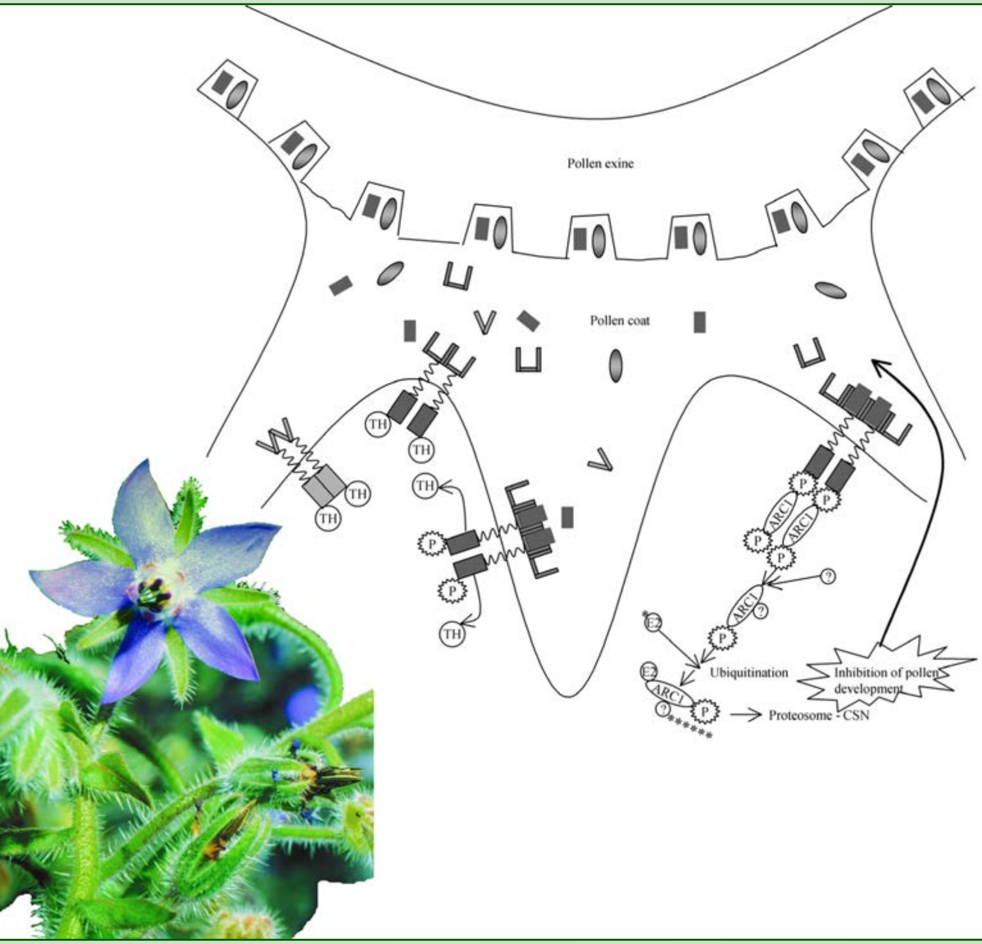
Outbreeding Mechanisms in Flowering Plants

An evolutionary perspective from Darwin onwards

by Carolyn Leach and Oliver Mayo



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with 20 figures and 28 tables

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Cover pictures:

- Color figure: Borage *(Borago officinalis)*
- Diagram: Model for the mechanism of sporophytic self-incompatibility (SSI) in Brassica (Fig. 15, p. 76). Redrawn from Hiscock and McInnis 2003. *Trends in Plant Science* **8** 606-13

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Preface

Darwin initiated the systematic study of mechanisms that encourage or enforce outbreeding in plants, at least in the English-speaking world. He was also the most important pioneer in the investigation of the direct effects on vigour and fertility (biological fitness) on inbreeding and outbreeding in plants.

