

SPECIAL INVITED PAPER

“A CASE TO WHICH NO PARALLEL EXISTS”:
 THE INFLUENCE OF DARWIN’S *DIFFERENT FORMS OF FLOWERS*¹

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- *Premise of the study:* Research on the subject of heterostyly is often traced back to 1877 when Charles Darwin published the landmark book *The Different Forms of Flowers on Plants of the Same Species*. This book synthesized heterostyly research at the time, much of which Darwin conducted, and it continues to be a major contribution to the study of the breeding system. In this book, Darwin discussed the ecology, morph-specific differences, self- and intramorph-incompatibility, evolution and origin, and floral development of heterostyly. Many of the hypotheses he proposed have been and continue to be tested.
- *Key results:* Throughout the 20th and 21st centuries, researchers have continued to identify new and different morph-specific floral characters, discover the mechanisms that underlie heteromorphic self-incompatibility, use phylogenies to examine the evolution of heterostyly, and determine novel floral developmental patterns in heterostylous species. From all of these studies, we have learned a great deal about the function, evolution, and development of heterostyly.
- *Conclusions:* However, almost 150 years after Darwin’s publications on the subject of heterostyly, we still have a great deal to learn concerning the breeding system, and new technologies and techniques are allowing for new advances in heterostyly research to occur.

Key words: breeding system; Darwin; distyly; floral development; herkogamy; heteromorphic self-incompatibility; heterostyly; tristily.

“It is ironic that although *Forms of Flowers* is one of Darwin’s lesser known books, it summarizes work about which he wrote ‘I do not think anything in my scientific life has given me so much satisfaction as making out the meaning of the structure of these plants’.” —R. Ornduff, 1992, p. 34

Since the 16th century, botanists have known that different forms of hermaphroditic flowers can occur in one species (Clusius, 1583; van Dijk, 1943; Ornduff, 1992), but the first comprehensive study of species with these di- and trimorphic flowers was made during the latter half of the 19th century. In 1877, at the age of 68, Charles Darwin published the landmark book *The Different Forms of Flowers on Plants of the Same Species*. Darwin devoted the majority of this book to studies of heterostyly, a complex and elegant breeding system, that he (Darwin, 1862, 1864, 1865, 1869, 1877) and his colleagues, such as Friedrich Hildebrand (e.g., 1863, 1867), Fritz Müller (1869), and Asa Gray (1842) (to whom the book is dedicated), conducted. This book has influenced many studies of heterostyly,

and although numerous books and papers have been written since, many currently accepted ideas trace their origin back to *Different Forms of Flowers*, published over 140 years ago.

Since Darwin’s time, researchers have added considerably to our understanding of heterostyly through hundreds of studies on the subject of the evolution, genetics, ecology, morphology, and development of the heterostylous breeding system (e.g., Barlow, 1923; Ernst, 1928; Newton and Pellew, 1929; Dowrick, 1956; Dulberger, 1975; Ganders, 1979a; Barrett, 1992; Kohn et al., 1996; Faivre, 2000; Barrett and Shore, 2008; Hodgins and Barrett, 2008; Weller, 2009). Many of the hypotheses Darwin proposed have held up to rigorous examination, while others appear to be more complex than he initially considered (Ganders, 1979a; Dulberger, 1992). The goal of this paper is to address the ideas Darwin discussed in *Different Forms of Flowers* as they relate to multiple aspects of heterostyly, including polymorphic characters, genetics, physiology, and molecular biology, phylogenetic distribution and evolution, and floral development. Some topics related to heterostyly, such as the morphology and function and the physiology of the breeding system, have been abbreviated in this paper; recent reviews by Barrett and Shore (2008) and Weller (2009) focus on these topics in more detail.

MORPHOLOGY AND FUNCTION OF HETEROSTYLY

“The benefit which heterostyled dimorphic plants derive from the existence of the two forms is sufficiently obvious, namely, the intercrossing of distinct plants being thus ensured. Nothing can be better adapted for this end

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than the relative positions of the anthers and stigmas in the two forms..."—Darwin, 1877 (p. 30)

Heterostyly is defined as the occurrence in a species of two or more floral morphs that exhibit reciprocal herkogamy (cf. Barrett and Shore, 2008) ("Herkogamy is the spatial separation of pollen presentation and pollen receipt within or between blossoms of an individual plant." [Webb and Lloyd, 1986]). Heterostyly is often associated with other physiological and structural characters, such as self- and intramorph-incompatibility (SI) and pollen and stigma polymorphisms (Richards, 1997; Barrett et al., 2000a; Barrett and Shore, 2008). Indeed, Darwin's definition of heterostyly (1877) specified the presence of self- and intramorph-incompatibility in addition to the morphological differences. Furthermore, the features of the heterostylous syndrome only rarely disassociate in the progeny of heterostylous species (Darwin, 1877; Ernst, 1928, 1936a, b). Researchers have demonstrated that in some taxa this tight linkage is due to the presence of a supergene, which they hypothesize to include the genes involved in the development and function of heterostyly (e.g., Dowrick, 1956; Barrett and Shore, 2008), although in other taxa, the tight linkage between the morphology and SI may have other explanations (see Genetics, Physiology, and Molecular Biology of Heterostyly later).

