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Occurrence of unreduced pollen in diploid blueberry species, *Vaccinium* sect. *Cyanococcus* *

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Summary. A total of 1475 individuals belonging to 43 natural populations of seven diploid (2x) blueberry species (*Vaccinium* section *Cyanococcus*) and two natural interspecific 2x hybrid populations were evaluated for unreduced pollen production. Significant differences were found in the frequency of unreduced pollen producers between species and within and between populations of the same species. Individuals with 1% or more unreduced pollen were considered unreduced pollen producers. The average frequency of unreduced pollen producers in these diploid species was 13.5%, ranging from 7.4% (*V. corymbosum*) to 18.4% (*V. darrowii*). The frequency of unreduced pollen grains in individual clones varied from ≤1% to 28.6%. The production of unreduced pollen was not associated with male fertility. The widespread occurrence of unreduced pollen in the diploid species should allow the introgression of this germ plasm to the tetraploid level via unilateral sexual polyploidization.

Key words: Blueberry – Unreduced gametes – 2n gametes – First division restitution – Sexual polyploidization

Introduction

The cultivated blueberry belongs to section *Cyanococcus* of the genus *Vaccinium* L. (Ericaceae). The North American blueberry consists of diploid (2x), tetraploid (4x), and hexaploid (6x) species. The main cultivated types are 4x (*V. corymbosum* L. and *V. angustifolium* Aiton) and 6x (*V. ashei* Reade). The 2x species are: *V. boreale* Hall and Alders, *V. corymbosum* L., *V. darrowii* Camp, *V. elliottii* Chapm., *V. myrtilloides* Michx., *V. pallidum* Ait., and *V. tenellum* Ait. These 2x species are an important source of favorable alleles for blueberry breeding (Sharpe and Sherman 1971; Galletta 1975; Lyrene and Sherman 1980, 1983; Ballington et al. 1984a, b; Hancock 1989). For example, *V. darrowii* was used in the development of varieties with low-chilling requirement (Sharpe and Sherman 1971) and could expand temperature tolerance ranges (Moon et al. 1987a, b) and drought tolerance (Erb et al. 1988).

Differences in ploidy level between the 2x species and the cultivated types pose a problem for the efficient utilization of the 2x germ plasm in blueberry breeding. The use of 2n gametes (gametes with the sporophytic chromosome number) can be used to overcome this problem (Peloquin and Ortiz 1991). Tetraploid hybrids can be derived by crossing tetraploids with diploids producing 2n gametes.

The production of unreduced pollen in blueberry has been reported in a small number of individuals from different 2x species (Sharpe and Sherman 1971; Cockerham and Galletta 1976; Megalos and Ballington 1987, 1988) and a few interspecific hybrids (Lyrene and Sherman 1983; Vorsa 1986; Dweikat and Lyrene 1988; Vorsa and Ballington 1991). However, there has not been a systematic effort to survey the production of unreduced pollen among all the 2x species of blueberry.

It has been suggested that the production of 2n pollen could be under genetic control (Cockerham and Galletta 1976; Megalos and Ballington 1987). The cytology of 2n pollen production has been studied by Vorsa (1986) in a *V. ashei/corymbosum* hybrid derivative. The steps leading to 2n pollen formation were: (1) desynapsis before metaphase I, (2) equational division of sister centromers at

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anaphase I, and (3) cytokinesis immediately after telophase I. This mode of 2n pollen formation is genetically equivalent to a first division restitution (FDR) mechanism. The lack of crossing-over provides the opportunity for this FDR 2n pollen to transmit intact the genotype of the 2x parent to the 4x offspring. Thus, both the heterozygosity and the epistasis present in the 2x parent would be transferred to the 4x offspring after $4x \times 2x$ crosses.

In blueberry, the four pollen grains from a single normal meiotic event are held together as a tetrad (Stushnoff and Hough 1968; Stushnoff and Palser 1969). The production of dyads, triads, and monads have been considered to be evidence of 2n or unreduced pollen formation in blueberry (Cockerham and Galletta 1976; Megalos and Ballington 1987, 1988; Dweikat and Lyrene 1988). Based on the cytological observations of dyad formation in blueberry (Vorsa 1986), the two pollen grains of a dyad should have or be near the 2n chromosome number. Monads might be expected to have twice the 2n number.

The objective of the research reported here was to conduct a systematic and complete survey of unreduced pollen production in natural populations of the seven 2x *Vaccinium* species.

