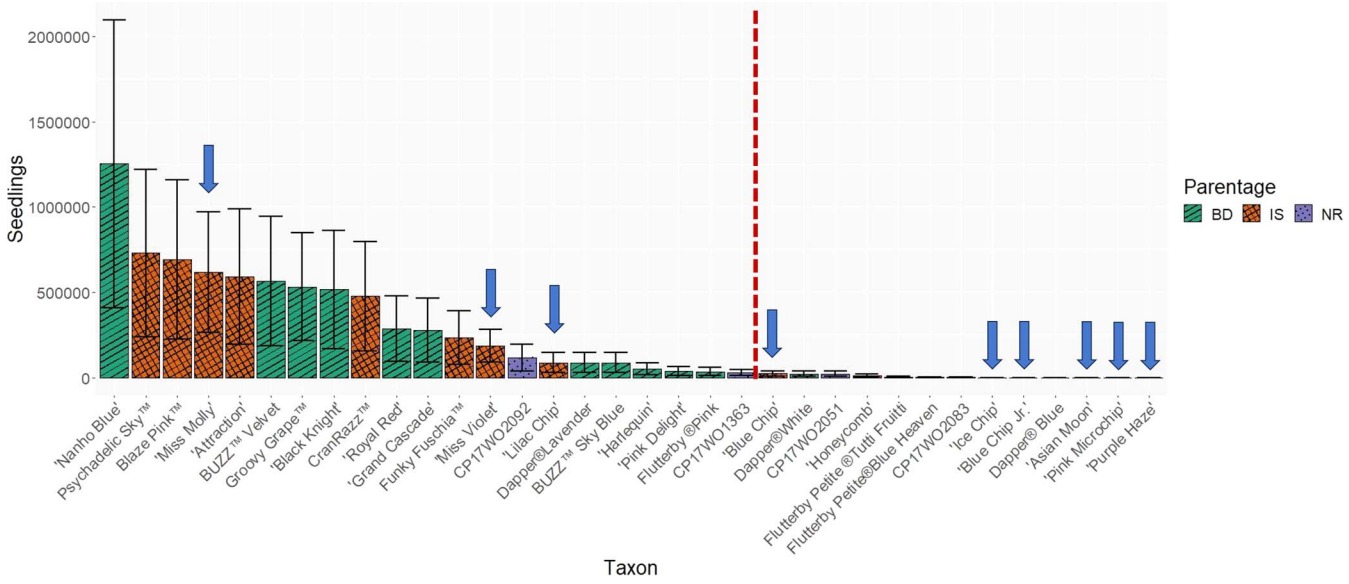

Fig. 3. Histogram of mean *Buddleja* seedling counts per cultivar in 2020, arranged from most to fewest seedlings. Error bars are standard error. Color and texture legend indicates interspecific status. Dashed line indicates 98% reduction in full fecundity compared to the most fecund cultivar. Arrows indicate cultivars currently allowed for sale in Oregon under ODA regulation that permits cultivars confirmed to have 98% reduction in fertility or to be of hybrid origin. BD = *Buddleja davidii*, IS = interspecific hybrid, NR = not reported.

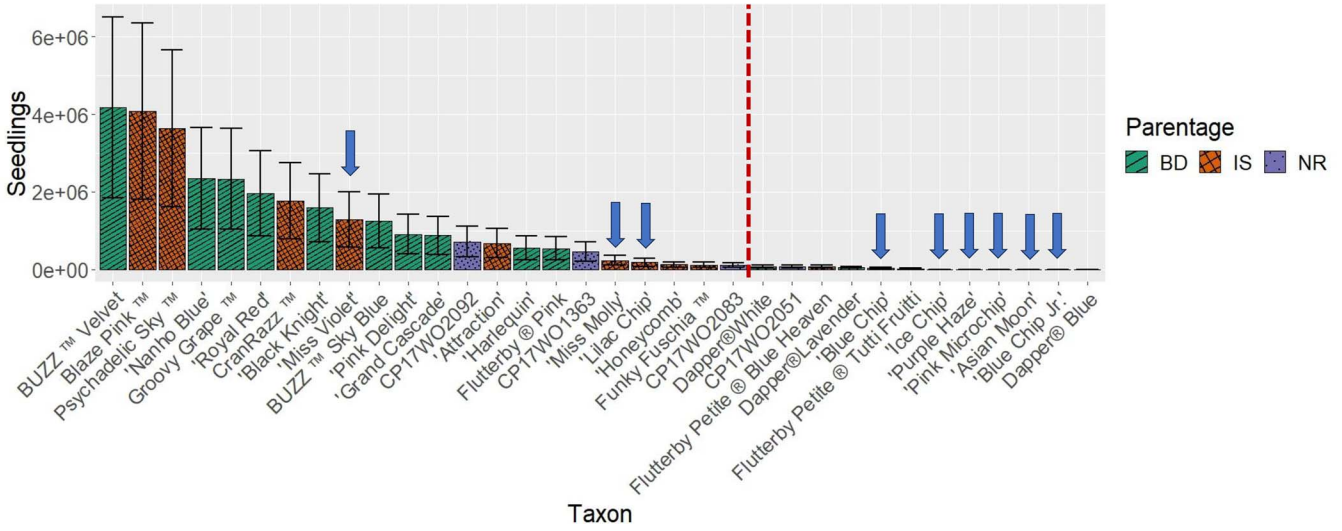

Fig. 4. Histogram of mean *Buddleja* seedling counts per cultivar in 2021, arranged from most to fewest seedlings. Error bars are standard error. Color and texture legend indicates interspecific status. Dashed line indicates 98% reduction in full fecundity compared to the most fecund cultivar. Arrows indicate cultivars currently allowed for sale in Oregon under ODA regulation that permits cultivars confirmed to have 98% reduction in fertility or to be of hybrid origin. BD = *Buddleja davidii*, IS = interspecific hybrid, NR = not reported.

Seeds mixed in vermiculite were spread across the top of the growing medium surface to create a thin layer and evenly distribute seeds. Containers were watered in lightly once more within hours of sowing. Additional fertilizer was not applied, as these containers were only maintained for four weeks in most cases, or six in few instances.

Seedling flats were maintained in a glasshouse under 12-h photoperiod and controlled temperature conditions at 20 C (68 F) days and 15 C (59 F) nights and hand watered as needed to maintain surface soil moisture. After at least 2 weeks passed sow date, the first round of seedling counts began.

