A Single Dominant Gene, *Ef*, Confers Early Flowering in Acorn Squash (*Cucurbita pepo* subsp. *ovifera*)

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Introduction

Cucurbita species are monoecious and produce staminate flowers on basal nodes close to the crown of the plant and pistillate flowers on more distal nodes of the main stem and lateral branches. However, there is considerable variability in flowering patterns in terms of node number of first flowers reaching anthesis and the time course for initiation of flower buds. For example, Hassan et al. (2016) reported that first female flowering among summer squash and acorn squash varieties occurred at nodes ranging from 7 to 33. Early and prolific flowering is characteristic of yellow summer squash and zucchini, *Cucurbita pepo* subsp. *ovifera* and *Cucurbita pepo* subsp. *pepo* respectively (Montero-Pau et al., 2017), whereby fruit sinks are continually removed, allowing continuous fruit set over an extended period. Early flowering in winter squash may be desirable in cultigens grown in short growing seasons like New England as it allows for full fruit maturation. Also, early flowering cultigens have proven useful as rootstocks for inducing early flowering in late flowering cultigens of squash (Ogden and Loy, 2018). On the other hand, excessively early flowering may result in nutrient sink competition between developing fruits and newly developing leaves, thereby suppressing development of the leaf canopy and resulting in a deficiency of photosynthate to support the developing fruit. Developing fruits and seeds may act as dominant sinks thereby limiting further vegetative development. This concept is well established in a variety of crops including members of the Cucurbitaceae family (Delesalle & Mooreside, 2020; El-Keblawy, 2020; Wardlaw, 1968).

Genomics research has revealed numerous quantitative trait loci (QTL) regulating flowering time in related crop species (Lu et al., 2014; Mcgregor et al., 2014). In cucumber, for example, Lu et al. (2014) identified a major QTL for earlier flowering. They speculated that early flowering in cucumber is caused by a single dominant gene designated *Ef1.1.* Also in Cucurbitaceae, a major QTL in watermelon regulating flowering time was recently discovered (McGregor, et. al, 2014). In squash, *C. pepo* subsp. *pepo*, SNP analyses conducted by Montero-Pau (2017) revealed at least two QTL regulating flowering time. This finding remains tentative and does not support that team's previous identification of a single major QTL regulating flowering time (Esteras et al., 2012). Despite advancement in the genomics field, we are not aware of any classical studies on inheritance of genes controlling flowering time in squash reported to date.

A better understanding of the genetics controlling flowering time in winter squash will contribute to plant breeders' ability to improve varieties of summer and winter squash and may provide insights into the genetic control of substances affecting photoperiodic and late flowering patterns. Season extension by using a mixture of early, mid, and late season cultivars is a popular practice among many vegetable growers and breeders must respond to this demand. This research could also provide germplasm to identify molecular markers for early flowering and better understand the genes involved in regulation of flowering time in squash.

At the University of New Hampshire, Dr. Brent Loy identified and generated two highly inbred lines of acorn squash which display early and late flowering. Because the two breeding lines display large differences in flowering time, they appeared to be good choices for an inheritance study on flowering time in acorn squash. Through examination of F_1, F_2, and backcross populations derived from two breeding lines with distinct flowering times, we sought to elucidate the genetic control behind early flowering.

Materials and Methods

Experimental site description. Experiments took place at the Kingman Horticultural Research Farm in Madbury, New Hampshire between the months of June to August during 2017, 2018, and 2019. All plants were grown on raised black plastic covered beds, 0.81 meters in width and 0.15 meters in height and fertilized with a pre-plant granular fertilizer with 90 kg/ha N and K. Standard pest and disease management methods were employed to control any pest or pathogen problems. Weed control between beds was provided with mechanical and manual cultivation.

