

Hybridization and pollinator behavior in *Castilleja* (Orobanchaceae)

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Abstract

Hybridization between interspecific angiosperms is common, yet the initial stages are rare (REF- is this even true????). One of the factors that affects the rate of hybridization is the behavior of pollinators responsible for transferring pollen and fertilizing the flowers – only for some plants. Pollinator behavior can facilitate assortative or disassortative mating. We studied pollinator behavior using artificial floral arrays to see if the presence of hybrids in an area changed – we actually did not look at this since we didn't do arrays w/o hybrids- the patterns of movement and proportion of time spent on certain flowers. We also performed hand-crossings to study hybrid fertility. Hybrids set fruit in combination with parent species and other hybrids. Pollinators generally lacked constancy in areas with hybrids- don't really have a basis for this since only did it in areas with hybrids-. Disassortative pollen transfer could result in the production of hybrid offspring (do you mean later generations?), increasing the rate of hybridization in the area.

Introduction

Hybridization is common among angiosperms and well studied by scientists seeking to understand speciation, fitness, and plant-animal interactions (Barton and Hewitt DATE). For plant populations, hybridization can be an important source of genetic variation, creating advantageous adaptive traits if repeated backcrossing with parents occurs (Anderson 1949). However, the initial stages of interspecific hybridization can be a rare occurrence (Hersch and Roy, in press; Wesselingh and Arnold, 2000), and because of this, it is especially curious why we see an abundance of

hybrids exist. ??? One aspect affecting this apparent “explosion” of hybrids is the way that pollinators’ behavior controls the movement of pollen. Plants that depend on animals to transfer pollen for fertilization are subject to selection from pollinator choices. One might suppose that since initial hybridization is rare, (this is not generally true it depends on the system!!) we would only see a few scattered patches of hybrids. But the rate of hybridization is not dependant alone on infrequent F-1 crosses. In order to explain hybrid affluence we must look at mechanisms of selection that affect hybrids, including abiotic, biotic, and genetic, as well as how hybrids may influence their own proliferation – sentence not clear. Could the presence of hybrids break down reproductive barriers set in place by pollinator behavior? The goal of this study was to determine if the presence of hybrids affects pollinator behavior in such a way that increases the frequency and advancement of hybridization among three species of Indian paintbrush (*Castilleja* spp.).

For animal pollinated plants, the frequency of hybridization can depend in part on how often and how well pollinators contribute to interspecific pollen transfer (Campbell et al., 2002). The movement of pollen and selection for certain traits or species is determined in part by the flower constancy and preference of pollinators (Waser, 1986; Melendez-Ackerman et. al, 1997). Flower constancy is the pollinator tendency to continue visiting one type of flower in sequence, even when other equitable options exist (Waser, 1986). By observing constancy, we can deduce the way pollen is moving between plant species in an area. Preference refers to a bias in pollinators to over-visit one species or phenotype more than other types in the area relative to their density (Cock, 1978). For example, if a pollinator shows a strong constancy pattern, but lacks preference, it will start visiting one type of flower and then continue visiting it for a

while, but will switch flower types enough that it will not visit one more than another overall. A pollinator exhibiting a lack of constancy but a strong preference may be hard to identify because it will stay relatively constant on the flower it prefers; however, it could visit less-preferred flowers randomly in its foraging sequence. In the absence of preference and constancy, pollinators will move randomly between plant types, increasing interspecific pollen transfer. If pollinators exhibit strong preferences for types of flowers, they can act as a selective force for certain traits or for creating or maintaining isolation between species or types (Campbell, 1997; Waser and Price, 1981). If pollinators lack strong selection tendencies (such as constancy and preference) for particular species or phenotypes, the likelihood of interspecific hybridization increases, and the subsequent mating with F1 hybrids could develop further hybrid generations (Wesselingh and Arnold, 2000).