Materials and methods

Forty-three natural populations of seven 2x *Vaccinium* species and three natural interspecific hybrid populations were evaluated for frequency of stainable pollen and unreduced pollen production during 2 consecutive years: 1990 and 1991. The populations, their taxonomic group, their site of collection, and the number of individuals sampled are indicated in Table 1.

Pollen was sampled from each plant that flowered in both years. Pollen from two flower samples were screened for each individual clone. Pollen was stained with 1% aceto-carmine glycerol jelly and microscopically examined at 200× magnification. At least 200 sporads (tetrads, dyads, and monads) were scored per clone in each year to determine unreduced pollen frequency (no triads were observed in the materials evaluated). For the estimate of unreduced pollen frequency to be consistent with previous reports in blueberry (Cockerham and Galletta 1976; Megalos and Ballington 1987, 1988; Dweikat and Lyrene 1988), the frequency of unreduced pollen was estimated as follows:

$$\text{unreduced pollen frequency} = (2D + M)/T$$

where D is the number of dyads, M is the number of monads, and T the total number of pollen grains examined. Individuals with 1% or more unreduced pollen production were considered unreduced pollen producers. Some of these unreduced pollen producers were used successfully in crosses with 4x parents to produce 4x offspring.

The frequency of unreduced pollen production was determined for the 46 populations and the seven species. The standard error of the phenotypic frequency was calculated as $\sqrt{[q(1-q)/n]}$, where q is the frequency of unreduced pollen producers and n is the number of individuals sampled.

The log-likelihood test, G-test (Fienberg 1977), was used to compare frequency distributions for unreduced pollen production among different populations within a species and between different species. The G-test involves the ratios between the observed and expected frequencies. The alternative hypothesis for this statistic is one of general association (non-independence of 2n pollen production with respect to the genotype: either a population within a species or the species itself). The theoretical distribution for the log-likelihood ratio is poorly known; however, twice this quantity, a value called G, approximates the χ^2 distribution (Sokal and Rohlf 1981). The G statistics for contigency tables takes the form:

$$G = 2\Sigma_i \Sigma_j [f_{ij} * LN(f_{ij})] - \Sigma_i [r_i * LN(r_i)] - \Sigma_j [c_j * LN(c_j)] + n * LN(n).$$

The χ^2 distribution has $(r-1)(c-1)$ degrees of freedom and is determined as:

$$Q_P = 2\Sigma_i \Sigma_j [n_{ij} * LN(n_{ij}/m_{ij})]$$

where r and c are the number of rows and columns in the contingency table, respectively, n_{ij} is the cell frequency in the ith row and the jth column, and $m_{ij} = [n_{i.} * n_{.j}/n]$, in which $n_{i.}$ is $\Sigma_i n_{ij}$ (column totals), $n_{.j}$ is the $\Sigma_j n_{ij}$ (row totals), and n is the $\Sigma_i \Sigma_j n_{ij}$ (overall total).

To evaluate if the production of unreduced pollen is associated with the frequency of stainable pollen, a Chi-square test of homogeneity was used in populations having frequencies of unreduced pollen producers greater than 15%: the Chi-square test was considered to be the more appropriate test due to the highly skewed appearance of the frequency distributions for both unreduced pollen producers and pollen stainability. The null hypothesis was that the production of unreduced pollen was independent of the level of male fertility as measured by the frequency of stainable pollen. The population was divided into two classes based on frequency of stained pollen: the low class included all individuals with values $\leq$ the population mean, while the high class included all individuals with values $>$ the population mean. The Chi-square was calculated using Yates' correction for continuity in small sample sizes (Yates 1934).

Results

Out of 46 populations 38 contained unreduced pollen producers (Table 1). There were significant differences in the frequency of unreduced pollen producers among the seven species. There were also significant differences for the frequency of unreduced pollen producers between populations of the same species and for the frequency of unreduced pollen between individuals of the same population.