Plant materials and data collected. The two parent lines, NH27-15-5-10 (hereafter P1) and NH8-17-12-7 (hereafter P2), along with their F1, F2 (2018 only), and backcross populations (2018 and 2019 only) were all seeded in 50 cell plug trays at the Macfarlane greenhouse in Durham, New

Hampshire and after germination, fertilized by hand watering fertigated water at a constant feed rate of 100 ppm-N with the fertilizer 17-4-17 (N-P_2O_5-K_20). P1 flowers early and initiates pistillate flowers as early as nodes three or four, whereas P2 often flowers 12 to 16 days later and initiates pistillate flowers at node 12 or later. Both parent lines have a bush growth habit and produce fruit with high starch content in the fruit mesocarp tissue. Seedlings were transplanted into the field at the 1 to 2 leaf stage. Plants were grown at a spacing of 0.6 meters between plants and 2.7 meters between raised beds. Upon reaching the 6-7 leaf stage, a plastic tag was placed around the petiole of the leaf at the 5th node from the cotyledons to facilitate counting of nodes. Daily observations of the plants enabled noting the date and the node number at which the first male and female flowers reached anthesis. Dates of flowering were converted to days to first male and female anthesis from transplanting.

Statistical design and analysis. Each year, a randomized complete block design was employed. A block was represented by a single row and there were eight rows. Treatments consisted of the genotypes, and each treatment was assigned randomly within each row and replicated once. For 2017, each row consisted of 5 F_1 plants, 5 plants of each parent, 10 backcrosses to the early parent (BCP1), and 10 backcrosses to the late parent (BCP2), for a total of 35 plants per block. For 2018, each row contained 4 F_1 plants, 4 plants of each parent (P1 and P2), 10 backcrosses to the early parent (BCP1), 10 reciprocal backcrosses to the late parent (BCP2R), and 24 F_2 plants for a total of 56 plants per row. In 2019, each row contained 4 F_1 plants, 4 plants of each parent, 4 backcrosses to the early parent (BCP1), 12 backcrosses to the late parent (BCP2), and 12 reciprocal backcrosses to the late parent (BCP2R) plants for a total of 40 plants per row.

Two-way ANOVA analyses were conducted to detect differences in flowering time as affected by genotype. Flowering time was represented as both days to first male and female flower reaching anthesis and node number of first male and female flower which reached anthesis. Each row contained a randomized complete block to enable assignment of some phenotypic variation to variability in field conditions. Frequency distributions were calculated for each genotype separately by year and compared. To test the single dominant gene model, χ^2 analysis was employed by categorizing each plant as either early or late. Because the female flower gives rise to the fruit, earliness was determined by the first female flower only. During 2017 and 2018, earliness was defined as having a female flower which reached anthesis at node number 12 or less and this flower reached anthesis in 37 days or less after transplant. In 2019, earliness was defined as having a female flower which reached anthesis at node number 15 or less and reached anthesis in 37 days or less after transplant. A later planting date and prolonged period in the greenhouse in 2019 likely caused more rapid shoot elongation than in 2017 and 2018. After classification based on female flowering patterns, male flowering was also compared between the classified segregants. We tested the hypothesis that early flowering was caused by a single dominant gene. Thus, the expected ratios were that all F_1 plants and all backcrosses to the early parent, BCP1, would be early flowering while backcrosses to the late parent, BCP2, would segregate at ratio of 1:1 early:late flowering and the F_2 population would segregate at a ratio of 3:1 for early:late flowering.

Results and Discussion

Flowering phenology, in general, among all genotypes occurred similarly to previous researchers' descriptions with male flowering beginning earlier and at lower node numbers than female flowering (Loy, 2004). Table 1 illustrates these differences. Both node number and days to flower are presented because initiation of flowering is both temporal and morphological and the two variables are highly correlated (data not shown). Variation in male flowering time was limited compared to female flowering across all genotypes. Male flowering initiated at nodes 1-5 between 24 and 30 days after transplant date. Female flowering initiated at nodes 9-22 between 29 and 46 days after planting. This greater variation in female flowering patterns was the justification for the use of female flowering time as an indicator of earliness of flowering.