Pollination studies using experimental floral arrays with mixed hybrids and parental species often observe an absence or reduction of constancy, sometimes to the point where a previously observed preference for a particular species was eliminated (Melendez-Ackerman et al., 1997; Leebens-Mack and Milligan, 1998; Wesselingh and Arnold, 2000). **THE INTRO SEEMS VERY ROUGH AND CIRCULAR – STICK WITH A POINT AND THEN MOVE TO ANOTHER TO BUILD A STRONG ARGUMENT FOR YOUR STUDY.**

Within the genus *Castilleja* **(DO YOU KNOW ABOUT THE WHOLE GENUS? NO ONLY 3 SPECIES IN A SPECIFIC AREA)**, different pollinator groups generally prefer different species, and tend to continue visiting those species in sequence, even in

mixed-species patches. Overall constancy (using experimental arrays with only parent species) is stronger in areas where the surrounding community lacks hybrids, and decreases in areas which include natural hybrids (Hersch and Roy, in press). Thus, the presence of hybrids within a *Castilleja* community may facilitate increased hybridization by reducing floral constancy of pollinators. However, Hersch and Roy (in press) only presented pollinators with a choice of parental species in the arrays, so constancy including hybrids was not tested. They looked at the formation of F-1 hybrids in the *Castilleja* system, but did not look at backcrossing or hybridization rates beyond the initial stages. In our study, we tested the influence of hybrids on pollinator behavior by including in our arrays hybrids and all parental species growing in the vicinity.

Inconstancy, as well as lack of preference, may only facilitate hybridization if pollen is transferred between plants that are reproductively compatible. It is necessary to assess the fertility and compatibility of both hybrids and all hybridizing species to determine what patterns of gene flow are possible in the population. *Castilleja* are self-incompatible, but easily produce F1 hybrids when crossed with another species (Hersch, unpublished manuscript). However, the fertility of hybrids and the potential for hybrid formation beyond the F1 generation, as well as backcrossing and introgression, is unknown in this system. **WHAT SYSTEM!!!!**

It is possible that once F1 hybrids of animal pollinated flowers are formed, the activity of pollinators increases crossing and accelerates the likelihood of subsequent generations of hybrids, forming a local hybrid “hot spot” (Hersch and Roy, in press). To test the hypothesis that hybrids affect pollinator behavior to increase pollen movement

between parental species, furthering hybridization, we asked three questions. 1) Do pollinators exhibit tendencies to visit one type of flower in sequence, in the presence of hybrids and parental species? 2) Do pollinators choose to visit hybrids more often than parental species? 3) How fertile are naturally occurring hybrids? Can they mate with themselves (self), other hybrids (outcross), and parent species (backcross)?

I predicted that pollinators would not visit hybrids more often than the parental species, but they would transition frequently between different species (we would see a lack of constancy). Because none of the parental species are self-compatible, I predicted that hybrids would be self-incompatible. However, the abundance and variation of hybrids in the community, as well as similar studies with other plant species, lead me to predict that hybrids would easily set fruit with all parental species and each other.

Study system

We investigated hybridization **AMONG** in three species of Indian paintbrush *Castilleja* (Orobanchaceae), *C. miniata*, *C. sulphurea*, and *C. rhexifolia*. These three species often co-occur and hybridize prolifically in meadows and open forests of the Gothic area of Gunnison County, Colorado. The showy racemes of this hemi-parasitic perennial forb attract many types of pollinators including hummingbirds, flies, hover flies, and bumblebees (Hersch and Roy, in press).

Because a previous study investigated patterns of constancy and preference among the three species in exclusion of hybrids, but in sites of varying species composition (Hersch and Roy, in press), this study did not attempt to duplicate their

findings. Instead, under similar methods and localities, we will look at pollinator behavior in relation to hybrids and parental species, and compared our results with the general knowledge previously gained about the system. IT IS NOT REALLY GENERAL KNOWLEDGE?

Methods and Materials

1) Do pollinators exhibit tendencies to visit one type of flower in sequence, in the presence of hybrids and parental species?

2) Do pollinators choose to visit hybrids more often than parental species?