In distylous taxa, one morph—the short-style (SS) morph—is reverse herkogamous, with anther(s) positioned above the stigma(s), and the other—the long-style (LS) morph—is approach herkogamous, with the stigma(s) situated above the anther(s). The anther(s) of each morph are at the same height as the stigma(s) of the other morph (Fig. 1). These morphs have often been referred to as thrum and pin, respectively. Darwin referred to the SS morph as thrum (or thrum-eyed [Darwin, 1862, 1877]) because the anthers at the apex of the corolla tube of this morph resemble "the ends of weavers' threads" (Darwin, 1877, p. 14), and the LS morph is called pin (or pin-eyed or pin-headed [Darwin, 1862, 1877]) because of the similarity of the capitate stigma(s) of some taxa, situated at or near the apex of the corolla tube, to the head of a pin. Darwin used this terminology because his initial studies of heterostyly focused on the species of *Primula* L. (Primulaceae), and the SS morph of these species actually produce flowers that resemble the ends of weavers' threads. He even stated, "I suppose that some weaver who cultivated the polyanthus invented this name [for the SS morph], from being struck with some degree of resemblance between the cluster of anthers in the mouth of the corolla and the ends of his threads" (Darwin, 1877, p. 14). However, the flowers of other heterostylous species take different forms, and even though Darwin initially used the names pin and thrum, he abandoned these terms in the majority of his discussions of heterostyly (Ornduff, 1992). However, some botanists continue to use "pin" and "thrum" to refer to the two distylous morphs (e.g., Wong et al., 1994; Yasui et al., 2004; Matsui et al., 2004).

In tristylous taxa, three morphs are present. In each of the three morphs, anthers are located at two of the three levels, and the stigma(s) is fixed at the third (Fig. 2). The heights of each of the two whorls of anthers of two morphs correspond to the height of the stigma(s) of the third morph. The terminology used to describe distylous taxa is also employed for morphs of tristylous taxa that have the stigma either above or below both sets of anthers, LS and SS, respectively, while the third morph, with the stigma(s) positioned at an intermediate height between the two sets of anthers, is called the mid-style (MS) morph. Despite Darwin's (1877) demonstration of the existence of

either two or three forms of flowers in heterostylous species, some of his contemporaries, such as Bonnier (1884), thought that many more forms existed in some species (Ornduff, 1992).

Darwin hypothesized that these structural features promote efficient pollen transfer (Figs. 2 and 3). The breeding system does not function perfectly because self and intramorph pollen can be found on stigmas, sometimes in large quantities (Müller, 1869; Darwin, 1877; Ganders, 1979a; Schou, 1983; Barrett and Glover, 1985; Piper and Charlesworth, 1986; Barrett, 1992; Lloyd and Webb, 1992b; McKenna, 1992). However, Ganders (1979a), Ornduff (1980), Lewis (1982), Piper and Charlesworth (1986), Lloyd and Webb (1992b), García-Robledo (2008) and others have demonstrated that the heterostylous morphology provides for the efficient transfer of pollen between and among the anthers and stigmas of different morphs. In addition, most heterostylous taxa possess an SI mechanism, and, along with reciprocal herkogamy, these incompatibilities promote cross-fertilization through the prevention of inbreeding. However, not all heterostylous species have an SI mechanism (e.g., *Salvia brandegeei* Munz [Lamiaceae] [Barrett et al., 2000b]), and SI barriers can either break down or may not be complete (Darwin 1877, Weller, 1979; Barrett, 1989, 1992). Darwin devoted much of *Different Forms of Flowers* to the description and discussion of crossing experiments that quantify the efficiency of SI in many heterostylous species.

THE POLYMORPHISMS OF HETEROSTYLY

"Village children notice this difference, as they can best make necklaces by threading and slipping the corollas of the long-styled flowers into one another. But there are much more important differences. The stigma in the long-styled form is globular; in the short-styled it is depressed on the summit, so that the longitudinal axis of the former is sometimes nearly double that of the latter. Although somewhat variable in shape, one difference is persistent, namely, in roughness: in some specimens carefully compared, the papillae which render the stigma rough were in the long-styled form twice to thrice as long as in the short-styled. The anthers do not differ in size in the two forms, which I mentioned because this is the case with heterostyled plants. The most remarkable difference is in the pollen-grains."—Darwin, 1877 (pp. 15, 16)

Darwin noticed both macro- and micromorphological differences between and among heterostylous morphs (Figs. 1–3). The micromorphological differences between the morphs tend to follow the same patterns observed in the gross morphology of the two or three morphs (Table 1). Although Darwin described micromorphological differences of several features between or among the morphs, such as pollen size and length of papillae on the stigma, subsequent researchers have described di- or trimorphisms in other characters, including pollen shape and stylar epidermal cell length (Johnston, 1952, 1954; Richards, 1997). Many morph-specific differences have been observed in heterostylous species, but only a few of these, such as pollen size and stylar epidermal cell length, are di- or trimorphic in most heterostylous species.

Corolla—In some species, such as *Sebaea grandis* Steud. (Gentianaceae) (Wolfe et al., 2009) and *Erythroxylum coca* Lam. (Erythroxylaceae) (Ganders, 1979b), gross corolla morphology