Although use of first female flowering as an indicator of earliness captured clear differences in flowering time among the different populations grown, this method was not without its limitations. Flowering time, while genetically controlled, is also highly influenced by other factors such as transplant vigor, field fertility and soil quality levels, pest and disease pressure, and environmental variables such as temperature, photoperiod, and light intensity. Use of first female flowering resulted in misclassification of some late flowering plants as early. This phenomenon was observed during the 2018 season when 29% of the late flowering parent (P2) plants were classified as early flowering (Table 1), based on a single flower reaching anthesis early. However, these precocious flowers did not typically set fruits and the subsequent flowering of these plants was late. In the future, days to two fruits set could be a possible way to eliminate this problem. Further, the P1 and P2 lines were not isogenic, and thus, it is possible that segregation of modifying genes affected flowering time to a minor extent.

There were clear differences between the flowering patterns of the two parent lines (Table 1). Most P1 plants initiated male and female flowering at earlier dates and at lower node numbers than P2. In 2017 and 2019, P1 male and female flowers initiated earlier and at lower node numbers than P2. In 2018, the node number of first male anthesis did not differ between P1 and P2 but days to male and female anthesis and node number of first female anthesis were all greater in P2.

Female flowering in the F_1 generation occurred simultaneously and at similar node numbers as P1 during 2017 and 2018. During 2019, F1 flowering was delayed compared to P1. In all three seasons, F1 progeny produced female flowers earlier and at lower node numbers than P2. Male flowering in F1 progeny was intermediate between P1 and P2 during 2017 while in 2019, F1 male flowering occurred simultaneously with P1, both earlier and at lower node numbers than P2. In 2018, F1 progeny produced male flowers at similar node numbers as P1 and P2 while days to male anthesis was delayed slightly (1 day) compared to P1 and more closely resembled P2.

Frequency distributions for female flowering patterns among parental genotypes for years 2017, 2018, and 2019 are depicted in Figures 1, 2, and 3, respectively. The clear difference between parent lines in the two response variables, days to first female flower reaching anthesis and node number of first female flower tor each anthesis, is shown in all figures. Frequency distributions of F_1 progeny also closely resembled those of the early flowering parent, P1, indicative of dominant gene action. In 2019 only, female flowering in the F_1 progeny occurred at an intermediate state between the two parents for both response variables. This is suggestive that partial dominance of the early flowering trait can occur under some environmental conditions.

Expected ratios for a single dominant gene conferring early flowering were met in both parent lines, F_1 progeny, and backcrosses to the early parent, BCP1, during all three years. Progeny of the F_1 backcrossed to each of the two parent lines initiated female flowering in two distinct patterns as depicted in Figures 1, 2, and 3. The backcross to P1, BCP1, flowered uniformly early during all three seasons. Also, in all three seasons, the backcross to the late parent P2, BCP2, segregated into two flowering groups, late and early. Individuals within the F_2 and backcross populations to the late parent, BCP2 and BCP2R, were classified as either late or early flowering based on previously described parameters. Only female flowering was used to classify plants as early or late because it was the most consistent flowering variable among genotypes across years. Early flowering plants showed both earlier male and female flowering although the difference was more pronounced in the female flowering pattern.

In 2017 and 2019, backcrosses to the late parent, BCP2 segregated according to expected ratios. In 2018 there was a lack of late flowering segregants among the BCP2 population and the F_2 population (Figure 3) and data failed to confirm the hypothesis that early flowering is caused by a single dominant gene during that season (Table 2). ANOVA analysis from 2018 revealed that P1 flowered earlier that season than the other two years, indicating a possible influence from environmental factors. Also, in 2018, 29% of the late flowering parent, P2, produced a single early female flower as mentioned previously. If a similar phenomenon occurred in the segregating populations, this could help to explain the large number of early flowering segregants observed that year. The frequency distributions for those two populations during 2018 (Figure 2) reveal that although there was a higher proportion of early flowering plants, segregation into two distinct groups of early and late flowering did still occur.

The hypothesis that cytoplasmic factors could have contributed to the observed results was tested in 2019. Data from reciprocal backcrosses to the late parent (P2) were compared during 2019 using t tests and revealed that female flowering patterns did not differ between the two populations (data not shown). This reduces the probability that significant cytoplasmic factors affected the observed female flowering patterns.