In these studies, he personally conducted experiments on over 70 species, some 55 in studies of inbreeding and outbreeding, 15 in studies of breeding mechanisms, not including his work on orchids. He observed or considered the work of others on at least 50 more species.

He was hampered throughout by lack of a workable theory or model for inheritance and by a lack of mathematical tools to analyse his very extensive quantitative data. The kinds of conclusion that he could draw were therefore those that required neither awareness of Mendel's already published work nor the development of small-sample statistical analysis.

Three important inferences illustrate his approach, the first two in regard to self-sterility accompanied by differences in floral morphology (which he named heterostyly), the third relating to inbreeding and outbreeding. "We may feel sure that plants have been rendered heterostyled to ensure cross-fertilisation, for we now know that a cross between the distinct individuals of the same species is highly important for the vigour and fertility of the offspring." (Darwin 1877, p. 258). He could draw this conclusion from the weight of evidence from many crossing and selfing experiments in several heterostyled species. Recognising that heterostyly was widespread in the plant kingdom, he wrote that, "We may therefore conclude that this structure has been acquired by various plants independently of inheritance from a common progenitor, and that it can be acquired without any difficulty". (Darwin 1877, p. 261) Again, Darwin confidently makes an inductive inference from a compelling mass of data. As to the effects of crossing and selfing, Darwin wrote, "that cross-fertilisation is generally beneficial, we have excellent evidence." (Darwin 1876, p. 442) This evidence lay in the results of his own extensive experimentation. That "self-fertilisation [is] often injurious to the offspring" (Darwin 1876, p. 443) he supported fair-mindedly with his own and others' experimental work, but noted some important exceptions, starting with the garden pea, *Pisum sativum* (with which Mendel had worked, of course, because it was true-breeding, but Darwin was not aware of this).

Over the intervening century and a quarter, scientists have taken up his leads time and time again, and have confirmed many of his boldest inferences and predictions. We have thought it time to try to review briefly the current understanding of the two linked fields of research, inbreeding depression and outbreeding mechanisms in plants, to assess what has been built on Darwin's strong foundations, and to point tentatively to some gaps that need to be filled. We have also attempted to review very briefly how subsequent workers have used the species that Darwin studied. Because our focus is genetical, we have not considered the relationship between pollinator and pollinated plant, another of Darwin's great themes, in any detail.

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1. Introduction

In his most important book, Darwin (1859) emphasised the importance of outbreeding, though, without a theory of inheritance, he was only able to call on empirical evidence of the benefits of crossing, as opposed to selfing, or close inbreeding, where selfing was impossible, as with most animals. Fisher, as he wrote to J. Davidson (17 April 1930, Bennett 1983, p. 191), had been "led to think that, while in a wholly parthenogenetic form evolutionary progress would not absolutely cease, yet that it would be enormously retarded." The negative and positive benefits of mechanisms, such as sex, that ensured outbreeding were thus recognised within a century of each other, and have been the subject of extensive investigation ever since.

Darwin (1876) began the systematic study of inbreeding depression in the context of evolution by natural selection. Although he had not determined the origin of species in any particular case, he had nevertheless recognised that the ability to cross within a species strengthened the individual members of the species. He had, like scientists from Linnaeus onwards, noticed that plants were almost always hermaphrodites, usually perfect hermaphrodites, whereas animals were almost always unisexual, these differences relating to the basic "strategies" of immobility and mobility.

Further, Darwin began the systematic study of mechanisms of outcrossing in the 1850s, as he recorded in his book on insect pollination of orchids (Darwin, 1862a). He closed it with the comment: "Nature tells us, in the most emphatic manner, that she abhors perpetual self-fertilization." Recognition, but not always understanding, of outcrossing mechanisms was much older, of course (Sprengel 1793, Brown 1833). Sprengel, for example, was the first to identify clearly that insects and other animals were necessary to ensure pollination and seed-set, and indeed felt that "the State should have a standing army of bees" but in this context did not go beyond the necessity for outcrossing. (Sprengel also studied pollen tube growth and many other aspects of pollination.)