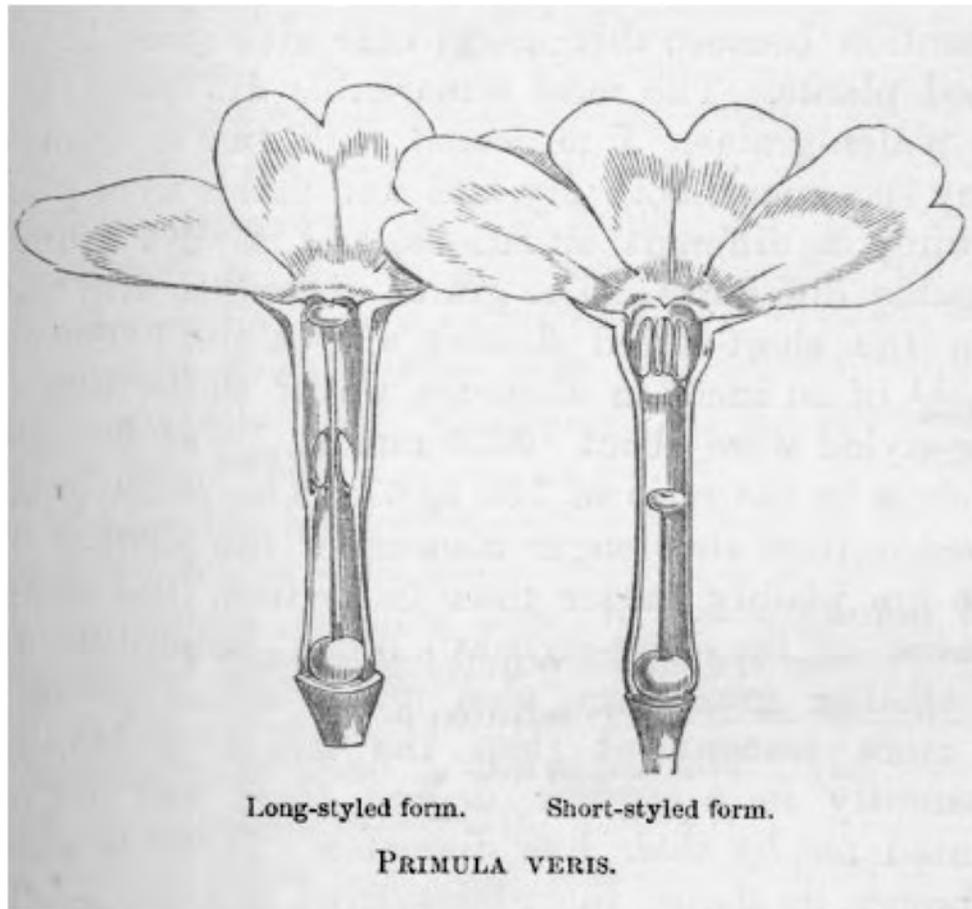


Fig. 1. Gross morphological differences of long-style (pin) and short-style (thrum) morphs of *Primula veris* (Primulaceae), a distylous species; fig. 1 of Darwin (1877).

is the same between the morphs; however, in other taxa, such as species of *Lithospermum* L. (Boraginaceae) (Cohen et al., 2008), *Amsinckia* Lehm. (Boraginaceae) (Ornduff, 1976; Ganders, 1979a), *Fagopyrum* Mill. (Polygonaceae) (Darwin, 1877; Ganders, 1979a), *Rudgea* Salisb. (Rubiaceae) (Baker, 1956; Ganders, 1979a), *Faramea* Aubl. (Rubiaceae) (Müller, 1869; Darwin, 1877), and *Palicourea padifolia* (Roem. & Schult.) T. N. Taylor & Lorence (Rubiaceae) (Hernández and Ornelas, 2007), the two morphs differ in corolla length. In many heterostylous species, the SS corolla tube is longer than that of the LS morph. This can be due to differences in the number of corolla cells, as occurs in species of *Lithospermum* (Cohen et al., 2008), or differences in the length of corolla cells, as observed in *Primula* (Richards, 1997, 2003; Webster and Gilmartin, 2006).

One of the more unusual differences in corolla morphology between morphs is trichome abundance and location. Darwin (1877) noted differences in the placement of trichomes in a species of *Borreria* G. Mey (Rubiaceae). He observed that the trichomes in the LS morph are situated at the apex of the corolla, but those of the SS morph are located in a more basal position, beneath the anthers. In *Lithospermum obovatum* J. F. Macbr., the LS morph bears trichomes on the adaxial surface, while the SS morph does not (Johnston, 1952), and in *Pentstemon* Harv. (Rubiaceae), the corolla tube of the SS morph produces more trichomes than that of the LS morph (Massinga et al., 2005). Only the taxa mentioned are known to differ in

corolla trichome abundance and/or position. Although the function of dimorphic trichome abundance is unknown, these observations suggest that this character is associated with the supergene in these taxa.

Stamens—Fixed difference in anther height between or among morphs is one of the main features that characterize heterostyly (Darwin, 1877; Barrett et al., 2000a), but multiple factors can contribute to the different anther heights. In some heterostylous species, the filaments are of negligible length (Faivre, 2000), so the point of attachment of the filaments to the corolla tube contributes to the majority of the anther height differences between morphs. In other taxa, the filaments are the main or only factor that determines anther height differences between or among morphs (Dulberger, 1975; Mal, 1998).

As Darwin mentioned, the anthers of the two morphs tend to differ in length, with SS anthers often longer than LS anthers. This pattern has been observed in species of Boraginaceae (Darwin, 1877; Johnston, 1952; Ornduff, 1976), Rubiaceae, Menyanthaceae, Pontederiaceae (Barrett, 1977), and Primulaceae, but members of Linaceae and Oleaceae exhibit the opposite arrangement (Darwin, 1877; Ganders, 1979a).

Gynoecia—One of the more consistent dimorphic characters in heterostylous species is stylar epidermal cell length. In most species, the LS morph has longer stylar epidermal cells than

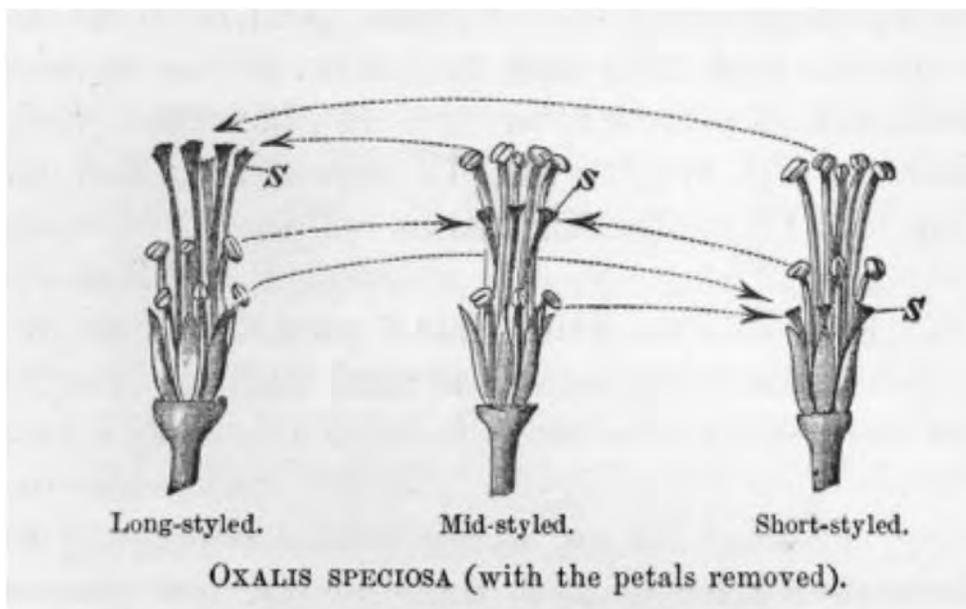


Fig. 2. Gross morphology of the sexual organs of *Oxalis speciosa* (Oxalidaceae) along with the legitimate pollinations for this and other tristylous species; fig. 11 of Darwin (1877).