Seedlings were counted by removing them gently from the soil with forceps (in such a way as to minimize soil disturbance), and then by counting seedlings removed. The flat was then returned to the greenhouse for two weeks, until more seedlings germinated. When additional seedlings germinated, they were counted and removed as described above. Flats with less than 50 seedlings were discarded after the second count, those with more than 50 were returned to the glasshouse once more to continue germination. Upon the third count, all flats were discarded four to six weeks after sowing.

Flow cytometry. A Quantum P flow cytometer equipped with blue laser and UV diode excitation sources (Quantum Analysis GmbH, Munster, Germany) was used to perform DNA content estimates of the cultivars in the study as well as selected *Buddleja* species. Open pollinated seedlings were also measured in summer seasons 2021 and 2022, by analyzing DAPI-stained nuclei prepared using Cystain™ UV Precise P kits (Sysmex) and protocol outlined below.

Pisum sativum. L. 'Citrad' (2C = 8.76 pg) was used as the internal standard for DNA content estimation. Leaf tissue of both plants of interest and the internal standard were selected off the last fully expanded leaves reared in a glasshouse. DNA content was estimated using two different fluorophore dyes, DAPI (4', 6-diamindino-2-phenylindole) and PI (propidium iodide) in separate analyses. Cultivars were measured with DAPI in the summer season 2019, and then measured with a PI protocol in summer season 2020. To prepare samples for analyses, about 0.5 cm^2 squares of leaf tissue from plants of interest and the internal standard were co-chopped with a double-sided razor blade in 500 µL nuclei extraction buffer (Cystain™ UV Precise P and Cystain™ PI Absolute P, Sysmex). Leaf tissue was selected such that major veins were avoided to reduce release of secondary metabolites that might interfere with dye binding. Chopped leaf tissue and nuclei extraction buffer were then passed through a 50 µm CellTrics filter (Sysmex) into a Rohren tube (SARSTEDT, Germany). Extracted and filtered nuclei samples were stained with either DAPI staining buffer (Cystain™ UV Precise P, Sysmex) or PI staining buffer (Cystain™ PI Absolute P, Sysmex). PI samples were treated with RNAseA according to manufacturer instructions and incubated on ice in darkness for 30-45 minutes prior to analysis. DAPI-stained samples were excited using a UV diode (365 nm), and PI samples were excited using a blue laser (488 nm). At least 2,000 nuclei were analyzed in all samples and CV% was below 10% with few exceptions.

Sample peaks were gated to capture mean fluorescence for the experimental sample and the internal standard, which were used to calculate 2C (holoploid) DNA content using the equation:

2C DNA content = 2C of internal standard × (mean fluorescence value of sample/mean fluorescence value of internal standard).

Monoploid (1C*x*) DNA content values used to determine putative ploidy level of interspecific hybrids was calculated as follows. Pedigrees were examined for patented or otherwise published interspecific cultivars in the study to gather parent information. Pedigrees were recreated in a stepwise manner by including genome sizes of gametes from each parent to generate progeny with the observed DNA content. This often assumed unreduced gametes in

Table 3. Relative female fecundity of 34 *Buddleja* cultivars from greenhouse crossing.

Female parent	Male parents	Number of crosses	Total seedling count	Number seedlings per cross	Year	Relative female fecundity (%)
'Asian Moon'	'Black Knight'	9	0	0.0	2021	0.0
	Groovy Grape™	10	0	0.0	2021	
	'Nanho Blue'	11	0	0.0	2021	
'Attraction'	Groovy Grape™	11	0	0.0	2021	13.0
	'Nanho Blue'	11	0	0.0	2021	
	'Black Knight'	12	16	1.3	2021	
	Blaze Pink™	23	164	7.1	2020	
	Flutterby Petite® Tutti-Frutti	17	307	18.1	2020	
'Black Knight'	'Black Knight'	30	39	1.3	2020	40.0
	'Royal Red'	24	86	3.6	2020	
	'Nanho Blue'	12	241	20.1	2021	
	BUZZ™ Velvet	11	308	28.0	2021	
	'Royal Red'	12	341	28.4	2021	
Blaze Pink™	'Harlequin'	30	70	2.3	2020	24.7
	'Nanho Blue'	10	94	9.4	2021	
	'Black Knight'	10	105	10.5	2021	
	Groovy Grape™	10	137	13.7	2021	
	'Attraction'	11	158	14.4	2020	
'Blue Chip'	Groovy Grape™	11	2	0.2	2021	1.7
	'Nanho Blue'	10	2	0.2	2021	
	'Royal Red'	18	17	0.9	2020	
	'Black Knight'	10	15	1.5	2021	
'Blue Chip Jr.'	'Black Knight'	11	0	0.0	2021	0.1
	Groovy Grape™	10	0	0.0	2021	
	'Nanho Blue'	11	0	0.0	2021	
	'Pink Delight'	20	1	0.1	2020	
	Psychedelic Sky™	28	2	0.1	2020	
BUZZ® Sky Blue	'Harlequin'	26	2	0.1	2020	17.7
	'Black Knight'	10	63	6.3	2021	
	'Nanho Blue'	10	67	6.7	2021	
	'Royal Red'	23	217	9.4	2020	
	Groovy Grape™	11	148	13.5	2021	
BUZZ® Velvet	'Nanho Blue'	23	249	10.8	2020	53.4
	Groovy Grape™	31	531	17.1	2020	
	'Black Knight'	11	290	26.4	2021	
	'Nanho Blue'	11	291	26.5	2021	
	Groovy Grape™	12	334	27.8	2021	
CP17WO2051	'Black Knight'	11	45	4.1	2021	29.3
	Groovy Grape™	10	51	5.1	2021	
	'Nanho Blue'	12	325	27.1	2021	
	'Royal Red'	27	302	11.2	2020	
	'Black Knight'	30	364	12.1	2020	
CP17WO2083	'Black Knight'	12	9	0.8	2021	19.0
	'Nanho Blue'	10	56	5.6	2021	
	Groovy Grape™	13	117	9.0	2021	
	'Nanho Blue'	30	469	15.6	2020	
CP17WO2092	'Black Knight'	11	65	5.9	2021	24.2
	'Nanho Blue'	11	70	6.4	2021	
	Groovy Grape™	11	111	10.1	2021	
	'Pink Delight'	21	20	0.9	2020	
	Psychedelic Sky™	29	751	25.9	2020	
Dapper® Lavender	'Harlequin'	19	12	0.6	2020	20.7
	'Black Knight'	10	51	5.1	2021	
	Groovy Grape™	11	59	5.4	2021	
	'Nanho Blue'	11	248	22.6	2021	
Dapper® White	'Black Knight'	25	9	0.4	2020	3.4
	'Royal Red'	23	149	6.5	2020	
	'Black Knight'	10	0	0.0	2021	
	'Nanho Blue'	10	0	0.0	2021	
	Groovy Grape™	10	1	0.1	2021	

Table 3. Continued.

