On The Evolution of Distyly and Morph Ratio Distortion in *Lithospermum Caroliniense*

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Abstract

Two conflicting models presently exist describing the evolutionary history of *heterostyly*, a condition in which there presents two *morphs*-- different plant sexual organ structure that some individuals within the same species exhibit as a means of promoting outcrossing. The charlesworth model (1979) and the Lloyd-Webb model (1992) differ in that the latter assumes the ancestral character state was one of approach herkogamy, whereas the former assumes it was homostyly. One such heterostylic species, *Lithospermum caroliniense* (Walt.) MacMill. (Boraginaceae), is unique in that it additionally exhibits morph ratio distortion, that is, in natural populations, one morph is disproportionately represented over the other morph, which is of particular interest because inverse frequency dependent selection should presumably shape a 1:1 morph ratio. In this paper, we discuss the Levin (1968) model of morph ratio distortion in *L. caroliniense*, and propose the *superthrum* model, in which we hypothesize the existence of *superthrums*, a genetically unique form of the thrum morph that, after 5 generations, produces distortion to the degree recorded by several field studies (Cartmill & Murray 2011; Levin 1968; Levin 1972).

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Introduction

Heterostyly is the differential length of the style within the same plant species. Species that have two style lengths are said to be *distylic*, while species that have three style lengths are said to be *tristylic*. Species that to not exhibit heterostyly are said to be *homostylic*. Darwin first commented upon the different morphologies of flower types in his 1877 book *The different forms of flowers on plants of the same species*, where he used the term 'pin' and 'thrum', the former having stigma above the anthers by means of a longer style, and the latter having stigma below the anthers, by means of a shorter style.

Lithospermum caroliniense is a herbaceous distylic species exhibiting the two floral morphs pin and thrum. Pin morph pollen grains are relatively smaller and more elongate, whereas thrum pollen are relatively larger and more ovicular (Levin 1968). Dimorphism in plant sexual organs is adaptive in that it acts to reduce the prevalence of inbreeding; that is, negative assortative mating is established in plant populations that exhibit heterostyly because pins can only successfully mate with thrums, and thrums can only successfully mate with pins (Darwin 1877; Levin 1968).

L. caroliniense is of particular interest because it displays both chasmogamous and cleistogamous flowers. Chasmogamous flowers are open for pollination and viable for wind pollination, while cleistogamous flowers are closed and have high rates of selfing. The prevalence of cleistogamous flowers, which can be found on pin or thrum individuals, occur relatively later in the season and do not exhibit the stigma and anther

morph of the plant they are on. A 1:1 ratio is expected in distylus species because distyly is a self-incompatibility mechanism that prevents selfing. In other words, it promotes negative assortative mating, and the morph ratio is under the pressure of inverse frequency dependent selection. In *L. caroliniense*, several studies have found that the pin:thrum ratio deviates from the expected 1:1 (Cartmill & Murray 2011;Levin 1968; Levin 1972). The authors will propose a new model for understanding morph ratio distortion in *L. caroliniense* that predicts the existence of *superthrums*, thrums that exhibit dominant homozygosity in distylic-associated genes.

On the Evolution of Distyly in *Lithospermum caroliniense*

Heterostyly has evolved independently at least 23 times (Ganders 1979). While the evolution of heterostyly, specifically distyly, (in which there are two floral morphs), is obscure, there are two prevailing hypotheses regarding the evolution of distyly: The Charlesworth model maintains that self-incompatibility, not stigma height polymorphism, evolved first (Charlesworth & Charlesworth 1979). It posits that because of extensive inbreeding from homostyly, sterile pollen would arise. A subsequent mutation affecting the stigma, both altering its height and making it compatible with the previously sterile pollen, would generate dimorphism, and the dimorphic self-incompatibility system. There would be selective pressure on the degree of distinction between the morphs due to negative assortative mating, thereby further perpetuating and establishing dimorphic self-incompatibility.