Based on the preceding analyses of variance, frequency distributions, and inheritance data, we propose the naming of a new gene for acorn squash, *Cucurbita pepo* subsp. *ovifera,* as *Ef* for early flowering. Genotypes of acorn squash carrying the *Ef* allele typically produce mature female flowers approximately 14-18 days earlier and at node numbers approximately 7-11 nodes lower than genotypes homozygous for the recessive allele, *ef*. Male flowers of genotypes carrying the *Ef* allele may mature slightly earlier and at lower node numbers than genotypes homozygous for *ef* but the difference is small, as acorn squash tends to produce male flowers at low node numbers and often earlier than female flowers.

This finding should be useful for breeders aiming to breed acorn squash with varied maturation times. Future studies could include genomics research to map *Ef* on existing genetic linkage maps of *C. pepo.* The clear segregation into early and late flowering groups could enable approaches such as bulk segregant analysis for mapping purposes, and later, metabolomics research to help identify specific substances associated with the early versus late flowering responses.

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Table 1. Node number and days to male and female anthesis among parental lines and F1 hybrids of acorn squash, *Cucurbita pepo*. subsp. *ovifera,* grown and evaluated in field experiments conducted at the Kingman Research Farm in Madbury, NH.

Genotype[z]	Node number first male anthesis	Days after transplant to male anthesis	Node number first female anthesis	Days after transplant to female anthesis
2017				
P1	1.7 a[y]	24.7 a	10.4 a	32.6 a
P2	5.3 c	32.8 c	17.7 b	47.9 b
F_1	3.0 b	28.7 b	11.3 a	32.6 a
2018				
P1	1.5 a	26.3 a	4.3 a	24.4 a
P2	1.7 a	27.5 b	15.3 b	42.5 b
F_1	1.5 a	27.2 b	4.7 a	25.1 a
2019				
P1	2.2 a	22.6 a	12.5 a	29.2 a
P2	5.4 c	29.5 c	22.2 c	43.6 c
F_1	3.2 b	23.6 b	15.0 b	33.1 b

[z] P1 is an early flowering inbred bush breeding line designated NH27-10-5-10; P2 is a late flowering inbred bush breeding line designated NH8-17-12-7; the F1 is the cross of P1 x P2.
[y] Within a column, means followed by the same letter are not significantly different at $P \leq 0.05$ using Tukey's honestly significant difference test.

Table 2. Inheritance of the early flowering gene (*Ef*) in acorn squash, *Cucurbita pepo* subsp. *ovifera* grown at Kingman Farm in Madbury, New Hampshire during 2017, 2018 and 2019.

		2017		2018		2019	
Genotype	Expected ratio (early:late)	Number of plants (early:late)	χ^2 (p)	Number of plants (early:late)	χ^2 (p)	Number of plants (early:late)	χ^2 (p)
P1 (early)	1:0	32:0	1	30:0	1	24:0	1
P2 (late)	0:1	0:29	1	9:22	0.11	1:24	0.84
F_1	1:0	27:5	0.38	31:1	0.86	20:1	0.83
BCP1	1:0	74:5	0.57	76:0	1.00	32:0	1.00
BCP2	1:1	43:37	0.5	NA	NA	44:37	0.44
BCP2 R	1:1	NA	NA	59:16	<0.0001	50:33	0.06
F_2	3:1	NA	NA	160:24	<0.0001	NA	NA

[z] All genotypes are acorn squash breeding lines and offspring generated at the Loy cucurbit breeding laboratory at the University of New Hampshire. P1 is an early flowering line designated NH 8-27-15-5-10 while P2 is a late flowering line designated NH 8-17-12-7. F_1 is the cross of P1 X P2. BCP1 is P1 X (P1 X P2) and BCP2 is P2 X (P1 X P2). BCP2R is the reciprocal backcross (P1 X P2) X P2 and F2 is the F1 cross P1 X P2 which was then self-pollinated. F_2 population was grown only in 2018 and BCP2R population was grown in 2018 and 2019.