Female parent	Male parents	Number of crosses	Total seedling count	Number seedlings per cross	Year	Relative female fecundity (%)
CP17WO1363	'Nanho Blue'	23	129	5.6	2020	28.9
	Groovy Grape™	22	194	8.8	2020	
	'Nanho Blue'	11	120	11.0	2021	
	'Black Knight'	10	145	14.5	2021	
	Groovy Grape™	11	208	18.9	2021	
Dapper® Blue	'Harlequin'	15	0	0.0	2020	3.7
	'Attraction'	25	185	7.4	2020	
	'Black Knight'	12	0	0.0	2021	
	Groovy Grape™	10	0	0.0	2021	
	'Nanho Blue'	12	1	0.1	2021	
CranRazz™	'Black Knight'	25	31	1.2	2020	47.5
	'Royal Red'	23	183	7.9	2020	
	'Nanho Blue'	11	161	14.6	2021	
	'Black Knight'	12	180	15.0	2021	
	Groovy Grape™	12	693	57.8	2021	
Flutterby Petite® Blue Heaven	'Nanho Blue'	23	61	2.7	2020	3.2
	Groovy Grape™	23	80	3.5	2020	
	'Black Knight'	12	1	0.1	2021	
	Groovy Grape™	10	1	0.1	2021	
	'Nanho Blue'	11	2	0.2	2021	
Flutterby Petite® Tutti Fruitti	'Black Knight'	11	0	0.0	2021	0.3
	Groovy Grape™	9	0	0.0	2021	
	'Nanho Blue'	10	3	0.3	2021	
Flutterby® Pink	'Nanho Blue'	24	34	1.4	2020	9.4
	Groovy Grape™	29	75	2.6	2020	
	'Black Knight'	9	0	0.0	2021	
	'Nanho Blue'	11	49	4.5	2021	
	Groovy Grape™	10	107	10.7	2021	
Funky Fuchsia™	'Nanho Blue'	10	12	1.2	2021	3.0
	Groovy Grape™	11	23	2.1	2021	
	'Pink Delight'	25	16	0.6	2020	
	Psychedelic Sky™	24	24	1.0	2020	
'Grand Cascade'	'Pink Delight'	23	218	9.5	2020	15.1
	Psychedelic Sky™	30	446	14.9	2020	
	'Nanho Blue'	10	7	0.7	2021	
	'Black Knight'	10	16	1.6	2021	
	Groovy Grape™	11	45	4.1	2021	
Groovy Grape™	BUZZ™ Velvet	32	624	19.5	2020	100.0
	'Nanho Blue'	22	490	22.3	2020	
	'Grand Cascade'	11	449	40.8	2021	
	Funky Fuchsia™	10	418	41.8	2021	
	'Royal Red'	12	950	79.2	2021	
'Harlequin'	'Black Knight'	12	0	0.0	2021	0.1
	Groovy Grape™	12	0	0.0	2021	
	'Nanho Blue'	10	0	0.0	2021	
	Flutterby Petite® Tutti-Frutti	25	3	0.1	2020	
	'Grand Cascade'	28	4	0.1	2020	
'Honeycomb'	'Nanho Blue'	10	1	0.1	2021	0.8
	'Black Knight'	10	2	0.2	2021	
	Groovy Grape™	10	3	0.3	2021	
	Psychedelic Sky™	28	11	0.4	2020	
	'Attraction'	13	7	0.5	2020	
'Ice Chip'	Groovy Grape™	10	0	0.0	2021	0.3
	'Nanho Blue'	10	1	0.1	2021	
	'Black Knight'	11	2	0.2	2021	
	Groovy Grape™	21	5	0.2	2020	
'Lilac Chip'	'Black Knight'	25	1	0.0	2020	2.6
	'Royal Red'	23	17	0.7	2020	
	'Black Knight'	10	13	1.3	2021	
	Groovy Grape™	9	13	1.4	2021	
	'Nanho Blue'	10	17	1.7	2021	

Table 3. Continued.

Female parent	Male parents	Number of crosses	Total seedling count	Number seedlings per cross	Year	Relative female fecundity (%)
'Miss Molly'	Groovy Grape™	11	1	0.1	2021	5.2
	'Attraction'	21	7	0.3	2020	
	'Nanho Blue'	10	10	1.0	2021	
	'Harlequin'	25	36	1.4	2020	
	'Black Knight'	11	84	7.6	2021	
'Miss Violet'	'Black Knight'	10	1	0.1	2021	5.1
	'Pink Delight'	29	6	0.2	2020	
	Groovy Grape™	11	5	0.5	2021	
	'Nanho Blue'	10	7	0.7	2021	
	Psychedelic Sky™	23	205	8.9	2020	
'Nanho Blue'	BUZZ™ Velvet	25	74	2.9	2020	83.3
	Flutterby ®Pink	21	254	12.1	2020	
	BUZZ™ Velvet	13	615	47.3	2021	
	Dapper® White	12	588	49.0	2021	
	'Grand Cascade'	10	582	58.2	2021	
'Pink Delight'	'Nanho Blue'	10	4	0.4	2021	6.3
	Groovy Grape™	9	8	0.9	2021	
	'Black Knight'	10	15	1.5	2021	
	Psychedelic Sky™	20	87	4.4	2020	
	Grand Cascade™	24	138	5.8	2020	
'Pink Micro Chip'	'Black Knight'	10	0	0.0	2021	0.0
	Groovy Grape™	10	0	0.0	2021	
	'Nanho Blue'	10	0	0.0	2021	
	'Black Knight'	25	0	0.0	2020	
	'Royal Red'	23	0	0.0	2020	
Psychedelic Sky™	'Purple Haze'	27	400	14.8	2020	29.5
	'Grand Cascade'	28	559	19.9	2020	
	'Black Knight'	12	30	2.5	2021	
	'Nanho Blue'	10	50	5.0	2021	
	Groovy Grape™	11	196	17.8	2021	
'Purple Haze'	Psychedelic Sky™	24	0	0.0	2020	0.0
	'Pink Delight'	22	0	0.0	2020	
	'Black Knight'	11	0	0.0	2021	
	Groovy Grape™	12	0	0.0	2021	
	'Nanho Blue'	10	0	0.0	2021	
'Royal Red'	Groovy Grape™	12	6	0.5	2021	36.6
	'Black Knight'	12	13	1.1	2021	
	Dapper® White	24	294	12.3	2020	
	'Black Knight'	25	507	20.3	2020	
	'Nanho Blue'	11	445	40.5	2021	