Conversely, a model proposed by Lloyd and Webb (1992) suggests that dimorphism occured before self-incompatibility. Inherent in this model is the notion that the ancestral condition was one of herkogamy, which refers to the strategy of spatially separating anthers and stigma in order to reduce the prevalence of selfing, a form of inbreeding which is usually detrimental to a population. This is supported further by additional floral morphological characteristics, including, in species of 22 families that exhibit heterostyly, tubular corollas, an indicator of herkogamy (Webb & Lloyd 1986). If herkogamy, specifically, *approach* herkogamy, where the stigma is above the anthers, was the ancestral condition, then a mutation of this form causing opposite spatial separation, in what is called *reverse* herkogamy, could generate dimorphism, at which point negative assortative mating would act to reinforce the dimorphism.

In either model, it is supposed that the evolution of heterostyly did not occur via the variation or mutation upon stigma or anther height if the ancestral state was one without herkogamy. However, a difficulty in the Lloyd-Webb model (1992), from an evolutionary perspective, is that while it provides an answer and associated mechanism, it also begs further questions about how approach herkogamy could have fixated in ancestral populations. Given that the majority of angiosperms do not exhibit approach herkogamy, and indeed the most parsimonious explanation of heterostyly is one when it evolved independently in each instance, this in effect is pushing the answer further back in evolutionary time. The question of when approach herkogamy first evolved, sufficiently ancient so as to include all instances of present heterostyly, would be less parsimonious than to assume it evolved independently from an ancestral homostyly,

because it would require the evolution of homostyly from an ancestral heterostyly to have occurred more often than if heterostyly evolved from an ancestral homostyly. A phylogeny of heterostyly is necessary to address these questions, and a revision of these models may thusly occur.

 We contend that the Lloyd-Webb model, amid the aforementioned unresolved questions, is still a useful explanation for the evolution of heterostyly because of the additional morphological evidence, namely tubular corollas, that are present in the majority (88%) of families that have representatives of heterostyly, and that are indicators of herkogamy (Webb & Lloyd 1986).

 Evidence readily applicable to hairy (Carolina) Puccoon, *Lithospermum caroliniense*, a herbaceous distylic species found throughout the Midwestern United States, for the evolution of distyly must be considerate of the inherent assumptions that models make, particularly for selective pressure and fixation of heterostylic alleles, when those models use different vectors for pollen dispersal. Indeed, *L. caroliniense* are primarily pollinated by bee and lepidopteran pollinators, not wind (Kerster & Levin 1968).

 A study testing the Lloyd-Webb model by looking at bee pollination patterns between artificially constructed approach, reverse, and thrum herkogamy found that a stigma-height polymorphism could be established in a population because the total pollen transfer between morphs was greater than twice the total pollen transfer between members of the same morph (Stone & Thompson 1994). This indicates that should a mutation arise in stigma height, it could establish due to the differential pollen

deposition it would cause. This offers a pollinator-mediated mechanism that could explain the evolution of heterostyly via the Lloyd-Webb model, and is applicable to *L. caroliniense*.

On Morph Ratio Distortion in *Lithospermum Caroliniense*

Morph ratios operate like sex ratios insofar as they are subjected to the same type of selection-- inverse frequency dependent selection, which acts to maintain a 50:50, or 1:1 ratio by nature of the rarer morph or sex having advantage over the more common morph or sex. However, several studies have shown that in *L. caroliniense*, there is a distortion of the expected 1:1 ratio; thrums tend to represent a higher proportion of the overall population. In Levin (1968), a population of *L. caroliniense* in Zion Illinois consisted of 62% thrums and 38% pins; 12 other populations included in the study consisted of 55 - 67% thrum. In a study in northern lower Michigan, 4 populations were surveyed, and collectively they consisted of 70% thrum and 30% pin (Cartmill & Murray 2011).