We observed pollinator behavior using experimental floral arrays in two sites naturally occurring sites of *Castilleja* in wet subalpine meadows in the Copper Creek river basin near the Rocky Mountain Biological Laboratory, Gothic, Colorado. Site one was located about two thirds of the way up the four mile trail leading to Copper Lake. The immediate site consisted of naturally occurring hybrids and *C. sulphurea*; *C. miniata* grew in the vicinity but not the immediate site. Site two was at Copper Lake, and nearby at East Maroon Pass, where all three species and hybrids co-occur. Array design differed at the two sites to consciously reflect the differing *Castilleja* community compositions. Both sites consisted of arrays with 12 total plants, hand picked and placed in small plastic floral vases in a 4x4 grid (lacking the corner positions; see Figure 1). We watched four arrays simultaneously for a period of 40 minutes per replicate. After each replicate, individual flower position and array design position were randomly re-assigned. Replicates were repeated 1-4 times per day, weather allowing. We recorded data on

voluntary pollinator visits to flowers within an array. We recorded pollinator group (fly, bumblebee, syrphid fly, “other”), and species if possible; flower species; number of flowers per raceme **VISITED**; length of time per raceme **VISIT** while in contact with sexual parts; and the sequence of movements from the time a pollinator entered an array to its departure. The study took place three or four days a week during the month of July and the first week of August, while *Castilleja* was flowering and pollinators were active. **WE NEVER DID IT 4 DAYS – SENTENCES NEED PROOF READING THEY ARE RUN-ONS.**

At site one, arrays consisted of three plant types and four stems each, *C. sulphurea*, *C. miniata*, and hybrids (see Figure 1). We began the study at site two using the same array design as site one, but replacing *C. miniata* with *C. rhexifolia*. However, we switched designs in response to our discovery of a large patch of *C. miniata* near the lake. New arrays contained three stems each of all four plant types, *C. sulphurea*, *C. miniata*, *C. rhexifolia* and hybrids, still totaling twelve. Data was analyzed separately for all the designs. I will refer to the design with three flower at the lake “Copper Lake”, since most of those replicates were done at the lake, and the design with all four flower types “Maroon Pass,” since all of that design was done at the pass. We switched from the lake to the pass because of superior pollination at the pass (more visits and more bumblebees), and a higher density of *Castilleja* compared to the lake site.

Since hybrids are naturally extremely variable, we picked those representing a range of phenotypes. Though hybrids do not comprise a species, for the remainder of the

paper I refer to them as such when in combination with the parental species for simplicity's sake.

YOU SHOULD TALK ABOUT CONSTANCY FIRST SINCE THAT WAS HOW YOU LAID IT OUT IN THE BEGINNING OR YOU SHOULD ATLEAST BE CONSISTENT. To analyze whether pollinators have different preferences for particular species, I used a two-way ANOVA on the mean number of raceme visits in a replicate with the factors of plant species, pollinator group, and their interaction terms. Pollinator groups were flies, bumblebees, hover flies, hummingbirds, and other. However, we did not **received** any hummingbird visits this year and consequently dropped that category **why was it a category then?** I ran separate analyses for each site because the array designs differed.

I used G-tests of independence to analyze constancy patterns. Each G-test assessed the independence of movements by one pollinator group between two *Castilleja* species for each site. G-tests were performed for all combinations of plant species, when possible. I used the number of transfers between two particular racemes per pollinator group per site, independent of individual pollinator foraging sequences. **NOT EXACTLY CLEAR – WHAT DO YOU MEAN BY WHEN POSSIBLE ALSO LAST PART IS CONFUSING.**

3) How fertile are naturally occurring hybrids? Can they mate with themselves (self), other hybrids (outcross), and parent species (backcross)?

We performed hand pollination to address hybrid fertility and crossing potential. We applied pollen to 120 “virgin” stigmas in eight different treatments, first emasculating them using sterilized tweezers. Pollinated flowers were marked with color coded nail polish to specify the pollen donor type; the raceme was then bagged to prevent visitation. Treatments of hybrids to all parental species, all parental species to hybrids, hybrids with themselves, and hybrids with each other (see Figure 2) were spread between our sites along an elevational gradient. Three to six weeks after pollinating I collected data on presence or absence of fruit set. I used an ordinal logistic analysis (based on the Chi-square) in order to see if all three species and hybrids are equally fit as pollen donors and recipients. Seed set may be a more accurate way to assess relative fitness, but we chose fruit set on account of time and resource constraints. The proportion of fruit set will tell us simply if a cross between two particular types is probable.