cases of diploid species crossing to *B. davidii* as has been observed by several previous studies (Moore 1960, Van Laere et al. 2009) and for the interspecific hybrid Blaze Pink™ (Fig. 2). *Buddleja davidii* monoploid was taken from this study's measurement of *B. davidii* 'Nanho Blue' in both PI and DAPI. This study also generated monoploid values for *B. alternifolia*, *B. lindleyana* and *B. globosa* using both DAPI and PI protocols that were used in pedigree reconstruction.

No reported genome size exists for *B. asiatica* or *B. fallowiana*, both of which were contributing species to at least one complex interspecific in our study. *Buddleja asiatica* is only present in 'Asian Moon', a confirmed triploid by chromosome counts (Renfro et al. 2007). *Buddleja fallowiana* is a tetraploid species and a parent of 'White Ball', a cultivar derived from *B. davidii* and *B. fallowiana* (cross direction not reported) and commonly used in many of the pedigrees of our study population. Since *B. fallowiana* was not available at the time of this study, where 'White Ball' was included in a cultivar's pedigree the haploid value of *B. davidii* was used instead of a composite value between *B. davidii* and *B. fallowiana*.

Once composite monoploid estimations were calculated, they were applied to 2C values derived from flow cytometry of cultivars. The DNA content was divided by the estimated monoploid value to estimate ploidy of each cultivar. If the result was at least 3.5, the cultivar was counted tetraploid. All cultivars except 'Asian Moon', confirmed by chromosome counts, measured tetraploid using this method. DNA contents were then divided by ploidy level to estimate 1C*x* content for analyses. Given these results, all OP2 seedlings (progeny of study cultivars measured by flow cytometry) appeared to be tetraploid.

Data analyses. Data analyses were conducted in R [R ver. 4.2.1 (R Core Team 2022)]. Analysis of variance was conducted on the flow cytometry data and means separated using Tukey's Honestly Significant Difference ($\alpha = 0.05$).

Table 4. Relative male fertility of 34 *Buddleja* cultivars from greenhouse crossing.

Male parent	Female parent	Number of crosses	Total counts	Number seedings per cross	Year	Relative male fertility of cultivar (%)
'Asian Moon'	'Black Knight'	12	0	0.0	2021	0.0
	Groovy Grape™	11	0	0.0	2021	
	'Nanho Blue'	12	0	0.0	2021	
'Attraction'	Dapper® Blue	25	185	7.4	2020	41.5
	Blaze Pink™	11	158	14.4	2020	
	'Nanho Blue'	12	7	0.6	2021	
	'Black Knight'	11	126	11.5	2021	
	Groovy Grape™	10	385	38.5	2021	
'Black Knight'	CP17WO2051	30	364	12.1	2020	54.2
	'Royal Red'	25	507	20.3	2020	
	CranRazz™	12	180	15.0	2021	
	'Nanho Blue'	11	227	20.6	2021	
	BUZZ™ Velvet	11	290	26.4	2021	
Blaze Pink™	'Harlequin'	25	0	0.0	2020	26.7
	'Attraction'	23	164	7.1	2020	
	'Black Knight'	9	2	0.2	2021	
	Groovy Grape™	10	11	1.1	2021	
	'Nanho Blue'	10	380	38.0	2021	
'Blue Chip'	'Black Knight'	25	0	0.0	2020	0.0
	'Royal Red'	26	1	0.0	2020	
	'Black Knight'	11	0	0.0	2021	
	Groovy Grape™	12	0	0.0	2021	
	'Nanho Blue'	12	0	0.0	2021	
BUZZ™ Sky Blue	'Black Knight'	25	1	0.0	2020	10.2
	'Royal Red'	26	169	6.5	2020	
	Groovy Grape™	12	9	0.8	2021	
	'Nanho Blue'	12	20	1.7	2021	
	'Black Knight'	10	88	8.8	2021	
BUZZ™ Velvet	'Nanho Blue'	25	74	2.9	2020	70.8
	Groovy Grape™	32	624	19.5	2020	
	Groovy Grape™	12	307	25.6	2021	
	'Black Knight'	11	308	28.0	2021	
	'Nanho Blue'	13	615	47.3	2021	
CP17WO2051	Groovy Grape™	12	1	0.1	2021	6.6
	'Black Knight'	10	16	1.6	2021	
	'Nanho Blue'	10	18	1.8	2021	
	'Black Knight'	25	18	0.7	2020	
	'Royal Red'	28	204	7.3	2020	
CP17WO2083	Groovy Grape™	11	0	0.0	2021	6.6
	'Black Knight'	9	3	0.3	2021	
	'Nanho Blue'	10	105	10.5	2021	
	Groovy Grape™	25	1	0.0	2020	
	'Nanho Blue'	26	14	0.5	2020	
CP17WO2092	'Black Knight'	10	9	0.9	2021	51.3
	Groovy Grape™	12	274	22.8	2021	
	'Nanho Blue'	10	449	44.9	2021	
	'Pink Delight'	23	65	2.8	2020	
Dapper® Lavender	'Harlequin'	26	1	0.0	2020	5.5
	'Attraction'	25	81	3.2	2020	
	'Black Knight'	10	0	0.0	2021	
	Groovy Grape™	11	2	0.2	2021	
	'Nanho Blue'	12	73	6.1	2021	
Dapper® White	'Black Knight'	25	5	0.2	2020	51.8
	'Royal Red'	24	294	12.3	2020	
	'Black Knight'	12	126	10.5	2021	
	Groovy Grape™	12	219	18.3	2021	
	'Nanho Blue'	12	588	49.0	2021	
CP17WO1363	'Nanho Blue'	20	0	0.00	2020	0.9
	Groovy Grape™	28	17	0.6	2020	
	'Black Knight'	11	0	0.00	2021	
	Groovy Grape™	12	0	0.00	2021	
	'Nanho Blue'	10	10	1.00	2021	

Table 4. Continued.