There presently exists a model that succeeds in predicting this distortion. Levin (1968) proposes a model in which there are 6 assumptions. These assumptions will be listed and directly quoted to ensure proper representation. (1) thrum is heterozygous, (2) both forms produce 27% fo their seed via cleistogamy, (3) 18% of the thrum seed is from chasmogamic selfing, (4) outcrossing is between pin and thrum, (5) SS genotypes are inviable, and (6) gene frequencies are not altered by selection or drift. Given population structure noted in Levin's study, this model, upon the fifth iteration,

calculates 45.7% thrum and 54.3% pin. Given in the above model that the pin is represented genotypically as *ss*, and the thrum *Ss*, the observed ratio was not obtained. Levin noted that if a single assumption were changed and all else held constant, that the thrum is a homozygote and not a heterozygote, the results after 5 generations were drastically different; the thrum proportion reaches 62.3% and the pin proportion 37.7%.

While this model seems sufficiently explicative of observed ratios, we propose a slightly different hypothesis that supposes that thrums can genotypically present both in the dihybrid and in the dominant homozygous condition. Additionally, using the *S/s* system of discussing the genetics of distyly is misleading because while the two loci controlling stigma and anther height (alleles *G/g,* and *A/a* respectively) are in linkage disequilibrium, the results of selfing are difficult to see using a single letter representation (Futuyma 2013). Therefore, for the duration of this paper, the pin morph will be represented as *ga/ga,* that is, homozygous recessive at both loci, and the thrum morph will be represented as *GA/ga*. Note that individuals that have *Ga* or *Ag* are homostylous, and are very rare in populations of *L. caroliniense*

The *superthrum* model of *L. caroliniense* morph ratio distortion

The thrum is *GgAa* and the pin is *ggaa*. In a single gene cross between genotypes *Gg* and *gg*, the resulting ratio will be 1:1 *Gg:gg*. When a thrum crosses with a pin, the resulting ratio is a 1:1:1:1 ratio between 4 genotypes: *GgAa, Ggaa, ggAa,* and *ggaa*. The first genotype is a thrum, and the fourth genotype is a pin, the middle two genotypes are a homostylic individuals, and due to distyly, these resulting genotypes are very rare. If

the homostylic genotypes are removed from consideration, the resulting ratio is a 1:1 thrum:pin. In this way, there must be some piece missing. This is where the idea of the *superthrum* is useful.

Pins are unable to self cross. In one study, Levin found that pin selfing contributed 0% to the selfed seed set, indicating complete inbred breakdown (1968). In the same study, Levin found that thrum selfing contributed approximately 25% to the selfed seed set (1968). If thrums are able to self cross, understanding the resulting genotypes ratios that can arise is useful. When two thrums cross, a 9:3:3:1 genotype ratio is observed because it is a dihybrid cross (Fig. 1 & 2)

Cross:
GgAa × GgAa

	GA	Ga	gA	ga
GA	GGAA	GGaA	gGAA	gGaA
Ga	GGAa	GGaa	gGAa	gGaa
gA	GgAA	GgaA	ggAA	ggaA
ga	GgAa	Ggaa	ggAa	ggaa

In *figure 1*, the phenotypic results of a thrum self cross are shown. 9/16ths would be a thrum, 6/16ths would be homostylic, and 1/16th would be a pin. The 6/16ths that are homostylic and can be expected to always self, therefore, in any thrum self cross, 9/10ths would be viable thrum, and only 1/10th would be viable pin. Every time a thrum self crosses, it will have a 90% chance

Figure 1[1]: Phenotype ratios of thrum self cross.

to produce a thrum nutlet. Considering several inflorescences per individual, a thrum self cross would contribute disproportionately to the thrum population.