In our book, we aim to describe the present state of understanding of the effects of inbreeding, and of the genetics of the outcrossing mechanisms that Darwin (1877) studied, together with related mechanisms that he could not investigate without statistics or Mendelian genetics or molecular biology. In 1930, Brieger published an excellent book that covered this ground, though not confining himself to the flowering plants, but there appears to have been no equivalent review since.

We shall first consider the effects of selfing (and other inbreeding) and crossing in plants, to assess the possibility that these were the drivers of the development of genetical systems that ensure, or at least favour, outcrossing.

We shall then consider self-incompatibility systems, aiming to clarify their genetical patterns of evolution, their population genetics, and their biochemical and physiological determination. It has been noted that "self-incompatibility (SI) systems are unique among self/nonself recognition systems in being based on the recognition of self rather than nonself" (Nasrallah, 2002). In principle, this should be expected since pollination, as a process that in general cannot be actively initiated by the female plant, requires species (self) recognition for effective reproduction. Hence, self-incompatibility[1] must be understood within the context that every plant that can be outcrossed requires a means to identify and accept pollen from its species alone and to identify and reject pollen from any other species.

Darwin (1875, vol. 2, p. 171) wrote:
"…we have conclusive evidence that the stability of crossed species must be due to some principle, quite independent of natural selection. [There are species that when crossed yield no seed,] but yet are affected by the pollen of other species, for the germen swells. It is here manifestly impossible to select the more sterile individuals, which have already ceased to yield seeds; so that this acme of sterility, when the germen alone is affected, cannot have been gained through selection….

"As species have not been rendered mutually infertile through the accumulative action of natural selection, and as we may safely conclude, from the previous as well as from other and more general considerations, that they have not been endowed through an act of creation with this quality, we must infer that it has arisen incidentally during their long slow formation in connection with other and unknown change in their organisation."

It will be of particular importance to determine from the evidence now available whether Darwin was right in his inference and use of the word "incidentally". That is, did the mechanism of incompatibility (whether trans- or intra-specific) arise by chance, or was natural selection more important than chance following isolation?

1 We shall use the abbreviations SI for self-incompatibility, GSI for gametophytically manifested SI, and SSI for sporophytically manifested SI throughout the book, except in direct quotations.

2. The effects of inbreeding and outbreeding

2.1 What Darwin wanted to achieve

When Charles Darwin published his pioneering book on the effects of outbreeding and inbreeding in 1876, he was building on almost a century of study of pollination mechanisms, beginning with the work of Sprengel (1793). He, like his predecessors, contributed to the convincing evidence that the flowers of most plants were constructed as if they have been designed to be pollinated by pollen from other flowers, whether on the same plant, or from a separate plant. Darwin made several distinctive and lasting contributions. First, he showed that cross-fertilisation was generally by pollen from a separate plant, rather than simply from a separate flower on the same plant. Secondly, and perhaps more important, he recognized that the widespread existence of mechanisms for out-crossing invited the inference that plants derive some advantage from out-crossing, and undertook to provide the evidence for such advantage.

Darwin appears to have set himself several tasks. First, he wished to conduct experiments to identify the effects of self- and cross-pollination and to distinguish between cross-pollination by pollen from flowers of the same plant and that from distinct plants. Secondly, he certainly wished to identify whether some of the different outbreeding mechanisms had different effects from others; he made a particular study of different forms of flower within the same species. (This was a rational approach, since he lacked the basis for investigation of homomorphic systems.) Thirdly, he wished to determine whether the effects were the same across a wide selection of taxa from the plant kingdom. (In this context, it is noteworthy that, while he considered some 80 genera of orchids in showing that these flowers were in the main highly specialised for insect pollination (Darwin 1862a), and hence outcrossing, he referred to only about 5 of those genera in his 1876 book.) Always, of course, he wished to compare his work with that of others.