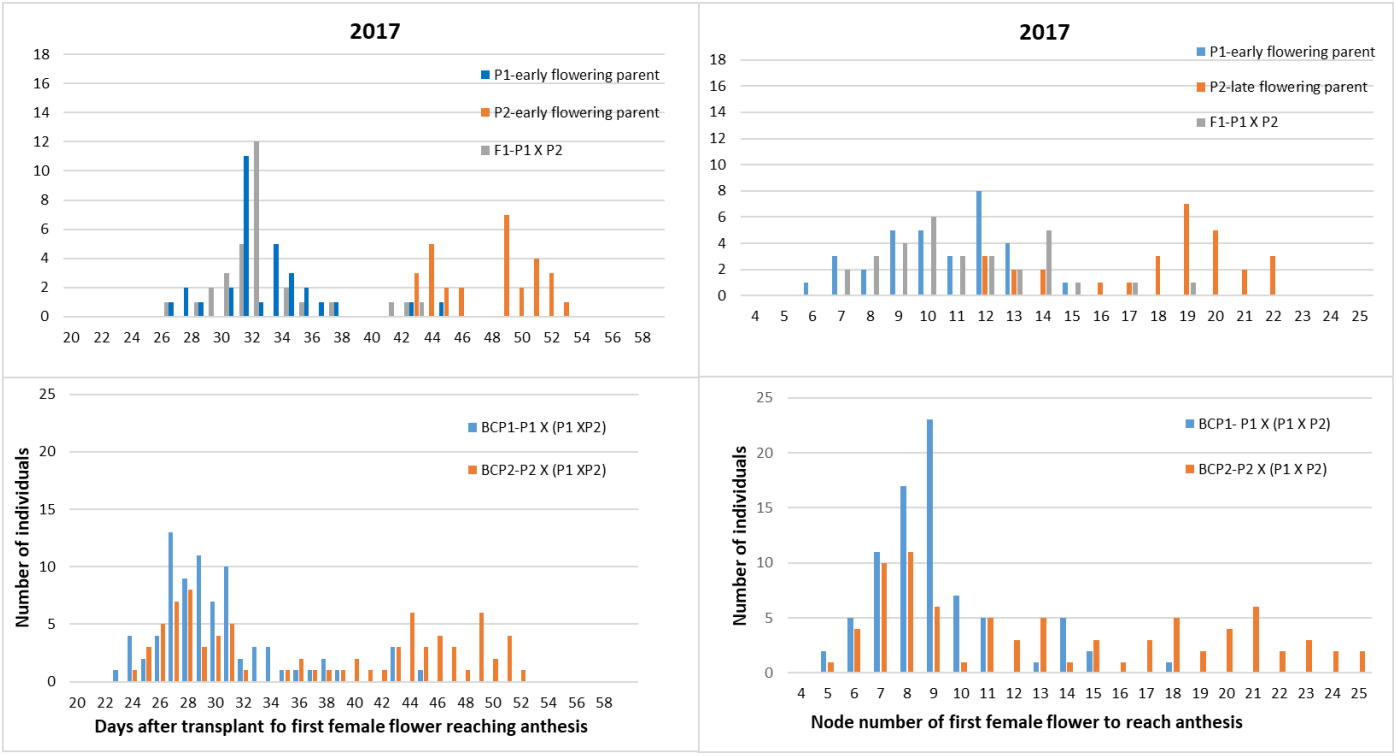

Figure 1. Frequency distributions of days after transplant to first female flower and node number of first female flower among acorn squash, *Cucurbita pepo* subsp. *ovifera,* breeding lines and F1 offspring. P1 is an early flowering line designated NH 8-27-15-10 while P2 is a late flowering line designated NH 8-17-12-7. F1 is the cross of P1 X P2. All backcross generations are derived from crossing the F1 with each of the two parent lines. All plants were grown under field conditions at Kingman Farm at the University of New Hampshire and the New Hampshire Agriculture Experiment Station in Madbury NH during 2017.