Genotypically, something more interesting happens. While there are still 6/16ths homostyly and 1/16th pin, 1/16th of the 9/16ths thrum will be homozygous dominant for *G and A*, this individual can be found in the top left square in *Fig. 1 & 2*. Notice in *Fig. 2*, it is the only dark red color, indicating that it is the only individual of that genotype. This is the superthrum, *GGAA*. The superthrum represents 1/16ths of the proportion of seeds produced via thrum selfing. When the superthrum is crossed with a pin, all offspring, or in other words, 16/16ths of offspring will be *GgAa,* or a thrum (*Fig. 3*). This distorts the morph ratio because when a *superthrum* mates with a pin, the resulting probability of thrum is 100% versus 0% probability of a pin. This is significantly different than a 50:50 probability when a regular thrum and pin cross. Therefore, as the number of superthrums increases in a population, the distortion effect increases (*Appendage A*).
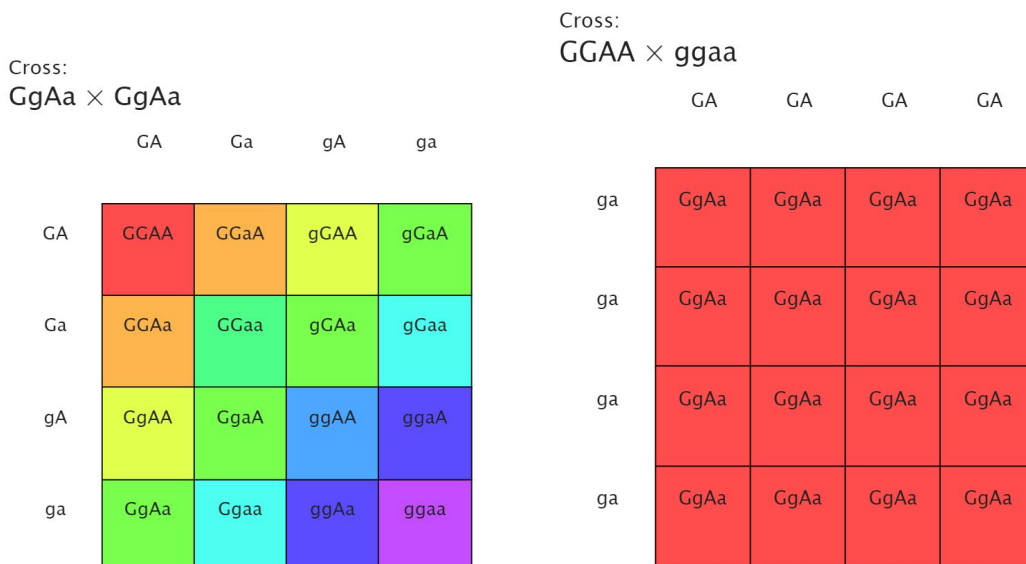
Cross:
GgAa × GgAa

	GA	Ga	gA	ga
GA	GGAA	GGaA	gGAA	gGaA
Ga	GGAa	GGaa	gGAa	gGaa
gA	GgAA	GgaA	ggAA	ggaA
ga	GgAa	Ggaa	ggAa	ggaa

Cross:
GGAA × ggaa

	GA	GA	GA	GA
ga	GgAa	GgAa	GgAa	GgAa
ga	GgAa	GgAa	GgAa	GgAa
ga	GgAa	GgAa	GgAa	GgAa
ga	GgAa	GgAa	GgAa	GgAa

Figure 2[2]: Genotype ratios of thrum self cross. cross

Figure 3[3]: Genotype/phenotype ratios of between *superthrum* and pin.

Discussion

The evolutionary context of heterostyly is not of particular importance to the *superthrum* model; indeed, we chose to discuss it as a means of introducing the unique population genetics and ecology of distylic species. Understanding the evolutionary history of heterostyly is important, nonetheless, because the self-incompatibility system put it place as a result of heterostyly is an adaptation that reduces inbreeding, and therefore the inbreeding depression that afflicts many homostylic plant species. We propose a study looking into the relative fitness benefits derived from having a self-incompatibility system, whether other such systems exist that do not use heterostyly as a means of achieving it, and a phylogeny of heterostyly in angiosperms.