Male parent	Female parent	Number of crosses	Total counts	Number seedings per cross	Year	Relative male fertility of cultivar (%)
CranRazz™	'Black Knight'	25	3	0.1	2020	7.6
	'Royal Red'	30	27	0.9	2020	
	Groovy Grape™	10	0	0.0	2021	
	'Black Knight'	12	20	1.7	2021	
	'Nanho Blue'	12	126	10.5	2021	
Flutterby Petite® Blue Heaven	Groovy Grape™	26	17	0.7	2020	29.0
	Groovy Grape™	10	0	0.0	2021	
	'Black Knight'	11	188	17.1	2021	
	'Nanho Blue'	11	250	22.7	2021	
Flutterby Petite® Tutti Fruitti	'Black Knight'	12	0	0.0	2021	11.1
	'Nanho Blue'	11	0	0.0	2021	
	Groovy Grape™	12	13	1.1	2021	
	'Harlequin'	25	3	0.1	2020	
	'Attraction'	17	307	18.0	2020	
Flutterby® Pink	Groovy Grape™	19	13	0.7	2020	10.2
	'Nanho Blue'	21	254	12.1	2020	
	'Black Knight'	10	0	0.0	2021	
	Groovy Grape™	12	26	2.2	2021	
	'Nanho Blue'	10	28	2.8	2021	
Funky Fuchsia™	'Black Knight'	12	196	16.3	2021	55.1
	'Nanho Blue'	10	377	37.7	2021	
	Groovy Grape™	10	418	41.8	2021	
	Psychedelic Sky™	32	0	0.0	2020	
	'Pink Delight'	24	5	0.2	2020	
'Grand Cascade'	'Pink Delight'	24	138	5.8	2020	73.2
	Psychedelic Sky™	28	559	19.9	2020	
	'Black Knight'	10	27	2.7	2021	
	Groovy Grape™	11	449	40.8	2021	
	'Nanho Blue'	10	582	58.2	2021	
Groovy Grape™	CP17WO1363	22	194	8.8	2020	84.6
	BUZZ® Velvet	31	531	17.1	2020	
	BUZZ® Velvet	12	334	27.8	2021	
	'Nanho Blue'	11	394	35.8	2021	
	CranRazz™	12	693	57.8	2021	
'Harlequin'	'Grand Cascade'	26	57	2.2	2020	2.9
	Blaze Pink™	30	70	2.3	2020	
	'Black Knight'	11	0	0.0	2021	
	'Nanho Blue'	11	3	0.3	2021	
	Groovy Grape™	10	3	0.3	2021	
'Honeycomb'	'Harlequin'	25	0	0.0	2020	0.2
	'Black Knight'	10	0	0.0	2021	
	'Nanho Blue'	11	0	0.0	2021	
	Groovy Grape™	11	3	0.3	2021	
	'Attraction'	18	0	0.0	2020	
'Ice Chip'	'Nanho Blue'	19	0	0.0	2020	0.0
	Groovy Grape™	29	0	0.0	2020	
	'Black Knight'	11	0	0.0	2021	
	Groovy Grape™	10	0	0.0	2021	
	'Nanho Blue'	10	0	0.0	2021	
'Miss Molly'	'Harlequin'	26	0	0.0	2020	18.8
	'Attraction'	23	151	6.6	2020	
	Groovy Grape™	10	4	0.4	2021	
	'Black Knight'	9	92	10.2	2021	
	'Nanho Blue'	11	172	15.6	2021	
'Miss Violet'	'Pink Delight'	20	1	0.1	2020	2.3
	Psychedelic Sky™	30	120	4.0	2020	
	'Black Knight'	11	0	0.0	2021	
	Groovy Grape™	12	0	0.0	2021	
	'Nanho Blue'	10	0	0.0	2021	

Table 4. Continued.

Male parent	Female parent	Number of crosses	Total counts	Number seedings per cross	Year	Relative male fertility of cultivar (%)
'Nanho Blue'	CPWO2083	30	469	15.6	2020	75.7
	'Groovy Grape'	22	490	22.3	2020	
	BUZZ™ Velvet	11	291	26.5	2021	
	CP17WO2051	12	325	27.1	2021	
	'Royal Red'	11	445	40.5	2021	
'Pink Delight'	CP17WO2092	21	20	0.9	2020	35.9
	'Grand Cascade'	23	218	9.5	2020	
	'Black Knight'	11	136	12.4	2021	
	'Groovy Grape'	11	137	12.5	2021	
	'Nanho Blue'	11	301	27.4	2021	
Psychedelic Sky™	'Grand Cascade'	30	446	14.9	2020	32.5
	CP17WO2092	29	751	25.9	2020	
	'Black Knight'	10	25	2.5	2021	
	Groovy Grape™	12	30	2.5	2021	
	'Nanho Blue'	11	120	10.9	2021	
'Purple Haze'	'Pink Delight'	26	26	1.0	2020	15.4
	'Psychedelic Sky'	27	400	14.8	2020	
	'Black Knight'	11	0	0.0	2021	
	Groovy Grape™	10	1	0.1	2021	
	'Nanho Blue'	10	109	10.9	2021	
'Royal Red'	BUZZ™ Sky Blue	23	217	9.4	2020	100.0
	CP17WO2051	27	302	11.2	2020	
	'Black Knight'	12	341	28.4	2021	
	'Nanho Blue'	12	552	46.0	2021	
	Groovy Grape™	12	950	79.2	2021	

Due to unequal variance in the seedling count data, ANOVA was not appropriate to use for that data set. Generalized linear regressions with a negative binomial distribution and log link function and estimated marginal means were generated to test whether there were differences among cultivars for field seedling counts, inflorescence counts, and seedling multiplied by inflorescence for each location. Years were analyzed separately because there were differences in number of replicates per cultivar between years because some replicates died. Additionally for inflorescence counts, there was only enough labor available to count 3 of 6 replicates of the field because of increased plant size in 2021. Percentage relative fecundity was calculated separately for both field data years, female fecundity and for male fecundity to account for shifts in relative cultivar fecundity from year to year and experiment to experiment. Full fecundity for percentage relative fecundity was the most fecund cultivar of each experiment in each study year.