Results

1) Do pollinators exhibit tendencies to visit one type of flower in sequence, in the presence of hybrids and parental species?

Maroon Pass site—Preference. Bumblebees visited a total of 71 racemes, flies visited 109, syrphids visited 96 and “other”, 29. Pollinator visits did not significantly differ from one another in number ($P=0.1839$). However, plants received significantly different visitations from each other ($P<0.0001$). Based on tukey tests, *C. rhexifolia* and *C. sulphurea* received the most visits and were not significantly different from each other. Hybrids and *C. sulphurea* did not differ from each other in average raceme visits, but hybrids got significantly less visits than *C. rhexifolia*. *Castilleja miniata* had the

fewest visits overall, but did not differ significantly from *C. sulphurea*. Pollinators did show preference patterns for the plant species they visited, because raceme visits by pollinator group depended on the plant ($P=0.0015$). Using Tukey tests, bumblebees preferred hybrids and *C. rhexifolia* over *C. miniata* and *C. sulphurea*. Syrphids preferred *C. rhexifolia* and *C. sulphurea* significantly over hybrids, and hybrids over *C. miniata*. Flies preferred *C. rhexifolia* and *C. sulphurea* overall, but hybrids were not distinguishable from *C. sulphurea*; *C. miniata* was least preferred, but not distinguishable from hybrids. Residuals were normal. **FIGURE WOULD BE NICE!!!!**

Copper Lake site—Preference. We observed 10 bumblebee visits, 65 fly visits, 59 syrphid fly visits, and 2 “other” visits. *Castilleja rhexifolia* received 66 visits to 102 flowers, *C. sulphurea* received 46 visits to 62 flowers, and hybrids received 24 visits to 33 flowers; however, none of these numbers were significantly different from each other. Our sample size was too small to get any conclusive results at this site

Copper Creek site—Preference. I excluded *C. miniata* because it looked like it was driving the analysis. Flies preferred *C. sulphurea* over hybrids. No other pollinator group showed preference that significantly differed between plant types. There was a significant difference in raceme visits by plant type with *C. miniata* in the model, but the interaction was insignificant without *C. miniata*. Significantly more flies visited than any other pollinator. Plant and pollinator interaction was significant (without *C. miniata*, $P=0.0003$; with *C. miniata* $P<0.0001$). **THIS SECTION IS VERY UNCLEAR. NEED FIGURES OR TABLES AND BETTER WORDING!!**

2) Do pollinators choose to visit hybrids more often than parental species?

Maroon Pass site—Constancy. *C. miniata* never received enough visits and transfers to perform G-tests on. Results are given where there were enough transitions made between two types to calculate G-tests and I use the first letter of the plant specific epithet to represent that species (for example, *C. sulphurea* is represented by S and hybrid by H). Flies—Out of a total of 20 transitions observed, 65% were interspecific.

Transitions between H/R were dependent ($G=7.23$, $P=0.01$) **WHAT????**.

Bumblebees—We observed 36 transitions, 61% were interspecific, tests revealed no dependence between any plant species (S/H: $G=0.05$, $P=0.80$; H/R: $G=0.87$, $P=0.35$; S/R: $G=0.96$, $P=0.33$). Syrphid flies—Total transitions equaled 31, 58% interspecific. H/R interaction was independent ($G=0.96$, $P=0.33$) where R/S interaction was dependent ($G=197.02$, $P<.0001$). (See Figure 3 and Table 1)

Copper Lake site—Constancy. Flies and syrphid flies were the only species abundant enough to calculate G-tests for. In all instances calculated, these two pollinator groups showed a lack of constancy (S/H: flies $G=0.037$, $P=0.85$; syrphid flies $G=0.4$, $P=0.53$; R/S: flies $G=0.78$, $P=0.38$; syrphid flies $G=0.084$, $P=0.77$; H/R: flies $G=0.096$, $P=0.76$).