does the SS morph, and in some species this difference can be quite drastic (Fig. 4). For example, in *Lithospermum cobrense* Greene, the LS stylar epidermal cells are more than four times as long as those of the SS morph (unpublished data, $147 \pm 24 \mu\text{m}$ vs. $34 \pm 10 \mu\text{m}$ long). Investigators have hypothesized that in most species, the LS style must consist not only of longer cells but also of more cells than that of the SS morph. Species of Pontederiaceae display this pattern of increased stylar epidermal cell length and number in the LS morph (Richards and Barrett, 1987, 1992), but in the three species of *Lithospermum* that Cohen et al. (2008) examined, the style of the LS morph is composed of half as many epidermal cells as that of the SS morph.

The stigma shape differences between the morphs of *Primula* that Darwin (Figs. 1 and 3) noted are not as prevalent in many heterostylous species as morph-specific differences in the stigmatic papillae. Most heterostylous species have LS stigmas with longer and/or larger papillae than those of SS stigmas (Darwin, 1877; Richards, 1997; Ünal and Yildirim-Fazla, 2007). Darwin observed that the stigmatic papillae of the LS morph of *Linum grandiflorum* Desf. (Linaceae) are longer than those of the SS morph. He suggested "the papillae, which render the stigma of the long-styled form of various species rough, serve to entangle effectually the large-sized pollen-grains brought by insects from the short-styled form, thus ensuring its legitimate fertilization" (Darwin, 1877, p. 254). Species of other genera, such as *Primula* (Darwin, 1877; Heslop-Harrison et al., 1981; Richards, 1997, 2003), display this pattern (Bahadur, 1970), but *Anchusa officinalis* L. (Boraginaceae) (Schou and Philipp, 1984) and species of *Limonium* Mill. (Plumbaginaceae) and *Decodon* J. F. Gmel. (Lythraceae) (Eckert and Barrett, 1994) exhibit the opposite morphology. Even though the lengths of the stigmatic papillae and the style tend to be correlated, Richards and Barrett (1992) point out that a developmental connection between the lengths of the cells of these organs is not absolute. If stylar length increased through a greater number of cell division events rather

than by cell elongation, then the lengths of the stigmatic papillae would not increase (Richards and Barrett, 1992). Therefore, understanding the developmental differences between or among the morphs allows for different interpretations of the morphology at anthesis. Apart from differences in the length of papillae, species of some taxa, such as *Armeria* Willd. (Plumbaginaceae) (Richards, 1997), *Plumbago* L. (Plumbaginaceae) (Dahlgren, 1918; Dulberger, 1975), and *Dyerophytum* Kuntze (Plumbaginaceae) (Dahlgren, 1970), produce different shapes of stigmatic papillae; while species of other taxa, such as *Linum*, deposit different combinations of cuticular substances, including lipids, pectins, and cellulose, on the stigmatic papillae of each morph (Dulberger, 1987; Ünal and Yildirim-Fazla, 2007). Dulberger (1975, 1992), Richards, (1997), McCubbin (2008), and others have hypothesized that these dimorphic stigmatic features relate to efficient pollen transfer between or among morphs.

Pollen—One of the most consistent and interesting features of heterostylous species is the presence of di- or trimorphic pollen (Fig. 4). Darwin (1877) observed these differences in species of *Primula*, *Linum*, *Faramea*, and other genera, and he found this feature of the heterostylous syndrome to be "most remarkable." In the majority of species, the SS morph produces larger pollen grains than the LS morph (Darwin, 1877; Richards, 1997). Even though SS pollen is often larger than LS pollen, investigators have described either the opposite pattern or a lack of size differences between the morphs in some species, including *Linum suffruticosum* L. (Punt and den Breejen, 1981) and *Cryptantha flava* Payson (Boraginaceae) (Casper, 1985; Dulberger, 1992).

The function of pollen grains of two or three different sizes has been and continues to be unknown. Darwin suggested, somewhat skeptically, "it is difficult quite to give up the belief that the pollen-grains from the longer stamens of heterostyled plants have become larger in order to allow for the development of longer tubes" (Darwin, 1877, p. 251). Although this theory,