Results and Discussion

Relative fecundity. There were significant differences between cultivars in the 2020 season in terms of seedling count per single inflorescence, inflorescence counts, and seedlings per entire plant (Generalized linear regression, p-values for all counts $< 2.2\ e^{-16}$; Table 2). The most fecund cultivars in 2020 included 'Nanho Blue' (*B. davidii*, mean 1,253,526 seedlings per plant), Psychedelic Sky™ (interspecific hybrid, 729,948 seedlings per plant), and Blaze Pink™ (interspecific hybrid, 691,702 seedlings per plant). The least fecund cultivars in 2020 included 'Asian Moon', 'Pink Microchip', and Dapper® Blue, all of which produced 0 seedlings in our counts. Several interspecific hybrids were above the 98% reduction required for *B. davidii* cultivar approval in the state of Oregon, including Blaze Pink™, CranRazz™, Funky Fuchsia™, 'Lilac Chip', 'Miss Molly', 'Miss Violet', and Psychedelic Sky™ (Fig. 3). Cultivars that did present a 98% or greater reduction in fertility in 2020 included *B. davidii* Dapper® White, and interspecific hybrids 'Asian Moon', 'Blue Chip Jr.', 'Blue Chip', Flutterby Petite® Blue Heaven, Flutterby Petite® Tutti-Fruitti, 'Honeycomb', 'Ice Chip', 'Pink Microchip', 'Purple Haze', Dapper® Blue, and unreleased Ball cultivars CP17WO2051 and CP17WO2083.

There were also significant differences between cultivars in 2021 in terms of seedlings per single inflorescence, inflorescence counts per location and seedlings per plant (Generalized linear regression, p-values for all counts $< 2.2\ e^{-16}$; Table 2). The most fecund cultivars in 2021 included BUZZ™ Velvet (*B. davidii*, mean of 4,179,478 seedlings per plant), Blaze Pink™ (interspecific hybrid, mean of 4,074,170 seedlings per plant), and Psychedelic Sky™ (interspecific hybrid, mean of 3,630,297 seedlings per plant). Least fecund cultivars in 2021 included 'Asian Moon', 'Blue Chip Jr.', and Dapper® Blue, all of which produced 0 seedlings. Many of the same cultivars showed a 98% reduction in full fertility in 2021 as they did in 2020 (Fig. 4). In 2021 this included 'Asian Moon', 'Blue Chip', 'Blue Chip Jr.', Dapper® Lavender, Dapper® White, Flutterby Petite® Blue Heaven, Flutterby Petite® Tutti Fruitti, 'Ice Chip', 'Pink Microchip', 'Purple Haze', Dapper® Blue, and CP17WO2051. 'Honeycomb' was just above the 98% reduction threshold, representing 2.8% of full fertility.

From these field results alone, we generated conclusive evidence that interspecific hybridization is no guarantee of

Table 5. Relative fecundity of *Buddleja* OP seedlings by single inflorescence, arranged by parent cultivar.

Original cultivar[z]	Interspecific status[y]	Mean[x]	Relative fecundity OP (%)[w]	Relative fecundity cv. (%)[v]
'Attraction'	BD	745.0 abc	28.9	41.3
'Blue Chip'[t]	IS	1.8 d	0.1	1.8
'Black Knight'	BD	644.8 abc	25.0	39.7
BUZZ™ Sky Blue	BD	926.3 abc	36.0	6.5
BUZZ™ Velvet	BD	1,151.0 abc	44.7	42.1
Dapper® Lavender	BD	805.0 abc	31.3	6.5
Dapper® White[t]	BD	71.3 bc	2.8	1.6
CP17WO2051	NR	68.3 bc	2.6	1.6
CP17WO1363	NR	443.3 abc	17.2	2.1
CP17WO2083	NR	1,052.8 abc	40.9	0.1
CP17WO2092	NR	199.2 abc	7.7	8.6
CranRazz™	IS	1,245.8 abc	48.4	35.5
Funky Fuschia™	BD	1,201.6 abc	46.6	17.4
Flutterby® Pink	BD	148.4 bc	5.8	2.6
'Grand Cascade'	BD	1,649.8 ab	64.1	20.8
Groovy Grape™	BD	3,936.4 a	152.8	39.6
'Harlequin'	BD	1,764.4 ab	68.5	3.5
'Honeycomb'	IS	0.0	0.0	1.0
'Ice Chip'[r]	IS	109.0[s]	4.2	0.0
'Lilac Chip'	IS	1,417.0 abc	55.0	6.5
'Miss Molly'	IS	685.0 abc	26.6	46.1
'Miss Violet'	IS	136.0 bc	5.3	13.9
'Nanho Blue'	BD	628.2 abc	24.4	100.0
'Pink Delight'	IS	1,179.0 abc	45.8	5.0
'Purple Haze'[t]	BD	59.6 c	2.3	0.0
Psychedelic Sky™	IS	1,505.4 ab	58.4	57.3
Flutterby Petite® Tutti-Fruitti[t]	IS	93.0 bc	3.6	0.4
'Royal Red'	BD	869.6 abc	33.8	19.8

[z]The female cultivar from which OP seedlings were collected.

[y]The interspecific status of the original cultivar. Codes are as follows: *Buddleja davidii* only (BD), interspecific hybrid (IS), not reported (NR).

[x]Mean number of seedlings and compact letter display of separation of means using a generalized linear model with a negative binomial distribution and the Tukey method for comparing a family using R V 4.2.1.

[w]% relative fecundity OP was calculated against the highest mean seedling count of the 2020 field season ('Nanho Blue' with 2,575.8 seedlings per inflorescence). This provides the relative fecundity of the OP generation as compared to their parent cultivars of similar size and maturity.

[v]% relative fecundity cv. is calculated using mean seedlings per plant in study year 2020. The fully fertile standard is the same used to calculate % relative fecundity OP.

[u]Numbers collected off a single seedling for 'Ice Chip'. n = 4-5 for all other cultivars.

[t]Represents a cultivar measuring a 98% reduction in fecundity across both study years and greenhouse crossing experiments.

[s]A single count from the only seedling derived from this cultivar, not a mean, and thus not included in mean separation.

reduced fecundity in *Buddleja*, with the interspecific hybrids Blaze Pink™ and Psychedelic Sky™ among the top three most fertile cultivars in both years of our field study. Under the current regulation structure, Blaze Pink™ and Psychedelic Sky™ could apply for an exemption to the plant ban on *B. davidii* because they are interspecific hybrids (Scott Trees 2015, Dirr and Kardos 2013). The current regulation appears to be ineffective in its goal to prevent further invasion and spread of *Buddleja*.