The *superthrum* model is the same from the Levin (1968) model in that both use *SS* or *GGAA* as a means of distorting the morph ratio, but the former uses it as a special case of thrum selfing, whereas the latter assumes all thrums are homozygous dominant. The implications of the *superthrum* model in *L. caroliniense* can be applied to any distlylic species, insofar as it predicts the existence of a special class of 1 of the 2 morphs that acts to distort the ratios of its progeny, and when members of this special class are breeding individuals within a population, they are the cause of the population tending toward more of its morph.

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[2] Image generated using Punnett Square Calculator; scienceprimer.com © Andrew Staroscik 2018.
[3] Image generated using Punnett Square Calculator; scienceprimer.com © Andrew Staroscik 2018.

Superthrums are necessary because without them the distortion functionally limits at a morph ratio significantly different than observed. Without *superthrums*, the thrum morph increased to 56.11% in F3 and 56.12% in F4-- the increase becomes negligible. But once *superthrums* are added to the population by a calculated amount, the proportion of thrum in the F5 generation increases to 58.31 %, with additional iterations further increasing that proportion (see *Appendage A*).

This model can be tested in the field with any distylic species with simple genetic crosses between thrums and pins, assuming the genotypes of the morphs are the same. While revisiting sites included in a study done in 2011 in northern lower Michigan by Cartmill and Murray, we did not observe any unique thrum phenotype that would indicate a *GGAA* genotype. However, a *superthrum* will produce only thrums when crossed with a pin, whereas a thrum will produce a 1:1 ratio of thrums:pins. In our model population, we calculated only 2.089 *superthrums* in the F5 generation, indicating that these individuals are very rare. Therefore, should a study be devised that crosses thrums and pins, large numbers of crosses would be necessary. We propose a study of this fashion in order to test the *superthrum* model. If *superthrums* could be found, subsequent crossing of these individuals with pins to ensure that no pins are created would offer compelling evidence in support of the model.

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Appendage A

A mathematical walkthrough of the *superthrum* model

In order to see the effects of thrum selfing and the *superthrum*, a mathematical model may be useful. Assuming a population of 100 individuals, where 50 are pin and 50 are thrum, and 20% of thrums undergo selfing, there will be 40 crosses between thrum and pin (hereafter abbreviated T and P) individuals, and 10 thrum x thrum selfing events. In the F1 generation, the 40 T x P crosses will yield equal numbers T and P (20 each assuming 1 offspring per cross), while the 10 T x T crosses will generate <1/16th P and 5.125 T. When we total the number of T, we get 20 + 5.125 = 25.625, and when we total the P, we get 20 + approximately 1 from the 1/16th = 21. Now to see the relative proportion of T, we divide T by (T+P), 25.625 / 46.625 = .5496 = 54.96% T. When we use the relative proportion of T as a starting number of T for the next generation, we can calculate F2 from a starting population of 55 T and 45 P. The crosses are changed from 40 T x P and 10 T x T to 39 T x P and 11 T x T calculated considering the higher proportion of T selfing, which is directly correlated to the higher proportion of T in the population. In F2, the proportion of T increases to 56% and P decreases to 44%. In F3, T = 56.11% and P = 43.89%. In F4 T = 56.12% and P decreases to 43.88%.

Just looking at these generations, the increase in the proportion T becomes negligible. The *superthrum* is a mechanism to increase further the proportion T beyond this apparent functional limit. If we were to add this variable in F5, we would need calculate the number of *superthrums* present in the T population. This calculation would multiply the number of T x T crosses by the proportion of the progeny exhibiting the *GGAA* genotype, which is 1/16, for each generation, and then sum the generations. Thus: 11(1/16) + 11.2(1/16) + 11.222(1/16) = 2.089 *superthrums*.

In the F5 generation, there are 54.12 T, 2 (rounded down from 2.089) *superthrums*, and 43.88 P. There are 38.778 T x P and 11.224 T x T crosses. In an interest to simplify the numbers, let us suppose instead we have 20 individuals, 10 T and 10 P, and of the 10 T, 2 of them are *superthrums*. As was previously stated, when a T x P, there is a 50% ratio of T and P in the progeny because in each T x P, there is a equal 50% chance that the cross will yield a T or a P. Therefore, if we look at 10 crosses between T and P, we should expect to see 5 T and 5 P because in each of the 10 crosses there is a 50% chance of a T or P, it is like flipping a coin, and so with enough iterations of 10 crosses, the mean T and P will be 5.