Copper Creek site—Constancy. Bumblebees between S/H showed a lack of constancy ($G=0.02$, $P=0.88$). Flies were also inconstant between S/H ($G=1.62$, $P=0.2$). Other interactions did not have enough transitions to calculate. (Constancy results see Table 2.)

3) *How fertile are naturally occurring hybrids? Can they mate with themselves (self), other hybrids (outcross), and parent species (backcross)?*

Out of 120 crosses, 112 were identifiable as either having set fruit or not. Most cross types were at least partially successful at setting fruit, with the exception of hybrids with themselves (selfing) (see Figure 4 and Table 4). **TELL US WHAT YOU FOUND.**

Discussion

The data reveals generally inconstant behavior by pollinator groups. Pollinators may indeed be facilitating the transfer of pollen between different species of *Castilleja* in the hybrid zone. The proportion of viable fruit set for all possible described mating combinations supports the possibility of hybrid proliferation because it implies that pollen transfer can result in subsequent generations of hybrids. Like I expected, *Castilleja* hybrids look to be mostly self-incompatible, similar to parental species. It is interesting to note that *C. rhexifolia* and hybrids may be experiencing asymmetrical gene flow because the proportion of fruit set for hybrid recipients and *rhexifolia* donors was 100%, while the reverse cross was 44%. This result could be due to random chance within the system, or due to mistaken *C. rhexifolia* identity if there are hybrid phenotypes that look extremely similar to *C. rhexifolia*. In general, hybrids do not appear to be more or less fit as pollen donors and recipients. It appears that genes can circulate readily throughout the population, which supports the idea of prolific hybridization, and a potential for a high rate of new genetic combinations. Backcrossing is common in species of *Ipomopsis* and their naturally occurring hybrids (Campbell et al., 2002). This could be a result of pollinator activity, if they tend to move pollen effectively between hybrids and their parents.

The implications of backcrossing could be providing the parental species with a selective boost of new genes (Anderson 1949).

Hybrids sometimes suffer from reduced fitness due to their intermediate phenotypic traits, which pollinators may select against if exhibiting strong preferences for certain parent species (Campbell, 2003). Pollinators in general did not show a stronger or weaker preference for hybrids over the other species, but they did retain some preference patterns. Those preference patterns varied between pollinator groups. More study is needed to determine hybrid fitness within this system.

Hybrids of *Baptisia* (Fabaceae) in experimental arrays with parental species have acted as “bridges” that increase pollen movement between parent species, facilitated by pollinators. In arrays with only parental species, pollinators tended to transition more often between plants of the same species while foraging; when hybrids were added into the arrays, they transitioned less often between members of the same species (Leebens-Mack and Milligan 1998). It appears that the presence of hybrids in this system also decreases pollinator constancy. We observed a high number of between species transfers as compared to previous conclusions based on flights between only parental species (hersch and Roy, in press). This could mean that hybrid areas are indeed facilitating increased pollen movement and an increased rate of hybridization. However, more study is needed with larger sample sizes to discern with certainty the patterns we observed in the field.

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Fig 1: Array designs, Copper Creek site. The four experimental array designs of cut floral racemes. M=*C. miniata*; S=*C. sulphurea*; H=hybrid. Flower stems were placed four inches apart in plastic floral vases; Array position was randomized, placed 7 meters apart in a square.



Fig 2: Crossing study design. All eight treatments were replicated 15 times.

Pollen donor→Pollen recipient

1. Hybrid→*C. miniata*
2. Hybrid→*C. sulphurea*
3. Hybrid→*C. rhexifolia*
4. Hybrid→Hybrid
5. Hybrid→Self
6. *C. miniata*→Hybrid
7. *C. sulphurea*→Hybrid
8. *C. rhexifolia*→Hybrid

Figure 3: Preference for Maroon Pass. Shows least square mean per replicate of raceme visits by pollinator group (see Table 1).