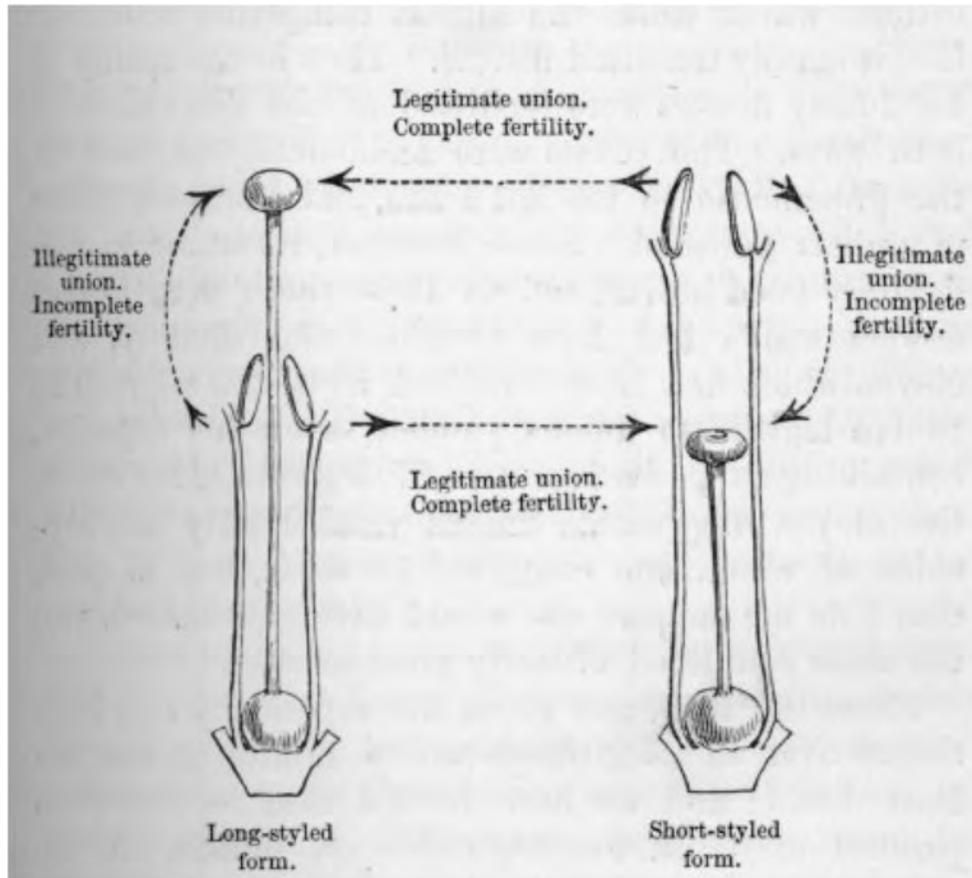


Fig. 3. Legitimate and illegitimate pollinations in *Primula veris* (Primulaceae) and other distylous species. Note the different morphologies of the long-style and short-style stigmas; fig. 2 of Darwin (1877).

originally advocated by Delpino (1867), has some intuitive appeal, no correlation between style length and pollen size exists (Darwin, 1877; Ganders, 1979a; Cruden, 2009). Cruden (2009) postulates that pollen grain size does not correlate with style length, but rather with stigma depth. Differences in stigma depth between morphs have not been well studied, but if there is a difference in the depths of the stigmas between the two morphs (and given the current information on morph-specific length differences, it seems a distinct possibility), this hypothesis could provide one explanation for the differences in pollen grain sizes between morphs. In addition, Darwin, (1877), Dulberger (1975, 1992), Richards (1997), and others have demonstrated that in some species, the different sizes of pollen grains are related to incompatibility reactions on the stigma. If a pollen grain is too large to fit between the stigmatic papillae, then larger pollen grains may not be able to reach the stigmatic surface or even adhere to the stigma (Fig. 4e) (Darwin, 1877; Dulberger, 1975, 1992; Richards, 1997).

Although a correlation between pollen grain size and style length does not exist, Darwin and others (e.g., Barrett and Glover, 1985; Dulberger, 1992; Richards, 1997) have observed a correlation between pollen grain size and number. Anthers of SS plants often produce fewer, larger pollen grains than those of LS plants. These different quantities of pollen produced by the LS and SS anthers have been hypothesized to be important to equalize male fitness between the morphs

(Piper and Charlesworth, 1986; Wolfe and Barrett, 1989; Richards, 1997); although, pollen flow between the shorter organs is more efficient in some taxa (e.g., Lewis, 1982; Massinga et al., 2005).

Apart from differences in size and number, some heterostylous species produce pollen with additional, unique dimorphic features. Müller (1869) and Darwin observed different types of exine ornamentation on the pollen of species of *Faramea*. According to Darwin, Müller hypothesized that “the little points on their [the SS pollen’s] surfaces cause them to cohere, and at the same time favor their adhesion to the hairy bodies of insects... On the other hand, the smooth grains of the long-styled flowers are safely included within the tube of the corolla, so that they cannot be blown away...” (Darwin, 1877, p. 130). Although exine ornamentation is one of the more common and apparent differences between morphs, there is often another, more subtle form of difference in exine sculpturing, with SS pollen grains exhibiting a coarser exine than the LS pollen grains (Dulberger, 1992; Richards, 1997). These dimorphic exine-sculpturing patterns have been observed in a variety of families, including Oleaceae (Pandey and Troughton, 1974), Plumbaginaceae (Richards, 1997), Gentianaceae (Wolfe et al., 2009), Rubiaceae (Bahadur et al., 1984; Naiki and Nagamasu, 2003, 2004) and Polygonaceae (Bahadur et al., 1984; Hong, 1999). Richards (1997) and others have proposed that these different exine patterns are involved in a type of lock-and-key

TABLE 1. Many characters are polymorphic within a heterostylous species, and this table (adapted from Richards, 1997) includes 16 of these characters and the manners in which they differ between or among morphs. Additionally, the characters that Darwin discussed in *Different Forms of Flowers* are noted, and examples of genera that include species that exhibit these polymorphic characters are provided.