However, we mentioned that 2 of the 10 T are *superthrums*, that, as we recall, are *GGAA*, and when they cross with pins (see *Fig. 3)*, they produce 100% thrums. If we go back to the 10 crosses between T and P, and we suppose that 2 of the 10 T are *superthrums*, than 2 of the 10 crosses do not have an equal 50:50 T and P, but instead a 100% chance at yielding a T. Therefore, there are 2 crosses that yield a 100% T, and 8

crosses that will yield 50:50 T:P. With the *superthrums* included, the end result is 6 T

and 4 P, and morph ratio distortion has occurred.

If we go back to the F5 data, recall that there are 54.12 T, 2 *uperthrums*, and

43.88 P. There are 38.778 T x P and 11.224 T x T crosses. If we apply the same methods

in the above, simple example, than we would take the number of crosses, 38.778,

subtract the 2 *superthrums*, and divide the difference by 2 to reflect the equal chance T

or P: 38.778 - 2 = 36.778, and 36.778 / 2 = 18.389 T and 18.389 P. Adding back the two

crosses we subtracted, which always produce thrums, we need to add 2 to the resulting

number of T: 18.389 + 2 = 20.389 T and 18.389 P. When we calculate the T and P

resulting from the 11.224 T selfing, we obtain 6.3135 T and .7 P. When we add the T

together we get 20.389 + 6.3135 = 26.7025 T, and when we add the P together, we get

18.389 + .7 = 19.089 P. When we calculate the relative proportion of T we get .5831 or

58.31 % T, an increase of over 2% from the rather stagnant previous generation where

we were reaching a limit of approximately 56.12%.

The proportion T would increase in the next generation the same way it increases

in F5, that is, the number of *superthrums*, would increase by a factor of the amount of T

selfing (recall, we let that be 20% of all T) multiplied by the proportion of the selfing

progeny that has the *superthrum* genotype (1/16), see the dark red square in *fig. 2*. In

other words, 20% of the T population in F6 is 58.31 * .2 = 11.662, and when we multiply

that by 1/16 we get .729, which we can add to the 2.089 in F5 to equal 2.818

superthrums in the next generation, which, running the model again, would calculate

59.48% T and 40.52% P. Sufficient iterations of the model will increase further the

proportion T. The proportion T will limit as a function of the average number of flowering seasons, which we presume to be sufficiently high to match T and P proportions observed in the field.

Appendage B

Mathematical terms

$\frac{1}{2}N_t$ is the number of thrums in population N. This term is equivalent to the number of pins because the proportions are assumed to be 50:50.

T_s is the number of superthrums in the population

T_n is the total number of thrums calculated using *formula B*, and is used as a term in *formula C*

P_n is the total number of pins calculated from *formula A*, and is used as a term in *formula C*

Formula A

$[\frac{1}{5}(\frac{1}{2}N_t) \cdot \frac{1}{16}] + \{[\frac{4}{5}(\frac{1}{2}N_t] - T_s\} \div 2 =$ Number of Pins from 80% dimorphic thrum crossing and 20% thrum selfing in a parent population with N individuals, assuming equal numbers thrum and pin, with superthrums T_s

Formula B

$[\frac{1}{5}(\frac{1}{2}N_t) \cdot \frac{9}{16}] + (\{[\frac{4}{5}(\frac{1}{2}N_t)] - T_s\} \div 2) + T_s =$ Number of thrums from 80% dimorphic thrum crossing and 20% thrum selfing in a parent population with N individuals, assuming equal numbers thrum and pin, with superthrums T_s

Formula C

$$100 \cdot \frac{T_n}{T_n \cdot P_n} = \text{percent } T_n \text{ in the next generation, necessary in calculating additional iterations.}$$