In evaluating Darwin's work, Whitehouse (1959) wrote that "Darwin considered that the morphological adaptations of flowers and also dichogamy and prepotency of cross- over self-pollen had evolved as devices to favour cross-fertilization, but dioecism, gynodioecism and self-sterility, whether associated with heterostyly or not, he regarded as largely incidental occurrences. He was unable to believe that unless the means for cross-pollination already existed these restrictions on self-fertilization could be anything but harmful to the species, although he admitted of course that once they had arisen, such conditions had led to regular cross-fertilization."

Whitehouse went further: he claimed that current thinking was that "the main advantage of cross-fertilization is the long-term advantage unknown to Darwin of increased opportunity for genetic recombination and hence increased hereditary variability and therefore adaptability."

What we first need to consider, therefore, is whether Darwin's work on the advantages of outbreeding was sufficient in itself for its purposes. Did Darwin demonstrate sufficient advantage for out-crossing to explain the manifold mechanisms that ensure such crossing? Do other evolutionary considerations better explain what he demonstrated? Has what he demonstrated stood the test of time, and where it has not, what has augmented or supplanted his findings?

2.2 What hampered Darwin

Darwin lacked a workable theory of inheritance throughout his career. The theory that he did develop, pangenesis, was no guide to him in his breeding work.

Darwin had no quantitative methods that would allow him to determine whether his experimental results or his observations did or did not fit his theory to any desired degree of precision. Indeed, he lacked completely any theory of variability, which no doubt contributed to his failure to make his theory of breeding quantitative.

2.3 Darwin's experimental designs

As already discussed, Darwin wished to determine what the advantages of cross-pollination as against self pollination might be, given almost a century's concordance of many botanists that outbreeding mechanisms were very widespread. Much of his eleven years of experimentation was driven by the result of one initial observation, but it is worth describing in detail the care with which he conducted his experiments. (The initial experiment involved cross-pollination of some flowers on a plant of *Linaria vulgaris*, the pollen for crossing coming from a separate plant. He found that the plants raised from the seed resulting from crossing were taller than those resulting from selfing.) He conducted other preliminary experiments, such as a careful examination of the effect of amount of pollen on pollination success. He found no effect, which made his subsequent experiments substantially easier, since equalizing the amount of pollen used would have been very difficult.

With a great deal of careful preparation, then, Darwin moved on to initiate his major experiments. He began with a species exotic to England, convolvulus or morning glory (*Ipomoea purpurea*), one plant of which was growing in his greenhouse. Because he began with this one plant in his greenhouse, there was no chance of contamination from pollen from other plants, but he did not have a baseline for his measurements of plant height at a given age. Other plants that he initially used for crossing were not grown in the same environment, for example.

He adopted a very straightforward experimental design for all species, though he did not of course always initiate work on one single plant in the greenhouse.

He self-pollinated ten flowers on his one convolvulus plant and cross-pollinated ten other flowers on the same plant with pollen from a separate plant. The flowers used as female in pollination were not emasculated, so some selfing could occur, but any effect on the outcome would be conservative: it would diminish the difference between self- and cross-pollination, if any. He appears to have used fresh pollen from flowers of similar age on flowers of similar age (time since opening etc.). He effected germination of self-pollinated and cross-pollinated seeds in one container with a partition, so that seeds germinating simultaneously would have had very similar experience. He then planted the seedlings that had germinated simultaneously as paired plants in a single pot. He used a soil as uniform as could be obtained, uniform watering and equal illumination of all plants. The main trait that he measured was height, and he also considered number and weight of seed produced at times. Most experiments were conducted in his extensive greenhouses, so that control over predation, unpredictable weather and the like was as strong as possible.