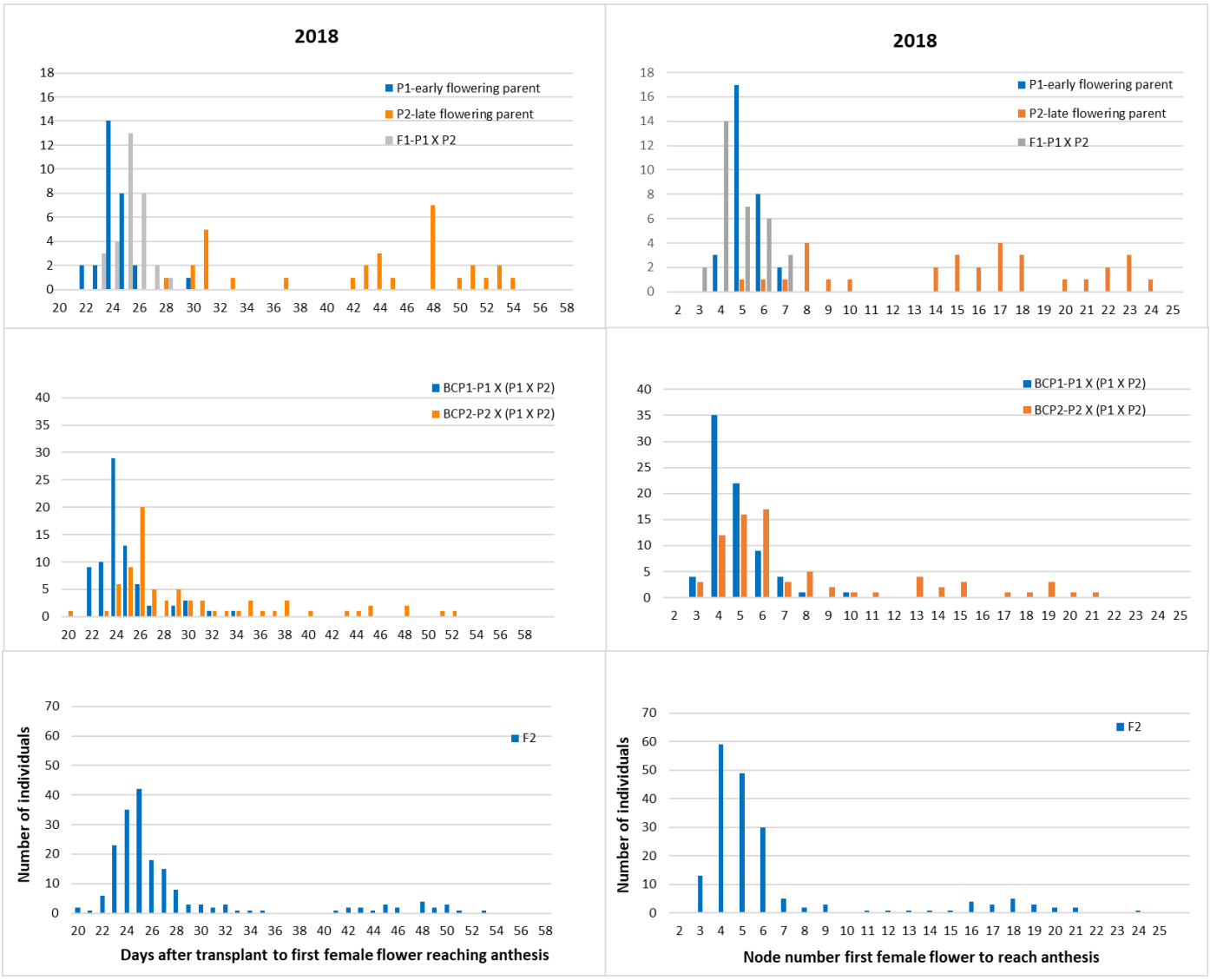

Figure 2. Frequency distributions of days after transplant to first female flower and node number of first female flower among acorn squash, *Cucurbita pepo* subsp. *ovifera,* breeding lines and F1 offspring. P1 is an early flowering line designated NH 8-27-15-10 while P2 is a late flowering line designated NH 8-17-12-7. F1 is the cross of P1 X P2. All backcross generations are derived from crossing the F1 with each of the two parent lines. The F2 is a self-pollinated selection derived from the F1 cross of P1 x P2. All plants were grown under field conditions at Kingman Farm at the University of New Hampshire and the New Hampshire Agriculture Experiment Station in Madbury NH during 2018.