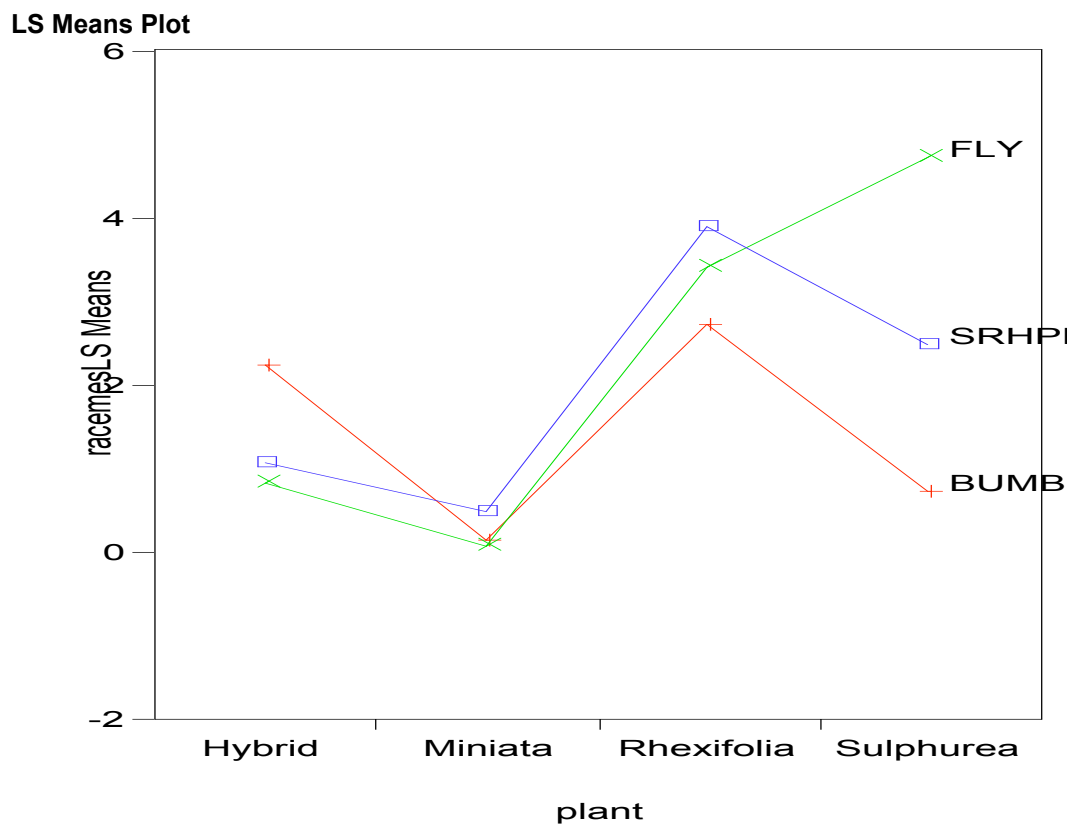


Table 1. LSMeans Differences Tukey HSD, Maroon Pass site. Shows relationship between average visits by pollinator group to plant species (see Figure 3). Levels not connected by same letter are significantly different.

Alpha=0.050

Level		Least Sq Mean
FLY,Sulphurea	A	4.7500000
SRHPHID,Rhexifolia	A B	3.9166667
FLY,Rhexifolia	A B C	3.4166667
BUMBLEBEE,Rhexifolia	A B C D	2.7500000
SRHPHID,Sulphurea	A B C D	2.5000000
BUMBLEBEE,Hybrid	A B C D	2.2500000
SRHPHID,Hybrid	B C D	1.0833333
FLY,Hybrid	C D	0.8333333
BUMBLEBEE,Sulphurea	C D	0.7500000
SRHPHID,Miniata	D	0.5000000
BUMBLEBEE,Miniata	D	0.1666667
FLY,Miniata	D	0.0833333

Table 2: Constancy patterns for pollinator groups across sites. Shows results of G-tests and P-values to determine independence of pollinator movements between racemes of certain Castillja species (S=C. sulphurea, H=hybrid, R=. rhexifolia).