In his statistical analysis, Darwin was handicapped, first, by not being very numerate in general and, secondly, by the fact that there was not a standard body of statistical methods for the experiments involving small numbers of observations. Darwin recorded and reported paired measurements by pot and considered the magnitude of differences between the two plants in a pot would be a good indication of positive or negative effects of selfing or crossing of the general vigour of plants. He tabulated arithmetic averages for selfed and crossed plants, and used these averages as his basis for comparison of the effect of pollination type.

At the end of his eleven years of experimentation, unsure that he had made the best use of his data, Darwin asked his pioneering statistician cousin, Francis Galton, for assistance. He printed Galton's response in his book, at sufficient length for Fisher (1935a) to show that Galton's analysis was invalid, Darwin's design requiring different treatment. (As noted above, Darwin used pairing of plants to minimise differences between selfed and crossed progeny as much as possible, and Galton ignored this fact in his analysis, which was therefore invalid, from a modern inferential point of view, as well as inefficient.) It is noteworthy that the set of data Galton discussed gave, when correctly analysed, a probability of precisely 5% of the difference between the selfed and crossed plants arising by chance, a result that would, in general practice today, need one to conduct further experiments. Darwin, of course, did conduct many further experiments, for the reasons given earlier. In total he presented a body of evidence proving to anyone's satisfaction that self-pollination is in the main deleterious as against cross-pollination.

We now consider the convolvulus experiment in more detail.

Darwin's generation means were as follows (Table 1); we have calculated the standard deviations. Analysis, as noted, requires pairing, but the tabulation shows the variability in the material and the means that were important to Darwin.

Table 1. Mean (± s.d. in brackets) heights of selfed and crossed convolvulus (*Ipomoea purpurea*) plants over ten generations (in inches)

Generation	Selfed plants	Crossed plants
1	65.7 (5.9)	86.0 (4.5)
2	66.3 (14.4)	84.2 (4.0)
3	52.8 (12.2)	77.4 (4.7)
4	60.1 (12.8)	69.8 (14.1)
5	62.3 (20.4)	82.5 (9.2)
6	63.2 (13.5)	87.5 (4.8)
7	68.3 (14.3)	83.9 (3.6)
8	96.7 (21.9)	113.5 (19.8)
9	64.1 (15.5)	81.4 (10.1)
10	50.4 (11.5)	93.7 (6.9)

Selfed plants were significantly shorter than crossed plants throughout the experiment. They were also significantly more variable. The results for generation 8 were exceptional, and Darwin was forced to consider it separately and identify reasons for the anomalous result (though crossed plants were consistent in their superiority in that generation as elsewhere).

Analysis of variance for height shown in Table 2 summarises the effects of selfing as against crossing, and generation.

Table 2. Analysis of variance for plant height in Darwin's experiments on convolvulus (*Ipomoea purpurea*)

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratios	Probability
Generation	9	18675.8	2075.1	13.53	<0.01
Pollination	1	13510.2	13510.2	88.10	<0.001
Gen x Poll	9	2257.1	250.8	1.64	>0.05
Residual	136	20856.4	153.4		
Total	155	55299.5			

The significant difference between selfing and crossing demonstrates clearly the superiority of the latter, but the difference among generations and the interaction are not as we would now expect: there is no overall trend down because of the peculiar results in generation 8.

If we remove generation 8 from the analysis, we still find crossed plants significantly taller than selfed plants, and still find a difference among generations, but do not observe significant divergence over time between crossed and selfed plants. Calculating the decline in height of the selfed plants as a regression on the inbreeding coefficient, F, we obtain a value of about -0.005 of the original mean plant height per 1% increase in F. (F is conventionally defined as the probability of homozygosity by descent for any gene in an organism whose parents have one of more common ancestors.) This is, as we would expect, not statistically significant, given that we have established that there is no trend in height with generation number, and is calculated by omitting generation 8. Nevertheless, if we compare this result with the twentieth century results given in Table 4, which are all statistically significant, we see that it is of the same order, and we can speculate that Darwin's scrupulous "fairness" has in fact masked the effects of inbreeding. This "fairness" included trying to use the best seeds from the best capsules, removing early seedlings that seemed likely to die, and measuring the "best" plants in competition experiments.