The average frequency of unreduced pollen producers in the 2x species was 13.49 ± 0.89 (Table 2). *V. darrowii* had the highest frequency of unreduced pollen producers (18.40 ± 2.18), while *V. corymbosum* had the lowest frequency (7.38 ± 1.83). The frequency distribution of unreduced pollen producers between these two species was significantly different ($G = 12.9$; $P < 0.01$). The frequency of unreduced pollen producers was significantly higher in *V. darrowii, V. elliottii, V. pallidum,* and *V. tenellum* than in *V. corymbosum. V. boreale* and *V. myrtilloides* did not differ significantly from either the high or low group. The three hybrid populations derived from *V. tenellum* and *V.*

Table 1. Unreduced pollen production in 2x *Vaccinium* species evaluated during 2 consecutive years: 1990 and 1991

Population	Location	Total number of individuals sampled	Unreduced pollen producers[a]		
			Number	Frequency	Range[b] (%)
V. boreale					
NJ 88.29	Cape Breton, NS, Canada	39	3	7.7	1.0– 2.0
NJ 88.30	Cape Breton, NS, Canada	28	5	17.9	1.0– 6.1
V. myrtilloides					
NJ 87.37	Washington Co, Me.	52	13	25.0	1.0– 5.7
NJ 87.43	Washington Co, Me.	37	0	0.0	–
NJ 88.26	Chippewa Co, Mich.	53	8	15.1	1.0– 5.7
NJ 88.27	Bayfield Co, Wis.	53	5	9.4	1.0– 1.9
NJ 88.28	Alger Co, Mich.	5	5	0.0	–
V. tenellum					
NC 78.8	Lexington Co, S.C.	32	2	7.2	1.0– 3.8
NC 83.9	Bladen Co, N.C.	37	2	5.4	1.0– 4.5
NC 87.9	Brunswick Co, N.C.	73	20	27.4	1.0–17.3
NJ 88.31	Turner Co, Ga.	51	6	10.0	1.0–14.5
NJ 88.33	Bacon Co, Ga.	37	5	13.5	1.0–14.5
NJ 88.34	Appling Co, Ga.	23	1	4.4	1.0
NJ 88.35b	Candler Co, Ga.	9	2	22.2	1.0– 2.0
V. pallidum					
NC 79.5	Montgomery Co, N.C.	37	9	24.3	1.0–13.8
NJ 88.18	Ocean Co, N.J.	66	8	12.1	1.0– 3.8
NJ 88.20	Sussex Co, Del.	34	4	11.8	1.0– 1.8
V. elliottii					
NC 79.24b	Pender Co, N.C.	9	1	11.1	28.6
NC 83.1	Richmond Co, N.C.	28	4	14.3	1.0
NJ 88.1	Bibb Co, Ga.	12	1	8.3	1.0
NJ 88.2	Evans Co, Ga.	34	9	26.5	1.0–18.7
NJ 88.3	Jackson Co, Fla.	21	1	4.8	1.0
NJ 88.4	Santa Rosa Co, Fla.	37	3	8.1	1.0–14.5
NJ 88.16	Stone Co, Fla.	6	0	0.0	–
NJ 88.19	Isle of Wight Co, Va.	31	6	19.4	1.0–15.2
V. pallidum × *V. tenellum*					
NC 79.5	Montgomery Co, N.C.	2	0	0.0	–
NC 79.76a	Montgomery Co, N.C.	64	4	6.2	1.0– 1.8
NJ 87.52	Southampton Co, Va.	74	9	12.3	1.0–12.3
V. corymbosum					
NC 79.5	Montgomery Co, N.C.	16	3	18.6	1.0– 2.9
NC 79.76b	Montgomery Co, N.C.	46	4	8.7	1.0– 1.9
NC 84.6b	Lake Co, Fla.	25	0	0.0	–
NJ 87.40	Southampton Co, Va.	26	0	0.0	–
NJ 88.8	Highlands Co, Fla.	28	2	7.1	2.0– 2.9
NJ 89.1a	Burlington Co, N.J.	62	6	9.7	1.0– 3.7
V. darrowii					
NC 84.6a	Lake Co, Fla.	44	3	6.8	1.0– 2.9
NJ 88.5	Covington Co, Fla.	36	7	19.4	1.0– 3.7
NJ 88.6	Santa Rosa Co, Fla.	37	6	16.2	1.0– 9.0
NJ 88.7	Highlands Co, Fla.	10	0	0.0	–
NJ 88.9	Polk Co, Fla.	23	2	8.7	2.0–12.2
NJ 88.10	Polk Co, Fla.	10	1	9.1	1.0
NJ 88.11	Highlands Co, Fla.	6	0	0.0	–
NJ 88.12	Liberty Co, Fla.	41	17	41.5	1.0–19.4
NJ 88.13	Liberty Co, Fla.	33	4	12.1	1.0– 2.0
NJ 88.14	Gulf Co, Fla.	6	2	33.3	1.0– 2.4
NJ 88.15	Okaloosa Co, Fla.	10	2	20.0	1.0– 2.0
NJ 88.17	Stone Co, Miss.	31	9	29.1	1.0– 2.4