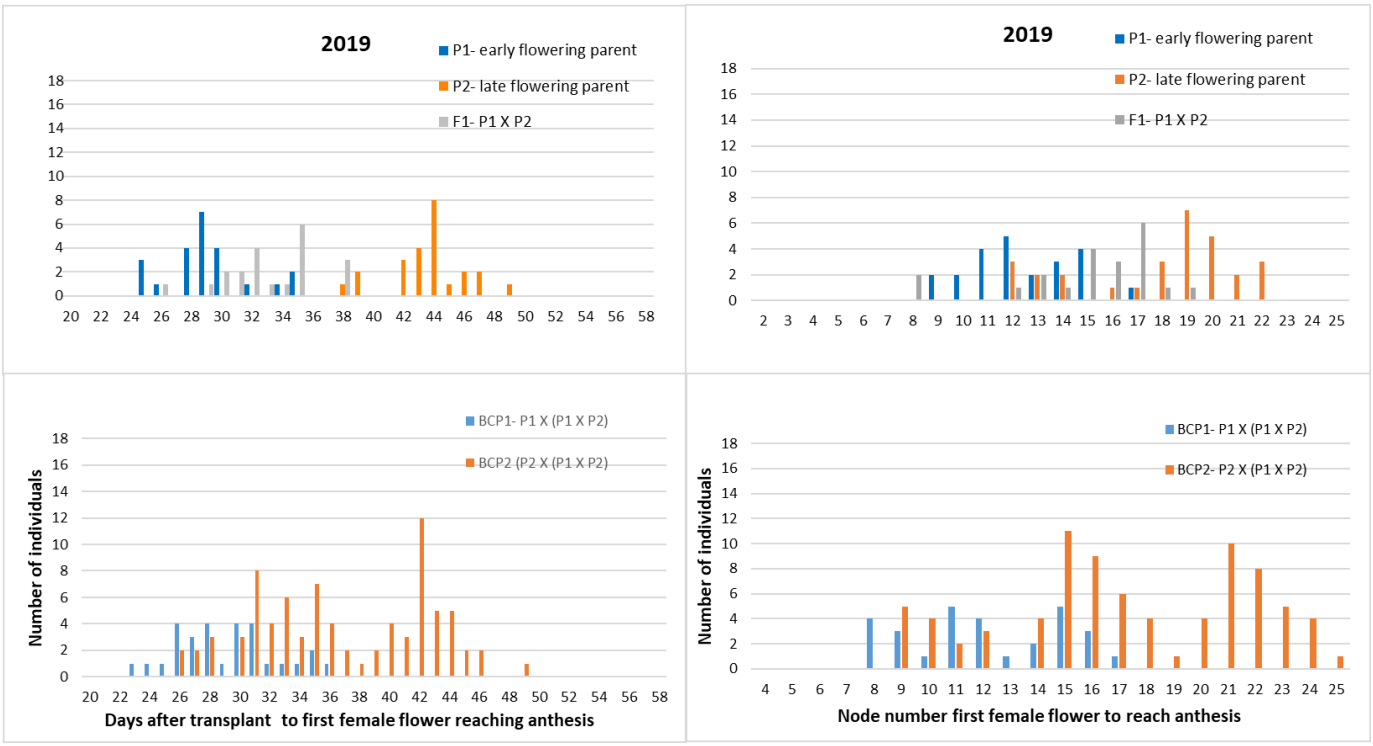

Figure 3. Frequency distributions of days after transplant to first female flower and node number of first female flower among acorn squash, *Cucurbita pepo* subsp. *ovifera,* breeding lines and F1 offspring. P1 is an early flowering line designated NH 8-27-15-10 while P2 is a late flowering line designated NH 8-17-12-7. F1 is the cross of P1 X P2. All backcross generations are derived from crossing the F1 with each of the two parent lines. All plants were grown under field conditions at Kingman Farm at the University of New Hampshire and the New Hampshire Agriculture Experiment Station in Madbury NH during 2019