Character	Polymorphic characters observed in heterostylous species			Darwin observed	Examples
	Long-style	Short-style			
Corolla length	shorter	longer		X	<i>Lithospermum</i> , <i>Rudgea</i>
Corolla diameter	narrower	wider			<i>Fagopyrum</i> , <i>Primula</i>
Corolla pubescence	no trend ^a			X	<i>Lithospermum</i> , <i>Pentanisia</i> , <i>Borreria</i>
Filament epidermal cell length	shorter	longer			<i>Primula</i> , <i>Lythrum</i> , <i>Pontederia</i>
Anther length	shorter	longer		X	<i>Eichhornia</i> , <i>Primula</i> , <i>Pulmonaria</i> , <i>Palicourea</i>
Pollen size	smaller	larger		X	<i>Armeria</i> , <i>Anchusa</i> , <i>Primula</i> , <i>Fagopyrum</i> , and many others
Pollen shape	prolate with a constricted equator	ellipsoid			<i>Lithospermum</i> , <i>Arnebia</i> , <i>Waltheria</i>
Pollen exine	finer reticulations	coarser reticulations		X	<i>Armeria</i> , <i>Sebaea</i> , <i>Faramea</i> , <i>Limonium</i>
Pollen color	no trend ¹			X	<i>Linum</i> , <i>Lythrum</i> , <i>Pentanisia</i>
Pollen starch	present	absent			<i>Lythrum</i> , <i>Jepsonia</i>
Pollen production	more	less		X	<i>Amsinckia</i> , <i>Cratoxylum</i> , <i>Cordia</i> , <i>Fagopyrum</i> , <i>Jepsonia</i> , and many others
Style epidermal cell length	longer	shorter			<i>Primula</i> , <i>Lithospermum</i> , <i>Lythrum</i>
Stylar conducting tissue area	smaller	larger			<i>Primula</i>
Stigma size and shape	no trend ^a			X	<i>Plumbago</i> , <i>Hedyotis</i> , <i>Rudgea</i>
Stigma papillae	longer, less crowded	shorter, more crowded		X	<i>Lythrum</i> , <i>Pulmonaria</i> , <i>Linum</i> , <i>Armeria</i>
Genetic control	homozygous	heterozygous			<i>Primula</i> , <i>Turnera</i>

^aThese traits do not exhibit any directional trend. Although differences have been observed between morphs, neither morph consistently produces structures that are larger/smaller or with lighter/darker characteristics.

pollen–stigmatic papillae interaction that is necessary for pollen grain germination.

Some heterostylous species exhibit different pollen shapes or colors between or among morphs. In *Arnebia* Forrsk. (Borraginaceae), *Lithospermum*, and *Waltheria* L. (Sterculiaceae), some species produce two different, morph-specific pollen shapes (Johnston, 1952, 1954; Köhler, 1973, 1976; Dulberger, 1992). In *Lithospermum caroliniense* MacMill. and *L. tubuliflorum* Greene, the LS morph produces prolate pollen with a constricted equator, and the SS morph produces ellipsoid pollen (Fig. 4a, b) (Johnston, 1952). Incorporating these dimorphic pollen shapes with phylogenetic information, it is possible to determine which morph of a heterostylous species is ancestral (if either). In *Lithospermum*, phylogenetic analyses suggest that the prolate pollen with a constricted equator produced by the LS morph is ancestral in *L. caroliniense*, but that this pollen type is the derived condition in *L. tubuliflorum* (J. Cohen and J. Davis, unpublished manuscript).

Pollen color only rarely differs between or among the morphs of heterostylous species. Darwin (1877), Barlow (1923), Dulberger (1992), and others have noted that species of *Lythrum* L. (Lythraceae) produce yellow pollen from the short- and mid-level anther levels, but olive green pollen from the long-level anthers. In *Linum grandiflorum*, SS pollen is dark blue, while LS pollen is dark gray (Dulberger, 1992), but in *Linum tenuifolium* L. SS pollen is cream-color or yellow, while LS pollen is red (Rogers, 1979; Dulberger, 1992). Recently, Massinga et al. (2005) observed that two species of *Pentanisia* produce yellow pollen from LS anthers and blue pollen from SS anthers. Not only does dimorphic pollen color provide information regarding a unique ancillary morphology linked or associated with the genes coding for the heterostylous morphology, but also researchers (e.g., Massinga et al., 2005) have used these color differences to identify morph-specific pollen grains in pollination biology studies of heterostylous species.

GENETICS, PHYSIOLOGY, AND MOLECULAR BIOLOGY OF HETEROSTYLY

“...an individual belonging to one form of the cowslip in order to be perfectly fertile must unite with one of the other form, just as a male quadruped must and can unite only with a female.” —Darwin, 1877 (p. 29)

History—In *Different Forms of Flowers*, Darwin recounts the multitude of crossing experiments he and other researchers conducted on heterostylous species. Much of Darwin’s effort focused on species of *Primula*, but he also explored the biosystematics of other taxa such as *Linum*, *Mitchella* L. (Rubiaceae), and *Lythrum*. In his studies of tristylous *Lythrum*, Darwin (1877, p. 149–150) stated, “Nothing shows more clearly the extraordinary complexity of the reproductive system of this plant, than the necessity of making eighteen distinct unions in order to ascertain the relative fertilizing power of the three forms.” These detailed experiments caused him to reevaluate his initial impression of heterostylous plants, that “they were tending to become dioecious, but [he] was soon forced to relinquish this notion” (Darwin, 1877, p. 257). These studies demonstrated that heterostyly was a stable condition, and differences between self, intramorph, and intermorph crosses exist; however, others botanists, such as Thomas Meehan (1876) and Edward Bell (1899), interpreted pollination and fertilization in a different manner. After the publication of *Different Forms of Flowers*, Meehan and Bell both published papers opposing the idea that plants outcross and do not naturally self-fertilize. Although Meehan’s view changed later in his career (1897), Bell’s did not. In 1899, Bell stated, “Radiation would likewise be almost entirely prevented by the net [covering the flowers in Darwin’s experiments], and the dew consequently would fail to fall on the anthers.... Thus the anthers could not attain under such conditions their natural condition for fertilisation” (pp. 213, 214).