Greenhouse crossing generally confirmed findings of field fecundity study when comparing female fertility (Table 3). The three most fecund female parents according to greenhouse crossing study were all *B. davidii* cultivars (Table 3), including Groovy Grape™ (19.5 to 79.2 seedlings per cross), 'Nanho Blue' (3 to 58.2 seedlings per cross), and BUZZ™ Velvet (10.8 to 27.8 seedlings per cross). The three lowest female fecund cultivars included interspecific hybrids 'Asian Moon', 'Pink Microchip', and 'Purple Haze', none of which produced seedlings as a female parent. Cultivars Flutterby Petite® Blue Heaven, Dapper® Lavender, Dapper® Blue, and CP17WO2051 measured at a female relative fecundity percent larger than 2% in greenhouse crossing, though according to field seedling counts they all represented a 98% reduction in fecundity.

Several cultivars varied in their relative male and female fertility (Tables 3 and 4). This may have been due, at least in part, because several had missing or malformed male organs but intact female organs. However, despite some variation, the three most male fecund cultivars are also highly female fecund *B. davidii* cultivars (Tables 3 and 4). These include 'Royal Red' (9.4 to 79.2 seedlings per cross), Groovy Grape™ (8.8 to 57.8 seedlings per cross), and 'Nanho Blue' (15.6 to 40.5 seedlings per cross) (Table 3). Least male fecundity cultivars were all interspecific or unreported cultivars that did not have anthers, including 'Pink Microchip', 'Blue Chip Jr.', Dapper® Blue, and 'Lilac Chip' (Table 4). Several interspecific cultivars that exhibited a 98% reduction in female fecundity across one or all three measurements of female fecundity exhibited higher male fertility. These include 'Purple Haze' (below 2% relative female fecundity in all 3

Table 6. **Genome sizes of *Buddleja* study cultivars and cultivar progeny determined using flow cytometry analysis of DAPI-or PI-stained nuclei and pea (*Pisum sativum* 'Ctirad'; 2C = 8.76pg) as internal standard.**

Tradename	Interspecific status[z]	Putative ploidy	1C*x* PI[y]	2C PI[y]	1C*x* DAPI[y]	2C DAPI[y]	Progeny 1Cx DAPI[y]
'Attraction'	BD	4*x*	0.74 f-i	2.97 e-h	0.84 h-j	3.31 g-j	0.82 c
BUZZ™ Sky Blue	BD	4*x*	0.74 f-i	2.97 e-h	0.87 e-i	3.48 d-h	0.85 c
BUZZ™ Velvet	BD	4*x*	0.73 f-j	2.93 e-i	0.84 g-i	3.37 g-j	0.87 a-c
Dapper® Lavender	BD	4*x*	0.72 ij	2.87 hi	0.75 kl	2.99 kl	0.83 c
Dapper® White	BD	4*x*	0.72 h-j	2.88 g-i	0.74 l	2.97 l	0.83 c
'Grand Cascade'	BD	4*x*	0.74 f-i	2.97 e-h	0.82 ij	3.27 h-j	0.81 c
Groovy Grape™	BD	4*x*	0.74 f-j	2.95 e-i	0.85 f-j	3.39 e-j	0.85 c
'Harlequin'	BD	4*x*	0.74 f-j	2.96 e-i	0.84 h-j	3.35 g-j	0.87 a-c
'Nanho Blue'	BD	4*x*	0.74 f-j	2.96 e-i	0.84 g-j	3.37 g-j	0.83 c
'Pink Delight'	BD	4*x*	0.75 f-h	3.00 e-g	0.84 h-j	3.35 g-j	0.88 a-c
'Royal Red'	BD	4*x*	0.73 g-j	2.91 f-i	0.82 ij	3.26 ij	0.84 c
'Black Knight'	BD	4*x*	0.73 f-j	2.93 e-i	0.85 e-j	3.42 d-i	0.85 c
'Asian Moon'	IS	3*x*	0.99 a	2.98 e-h	1.13 a	3.38 f-j	NA
Blaze Pink™	IS	4*x*	0.74 f-i	2.96 e-h	0.85 e-j	3.41 d-j	0.83 c
'Blue Chip Jr.'	IS	4*x*	0.84 d	3.36 c	0.91 de	3.62 cd	NA
'Blue Chip'	IS	4*x*	0.87 c	3.48 b	0.99 c	3.95 b	0.93 a
CranRazz ™	IS	4*x*	0.72 h-j	2.88 g-i	0.79 jk	3.19 jk	0.84 c
Flutterby Petite® Blue Heaven	IS	4*x*	0.74 f-j	2.85 i	0.84 g-j	3.38 f-j	0.88 a-c
Flutterby Petite® Tutti Fruitti	IS	4*x*	0.75 fg	3.02 ef	0.88 e-h	3.51 d-g	0.89 a-c
Funky Fuschia™	IS	4*x*	0.73 f-j	2.93 e-i	0.84 h-j	3.35 g-j	0.86 c
'Honeycomb'	IS	4*x*	0.94 b	3.74 a	1.04 b	4.18 a	0.93 ab
'Ice Chip'	IS	4*x*	0.79 e	3.19 d	0.89 d-g	3.59 c-f	0.93 a-c
'Lilac Chip'	IS	4*x*	0.76 f	3.05 e	0.87 e-i	3.48 d-h	0.84 c
'Miss Molly'	IS	4*x*	0.72 h-j	2.88 g-i	0.84 h-j	3.34 g-j	0.85 c
'Miss Violet'	IS	4*x*	0.80 e	3.22 d	0.90 d-f	3.61 c-e	0.86 bc
'Pink Microchip'	IS	4*x*	0.75 fg	3.00 ef	0.83 h-j	3.33 g-j	NA
Psychedelic Sky™	IS	4*x*	0.74 f-j	2.96 e-i	0.81 j	3.22 ij	0.82 c
'Purple Haze'	IS	4*x*	0.83 de	3.31 cd	0.95 cd	3.81 bc	0.92 a-c
CP17WO1363	NR	4*x*	0.75 fg	3.00 ef	0.83 h-j	3.30 g-j	0.82 c
Dapper® Blue	NR	4*x*	0.75 fg	3.00 ef	0.83 h-j	3.31 g-j	NA
CP17WO2051	NR	4*x*	0.76 f	3.05 e	0.81 j	3.25 ij	0.85 c
CP17WO2083	NR	4*x*	0.75 fg	3.02 ef	0.83 h-j	3.30 g-j	0.85 c
CP17WO2092	NR	4*x*	0.74 f-j	2.95 e-i	0.84 h-j	3.34 g-j	0.83 c

[z]IS indicates interspecific hybrid, BD indicates *Buddleja davidii*, NR indicates not reported.