[a] Individuals producing unreduced pollen at 1% or higher frequency

[b] Observed among the unreduced pollen producers

Table 2. Frequency of unreduced pollen producers among 2x *Vaccinium* species

Species	Number of populations	Number of individuals	Unreduced pollen reduced[a] %
V. darrowii	12	288	18.40±2.18a
V. pallidum	3	137	15.33±3.07a
V. tenellum	8	262	14.50±2.17a
V. elliottii	8	178	14.04±2.60a
V. myrtilloides	5	200	13.00±2.35ab
V. boreale	2	67	11.94±3.96ab
V. pallidum × *V. tennellum*	3	140	9.28±2.45ab
V. corymbosum	6	203	7.38±1.83 b

[a] Percentages followed by the same letter were not significantly different according to the G-statistics at the 0.05 probability level

pallidum (NC 79.5, NC 79.76a, and NJ 87.52) exhibited a lower value for unreduced pollen frequency than the parental species; however, the difference was not significant (Table 2). The frequency of unreduced pollen production per individual clone did not exceed 30% (Table 1).

Chi-square tests for homogeneity between frequency of stainable pollen and production of unreduced pollen for populations with greater than 15% unreduced pollen producers were not significant (Table 3). Therefore, the production of unreduced pollen does not appear to be associated with the frequency of stainable pollen: unreduced pollen producers were found in both low (≤population mean) and high (>population mean) classes for frequency of stainable pollen in all of the populations sampled.

Discussion

Meiotic abnormalities leading to the formation of unreduced pollen appear to be present in all seven 2x *Vaccinium* species of the section *Cyanococcus*. These results are very encouraging because this would allow the possibility of introgressing the 2x germ plasm to the 4x cultivated gene pool via 4x×2x crosses (unilateral sexual polyploidization). The fact that 2n pollen in blueberry seems to be genetically equivalent to a FDR mechanism (Vorsa 1986) increases the potential usefulness of this introgression technique. In this way the genes controlling low-chilling requirement (*V. darrowii*) or early fruit ripening and heat tolerance (*V. elliotii*) could be transferred almost intact from the 4x level via FDR 2n pollen.

Galletta (1975) indicated that polyploid induction following the use of colchicine either as a seed treatment (Aalders and Hall 1963; Rousi 1966a) or as a treatment of growing shoot tips (Moore et al. 1964; Rousi 1966b), was a useful tool for transferring genes from 2x to 4x species. The use of FDR 2n pollen represents an advantage over this methodology in blueberry breeding. The genetic consequences of the modes of polyploidization (sexual-FDR 2n pollen versus asexual-colchicine doubling) are quite different in relation to fitness and genetic flexibility of the newly arisen polyploid (Mackey 1970; Watanabe et al., 1991). The inbreeding coefficient is increased (F=1/3) after asexual polyploidization. This could result in reduced fertility in the colchicine-derived 4x parent (Krebs and Hancock 1988). It also complicates the pattern of segregation due to tetrasomic inheritance. In contrast, sexual polyploidization via FDR 2n pollen avoids inbreeding and transmits a high level of heterozygosity and a large fraction of the epistasis from the 2x parent to the 4x offspring.

Some of these unreduced pollen producers have been crossed with tetraploid cultivars to produce 4x offspring. The fact that the offspring were mainly 4x (data not shown) supports the existence of a strong triploid block following interploid crosses between blueberry species (Sharpe and Darrow 1959; Dweikat and Lyrene 1988).

Although the production of 2n gametes could represent a disadvantage in the conservation of the 2x level, it does provide a chance for sexual polyploidization. For example, it has been demonstrated that in potato sexual polyploidization constitutes an advantage as a result of the increase in fitness and flexibility achieved by the 4x progenies (Iwanaga and Peloquin 1982; Watanabe et al. 1991).

V. corymbosum, a crown-forming, highbush 2x species, had the lowest frequency of unreduced pollen producers. Reproduction within this species is obligately sexual. *V. corymbosum* lacks the rhizomatous habit found in the lowbush blueberry species. Therefore, a high frequency of unreduced pollen could represent a disadvantage for reproductive potential at the 2x level. Since reduced reproductive potential could restrict perpetuation and expansion of this species in natural habitats, selection pressure would reduce the frequency of alleles conferring 2n pollen production in the population.