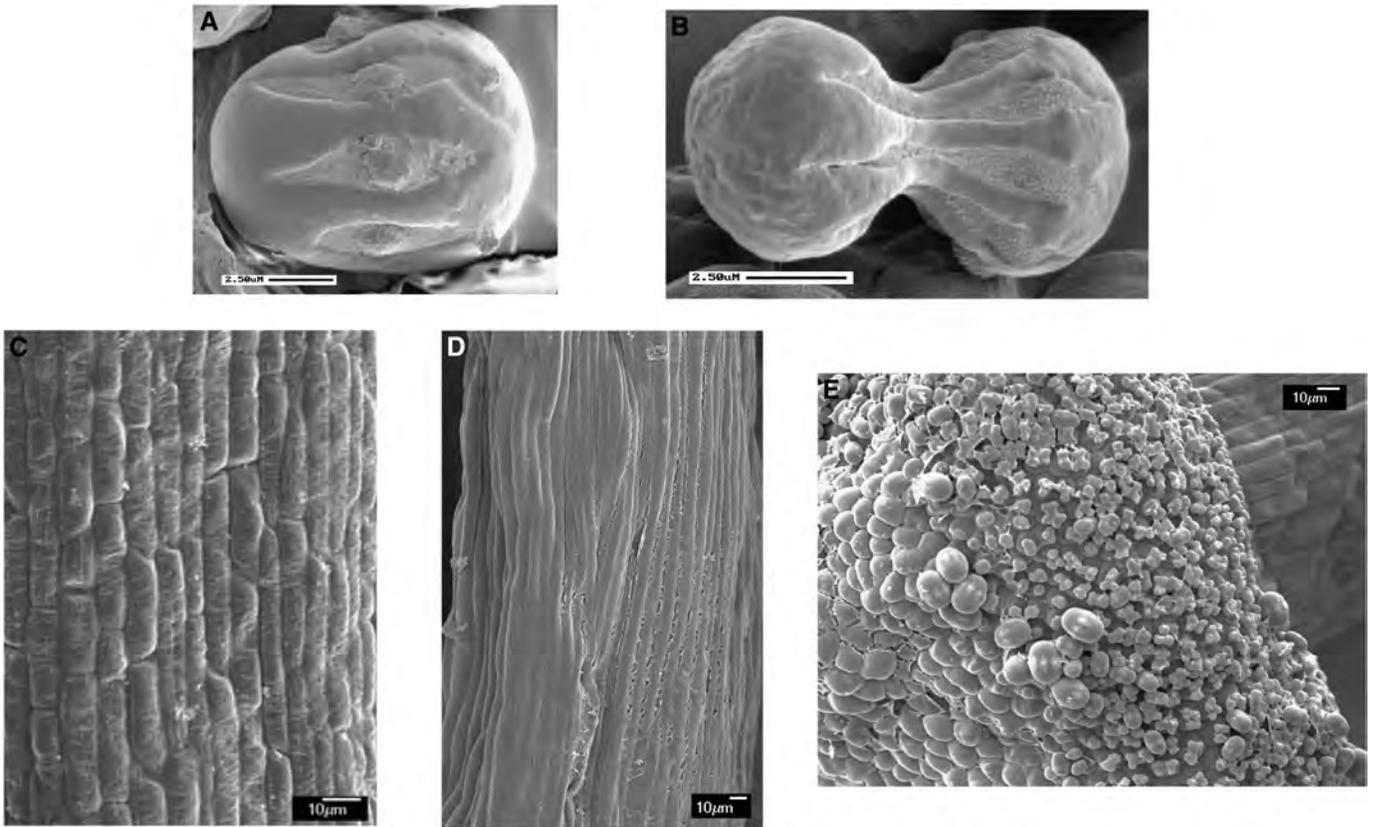


Fig. 4. Pollen and style polymorphisms in species of *Lithospermum* (Boraginaceae). (A) Pollen grain from the short-style morph of *L. caroliniense* (2000 \times), and (B) pollen grain from the long-style morph of *L. caroliniense* (2500 \times). Note the different shapes and sizes of the pollen grains between the two morphs. (C) Stylar epidermal cells of the short-style morph of *L. multiflorum* (750 \times) and (D) stylar epidermal cells of the long-style morph of *L. multiflorum* (350 \times). (E) Stigma of the short-style morph of *L. multiflorum* with larger short-style pollen grains on top of the papillae and smaller long-style pollen grains nestled among the papillae (500 \times). Scale bars: A, B = 2.5 μ m; C–E = 10 μ m.

Despite some early opposition to the ideas presented in *Different Forms of Flowers*, the studies by Darwin and others in the 1860s and 1870s formed the basis for future work on the genetics (or as Darwin termed it “transmission”) of heterostyly.

Different Forms of Flowers was published prior to the rediscovery in 1900 of Mendel’s pioneering work on genetic transmission. After this rediscovery, researchers such as William Bateson and R. P. Gregory (Bateson and Gregory, 1905), Nora Barlow (1913, 1923) (Darwin’s granddaughter), Caroline Pellew (Pellew and Durham, 1916; Pellew, 1928; Newton and Pellew, 1929), and Ronald Fisher and Kenneth Mather (Fisher and Mather, 1943) could interpret the inheritance of heterostyly in this framework. Shortly after 1900, Bateson and Gregory (1905, p. 581) stated, “In view of the results obtained by Darwin, Hildebrand, and others, it seemed likely that the characters long-style and short-style, well known in Primulaceae and other orders, might have Mendelian inheritance.” These authors went on to describe the genotypes of the SS and LS morphs. (This is also the case in most other heterostylous species that have been examined, with the exception of some members of Plumbaginaceae [Baker, 1966], *Hypericum aegypticum* L. [Hypericaceae] [Ornduff, 1979; Lewis and Jones, 1992], and several species of *Oxalis* [Oxalidaceae] [Von Ubsich, 1926].) Despite an early understanding of the genotypes of the two distylous morphs, the genotypes of the three tristylous morphs proved more difficult to unravel. The crossing studies of

Darwin (1865, 1877), Barlow (1913, 1923), and Von Ubsich (1921, 1926) provided much of the initial framework for the inheritance of tristylous, but these researchers were unable to satisfactorily determine the genotypes of each of the three morphs (Barlow, 1923). Subsequent investigators, including East (1927), Fisher and Mather (1943), and Fyfe (1950), continued to undertake crossing experiments of tristylous species, and through these and other studies, the genetics of tristylous and the genotypes of each of the three morphs were ascertained.