Darwin was particularly handicapped by the fact that he had not read Mendel's great work, though he had copies of two books that cited it (Hoffmann, 1869, Focke 1881; but note that neither work made clear what Mendel's achievement actually was: Olby and Gautrey 1968). When he studied the effect of inbreeding on plant height in *Mimulus luteus*, he observed that crossed plants were about 20–30% taller than selfed for four generations, but after this found for two generations a reversal. He also found that the taller selfed plants had white flowers. Today one would infer that segregation in inbred plants had uncovered rare recessive alleles of one or more genes influencing flower colour and height, but Darwin's conceptual framework lacked this fundamental insight, though following Knight (1799) and others he certainly recognised dominance (prepotency) and considered it important. Later in the course of the same experiment, Darwin obtained an outside plant ("Chelsea") that was taller than the crossed plants that were in turn taller than the selfed plants. He was therefore confident that the heights of plants seen in his experiment lay in the normal range; his experiment had not worked by virtue of anything abnormal.

Because some of Darwin's plant height results were difficult to interpret, he also measured seed weight, which followed the same general pattern: it was higher for crosses than for selfs.

2.4 Inheritance

Darwin was as much hampered by his lack of a workable theory of inheritance as by any other single factor, but this lack was not strictly separable from his lack of workable statistical tools. For example, in his long-running debate with Fleeming Jenkin over the possibility of really substantial change in an organism, change that would constitute a new species, the discussion was almost entirely abstract (see Darwin 1887, 1903). Had a sound understanding of variability been available to Darwin, he could have resolved the issue by appeal to data of the type that he did collect: on change in domesticated species.

Jenkin made a general criticism about the gaps in Darwin's argument: "He can invent trains of ancestors of whose existence there is no evidence; he can marshal hosts of equally imaginary foes; he can call up continents, floods, and peculiar atmospheres; he can dry up oceans, split islands, and parcel up eternity at will; surely with these advantages he must be a dull fellow if he cannot scheme some series of animals and circumstances explaining our assumed difficulty quite naturally. Feeling the difficulty of dealing with adversaries who command so huge a domain of fancy, we will abandon these arguments, and trust to those which at least cannot be assailed by mere efforts of imagination." Darwin's main answer to such criticism was that he was initiating new studies, and the gaps in his data would be filled over time by other workers. The specific difficulties that so troubled Darwin were, first, that rare advantageous variants would be genetically lost through blending inheritance and chance, and, secondly, that strong artificial directional selection, as with the speed of racehorses, had not been shown to be successful. (See Darwin 1887 vol. 3 p. 107, 1903 vol. 1 p. 282 for details and further references.) Mendelian population genetics has eliminated the former concern, research such as the Illinois maize selection experiment (e.g. Dudley and Lambert 1969) the latter. Indeed, current understanding of the ancestry of cultivated species (e.g. Simmonds 1976) shows that Jenkin's argument was wrong at the time that he made it.

2.5 What Darwin achieved

Darwin's achievements were based on experimentation that was in detail prodigious. He wrote, for example to J. D. Hooker (27 September 1862, Darwin 1903, vol. 2, p. 290): "I am rather disgusted to find I cannot publish this year on *Lythrum salicaria*; I must make 126 extra crosses." In this context, a remark of Darwin's youngest son Leonard to R. A. Fisher (4 December 1929?, Bennett 1983, p. 113) is of interest: "My father had only one old and inefficient gardener for his 'staff' for many years, and I believe his work was in some ways all the better in consequence."

He reached three really important conclusions by piling up the evidence until it was incontrovertibly massive. First, inbreeding lowered vigour and fertility; it was in general harmful. Secondly, outbreeding following inbreeding could restore some