[y]Means followed by different letters are different based on analysis of variance and separation using Tukey's HSD ($\alpha = 0.05$).

experiments but 15.4% relative male fecundity), Flutterby Petite® Tutti Fruitti (below 2% relative female fecundity in all 3 experiments but 11.1% relative male fecundity), and Flutterby Petite® Blue Heaven (below 2% relative female fecundity in field experiments only and 29% relative male fecundity). Here we conducted male fertility estimates for completeness and to fully understand the reproductive biology of these cultivars. However, when considering the ecological threat cultivars may pose, male fertility is a relatively unimportant consideration compared to seedling production.

The OP generation detected slight upticks in fecundity of low fecundity cultivar derived offspring in a few cases.

Table 7. **Genome sizes of select *Buddleja* species determined using flow cytometry with pea (*Pisum sativum* 'Ctirad'; 2C = 8.76pg) as internal standard.**

Taxon	Ploidy	1C*x* PI (mean ± SEM)[z]	1C*x* DAPI (mean ± SEM)
B. alternifolia	2*x*	0.90 ± 0.01c	0.95 ± 0.03c
B. globose	2*x*	1.13 ± 0.02a	1.28 ± 0.01a
B. lindleyana	2*x*	0.99 ± 0.02b	1.21 ± 0.03b

[z]Means followed by different letters are different based on analysis of variance and separation using Tukey's HSD ($\alpha = 0.05$).

A generalized linear regression confirmed differences in fecundity between OP seedlings ($P = 3.254\ e^{-16}$). Many of the highly reduced fecundity cultivars did not yield OP seedlings for the analysis, however of those that were measured, Dapper® White, Flutterby Petite® Tutti-Fruitti, 'Ice Chip', and 'Purple Haze' had relative fecundity levels slightly over the 2% threshold (Table 5). However, 'Ice Chip' only yielded one seedling where other OP seedling populations for this analysis were of n = 4 or n = 5. Additionally, there were some severe outliers in the OP seedling population of 'Purple Haze', some being quite fecund. These initial results are encouraging and indicate that sales of reduced fecundity *Buddleja* are not likely to contribute to invasive populations in Oregon; however, a larger study of seedling fecundity is warranted.

Flow cytometry. Though we did observe some reduction in fertility and even near hybrid sterility in the case of some interspecific hybrids, it did not appear that odd ploidy levels associated with chromosomal imbalance was the causal agent of the sterility. Given the difference in ploidy level between *B. davidii* (tetraploid) and many of the crossing partners (diploid), it was assumed that there would be more triploids found in the population. However, aside from the confirmed triploid result in 'Asian

Moon'(Renfro et al. 2007), calculations led us to conclude that most of these hybrids resulted from reduced gametes of diploid crossing partners, or that they may not be true hybrids at all (Table 6). This analysis was facilitated in part by determining genome sizes of selected *Buddleja* species used for pedigree reconstruction (Table 7). Across cultivars, genome size ranged from 0.711 pg/1C*x* for Flutterby® Pink to 0.994 pg/1C*x* in triploid 'Asian Moon' (expressed in PI measurements). While many of the interspecific hybrids had genome sizes that were not significantly different that *B. davidii* species types, 'Honeycomb' (0.935 pg/1C*x*), 'Blue Chip' (0.87 pg/1C*x*) and 'Blue Chip Jr.' (0.839 pg/1C*x*) were significantly larger than *B. davidii* species types as well as other interspecific hybrids. Differences between DAPI and PI estimates varied across taxa, indicating differences in GC content of genotypes. Across all cultivars, mean DNA content was 0.185 pg higher for DAPI estimates than PI.

OP seedlings collected from parent cultivars in our study varied less in differences between groups in terms of DNA content, with only 'Blue Chip'- and 'Honeycomb'-derived seedlings having significantly larger DNA content than other progeny. Monoploid genome sizes of seedlings ranged from a low of 0.808 pg/1C*x* from 'Grand Cascade' seedlings to 0.929 pg/1Cx in OP progeny of 'Honeycomb' and 'Blue Chip'.

These findings should be confirmed using more definitive means than inferred values from pedigree, such as chromosome counts from root tip squashes. However, it appears to support previous findings in *Buddleja* that putative interploid crosses could be mediated by unreduced gamete production in diploid crossing partners, at least in *B. globosa* and *B. lindleyana* (Van Laere et al. 2009). Pollen flow cytometry using a filter bursting nuclei extraction method was effective in *B. davidii* and could be used to study differences in species rates of unreduced gamete production (Kron and Husband 2012). However, impedance flow cytometry likely presents a simpler path, as it was designed specifically for pollen analysis. A study undertaking that comparison could track the likelihood that unreduced gamete production fosters interspecific crossing in *Buddleja*.

We recommend amendments to the current regulation on butterfly bush in Oregon. Though adjustments to the regulation, such as requiring a 98% reduction in fertility for interspecific hybrids as well as *B. davidii* cultivars, might narrow grower options in the short term, it would preserve public trust in the long term. Support is documented for breeding for reduced fertility to reduce invasive potential (Gagliardi and Brand 2007, Kelley et al. 2006), but for this strategy to be effective we must be sure we are not eroding trust of consumers by allowing cultivars on the market that do not represent the reduction in fertility needed to prevent spread from cultivation. Implications of this research reach beyond butterfly bush into other invasive crops with breeding projects for reduced fertility cultivars. Efforts in *Euonymus alatus* (Thunb.) Siebold(burning bush, Brand et al. 2012b, Thammina et al. 2011), *Berberis thunbergii* DC. (Japanese barberry, Brand et al. 2012a), *Acer ginnala* and *A. platanoides* L. (Amur and Norway maple, Contreras and Hoskins 2020), and *Pyrus* (flowering pear, Phillips et al. 2016) have successfully developed new cultivars with reduced fertility. If public trust in breeding for reduced fecundity is eroded by confusion created by regulation that is missing its intended goal, the substantial efforts and resources devoted to breeding new cultivars could be wasted. Our data indicates that for butterfly bush, and generally for all potentially invasive species, amendments to approve production and sale of specific cultivars should be based on data gathered through quantitative performance evaluation and not through predictions based on pedigree or other variable indicators of potential fertility.

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