	Bumblebees			Flies			Syrphid flies	
Site	E.M.	Cop. Cr		E.M.	Cop. Cr	Cop.	E.M. Pass	Cop.
	Pass			Pass		Lake		Lake
Total Trans.	36	48		20	88	24	31	28
% Btwn. Spp.	0.61	0.52		0.65	0.52	0.58	0.58	0.61
Trans.								
G value	0.05	0.02	*		1.62	0.037	*	0.4
(S/H)								
P	0.80	0.88	*		0.2	0.85	*	0.53

Figure 4: Frequency of fruit set. Bars represent proportion of successful fruits out of total crosses. The first letter represents the pollen recipient, the second is the pollen donor.

(See Table

4)

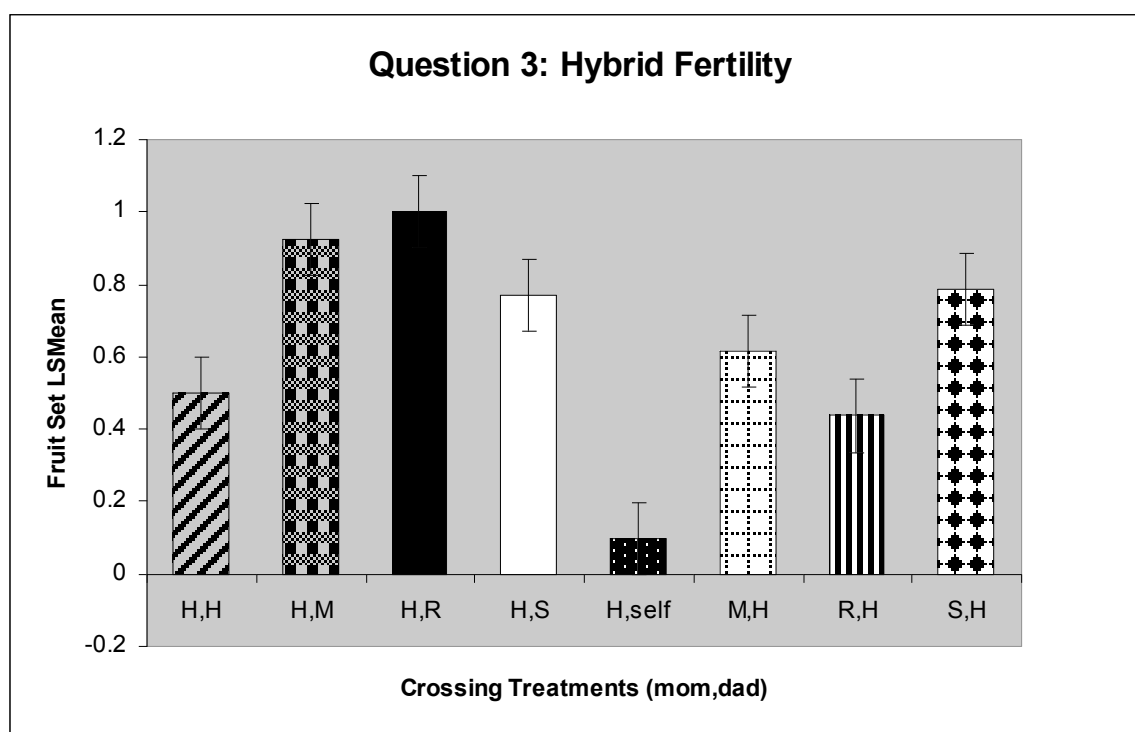


Table 4. Tukey similarity for the frequencies of fruit set. Crosses with the same letters are indistinguishable statistically from each other. If groups lack a common letter, they are significantly different (see Figure 4).

H,H	A,B,C
H,M	A,C
H,R	C
H,S	A,C
H,SELF	B
M,H	A,B,C
R,H	A,B
S,H	A,C