Vaccinium elliottii, a second crown-forming species, exhibits asexual propagation through tip layering. This species has been considered conspecific *V. corymbosum* by Van der Kloet (1988). However, the significant difference in the frequency of unreduced pollen producers in these 2x taxa further supports the recognition of *V. elliottii* as a distinct species. Bruederle and Vorsa (1990) have previously presented genetic data supporting the specific status of *V. elliottii*.

The expressivity for unreduced pollen production was variable, ranging from 1% to 28.6%. In effect, the unreduced pollen producers had both unreduced and n pollen. Variable expressivity and incomplete penetrance are characteristics of the alleles controlling 2n pollen

Table 3. Frequency of stainable pollen and unreduced pollen production in six 2x populations

Population	χ^2 test for association			
Population: NJ 88.30 (*V. boreale*)	Pollen stainability[c] (mean = 81.04%)			
Year: 1990; $n=28$	High	Low		
Unreduced pollen producers[a]	2	3		
Non-unreduced pollen producers[b]	19	4	$\chi^{2\,d}=2.0$	$P=0.154$
Population: NJ 87.37 (*V. myrtilloides*)	Pollen stainability[c] (mean = 86.76%)			
Year: 1990; $n=30$	High	Low		
Unreduced pollen producers[a]	5	5		
Non-unreduced pollen producers[b]	17	3	$\chi^2=2.9$	$P=0.089$
Population: NJ 87.9 (*V. tenellum*)	Pollen stainability[c] (mean = 78.77%)			
Year: 1991; $n=68$	High	Low		
Unreduced pollen producers[a]	5	7		
Non-unreduced pollen producers[b]	40	16	$\chi^2=2.9$	$P=0.087$
Population: NJ 79.5 (*V. pallidum*)	Pollen stainability[c] (mean = 63.85%)			
Year: 1990; $n=31$	High	Low		
Unreduced pollen producers[a]	2	6		
Non-unreduced pollen producers[b]	15	8	$\chi^2=2.9$	$P=0.089$
Population: NJ 88.2 (*V. elliotii*)	Pollen stainability[c] (mean = 69.81%)			
Year: 1991; $n=34$	High	Low		
Unreduced pollen producers[a]	2	6		
Non-unreduced pollen producers[b]	18	8	$\chi^2=3.8$	$P=0.052$
Population: NJ 88.17 (*V. darrowii*)	Pollen stainability[c] (mean = 53.70%)			
Year: 1991; $n=31$	High	Low		
Unreduced pollen producers[a]	6	6		
Non-unreduced pollen producers[b]	11	8	$\chi^2=0.1$	$P=0.737$

[a] Individuals producing $\geq 1\%$ unreduced pollen
[b] Individuals producing $< 1\%$ unreduced pollen
[c] Pollen stainability classes: low < mean of population; high > mean of population
[d] χ^2 calculated using Yates' correction for continuity in small sample sizes

production in alfalfa (McCoy 1982); similar phenomena have been observed in the production of 2n eggs in 2x potato (Ortiz and Peloquin 1991). This situation allows the maintenance of the 2x level by intercrossing with other 2x and a continous introgression from 2x species to 4x species via unilateral sexual polyploidization.

The production of unreduced pollen did not appear to be associated with male fertility: unreduced pollen producers were identified among clones with high or low pollen stainability. This could explain the wide distribution of unreduced pollen among the 2x self-incompatible outcrossing species. Bumblebees, which are their natural pollinators, are attracted by the production of viable pollen. In this way, the allele(s) controlling unreduced pollen production can be maintained in the 2x population.

The fact that male fertility was not associated with the production of unreduced pollen indicates that it would be possible to select a male parent combining both high male fertility with a high frequency of unreduced pollen production. This type of material could be very useful for crossing with 4x cultivars.

The selection and subsequent intercrossing of unreduced pollen producers with a high frequency of unreduced pollen is a potential method by which to develop a breeding population with higher frequencies of unreduced pollen. This methodology, recurrent selection, has been applied successfully for increasing the frequency of 2n pollen in red clover (Parrot and Smith 1986) and 2n eggs in potato (den Nijs and Peloquin 1977; Ortiz 1991).

In conclusion, a new strategy seems feasible in blueberry breeding. It consists in (a) identifying good attributes in the 2x germ plasm, (b) combining these attributes in a 2x population by breeding at the 2x level, and (c) subsequent transfer to the 4x level by FDR 2n pollen. This breeding scheme also broadens the genetic base of the blueberry crop due to an increase of the allelic variation in the cultivated gene pool.

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