Information on the genetics of heterostyly allowed for advances concerning the mechanism of SI in heterostylous species. Given the genetic constitution of the two or three heterostylous morphs, researchers such as Lewis (1943, 1954) and Dowrick (1956) concluded that the SI system that operates in most heterostylous species is under sporophytic control (Dulberger, 1992; Lloyd and Webb, 1992a, b; Richards, 1997; McCubbin, 2008); however, not only does some evidence contradict this claim, but also sporophytic SI in heterostylous species may function in a different manner than that of multiallelic homostylous species (e.g., Stevens and Murray, 1982; Gibbs, 1986; McCubbin, 2008) (see Self- and Intramorph-Incompatibility later).

One geneticist, Alfred Ernst, spent 30 years crossing morphs of species of *Primula* and observing the offspring (e.g., Ernst, 1928, 1933, 1936a, b). In the course of his numerous crossing experiments, Ernst (1933, 1936b) infrequently discovered

plants with homostylous flowers (flowers with anthers and stigmas at the same height, or nonherkogamous). These aberrant individuals tended to have one suite of characters (style length and gynoeceum incompatibility type) in common with either the LS or SS morph, and one character (anther height) and one suite of characters (pollen size and pollen incompatibility type) in common with the other morph. Additionally, Ernst found plants with anomalous heterostylous flowers (e.g., flowers with style length and gynoeceum incompatibility type and anther height of the SS morph in combination with pollen size and pollen incompatibility type of the LS morph) (Ernst, 1933, 1936b; Dowrick, 1956; Richards, 2003). From these experiments, Ernst (1936a) hypothesized that three loci are involved in the control of heterostyly, with one locus controlling the development and physiological properties of each character or suite of characters. These loci are known as G/g (gynoeceum and gynoeceal incompatibility type), A/a (androecium), and P/p (pollen and pollen incompatibility type), and the recognition of these three loci has been extremely, if at times paralyzingly, influential. Ernst thought that the homostylous and anomalous heterostylous individuals he discovered developed via mutation(s) in these loci (Ernst, 1936a; Dowrick, 1956; Richards, 2003), but when Dowrick (1956) reevaluated Ernst's data, she hypothesized that these atypical individuals arose via recombination among these loci. In addition, given Ernst's data, both Dowrick (1956) and Lewis (1954) proposed an arrangement of the three linked loci: GPA. This arrangement is important for the interpretation of atypical individuals if recombination causes them to form, but it matters less if mutation is the cause (Dowrick, 1956). Ernst's experiments demonstrated the tight linkage among these three loci, and he along with others, such as Newton and Pellew (1929), Mather and De Winton (1941), Lewis (1954), and Dowrick (1956), outlined, elaborated, and refined the underlying causes of this linkage. This led to the hypothesis that these loci act like a supergene (also called the *S*-locus because the *SI* gene(s) is included in it), and heterostyly has become one of the most oft-cited examples of a supergene (e.g., Halliburton, 2004; Chen et al., 2007). Despite more ambiguous evidence outside of *Primula* for supergenic control of heterostyly (Barrett and Shore, 2008), many researchers (e.g., Matsui et al., 2004; Wang et al., 2005; Shore et al., 2006) appear to discuss the inheritance and development of heterostyly in these terms.

Current research—Through investigations of the genetics, transcriptomics, and proteomics of species of *Turnera* L. (Turneraceae), *Averrhoa* L. (Oxalidaceae), *Lythrum*, *Fagopyrum*, and *Primula*, researchers have added considerable information and complexity to our understanding of the molecular biology of heterostyly. In *Turnera*, Shore and his colleagues have examined the proteomics of heterostyly. With this approach, they have identified proteins unique to the SS style (Athanasίου and Shore, 1997; Khosravi et al., 2003, 2006), SS stamens (Khosravi et al., 2006), SS pollen (Athanasίου and Shore, 1997; Khosravi et al., 2006), and LS stamens (Khosravi et al., 2006). Although Shore and his colleagues have not identified all of the differentially expressed proteins, they have determined the identity of each of four proteins as a polygalacturonase (Athanasίου et al., 2003; Khosravi et al., 2003; Tamari and Shore, 2006), an α -dioxygenase (Khosravi et al., 2004), a β -expansin (Khosravi et al., 2006), and a cysteine protease (Khosravi et al., 2006). Wong et al. (1994) have also taken a proteomics approach to the study of heterostyly in *Averrhoa carambola* L., the star fruit. The authors observed different proteins between

the stamens and styles of the LS and SS morphs, both in immature and mature flowers, though the authors noticed a greater number of protein differences between the organs of the two morphs at anthesis than earlier in development. A recent proteomics study on *Lythrum salicaria* by Kalinowski et al. (2007) identified different protein profiles for pollen from each whorl of anthers in each morph. For example, the authors found quantitative differences of esterases on pollen grains from the long anthers of the MS and SS morphs and the mid anthers of the LS and SS morphs.

It is thought that these morph-specific proteins in *Turnera*, *Averrhoa*, and *Lythrum* are involved in either the incompatibility reactions and/or the development of the SS style (Wong et al., 1994; Athanasίου et al., 2003; Khosravi et al., 2003, 2004, 2006; Tamari and Shore, 2006; Kalinowski et al., 2007). This latter hypothesis is consistent with Lloyd and Webb's (1992b) proposed series for the evolution of heterostyly (see Phylogenetic Distribution and Evolution of Heterostyly section). In their model, the SS morph is the derived condition; therefore, suppression of the growth of the SS style would be necessary in order for two different floral morphs with different style lengths to develop. Under this model, style length of the LS morph would not require any additional proteins for elongation, as this is the ancestral condition.

Much research has been undertaken to understand the genes controlling heterostyly in buckwheat, *Fagopyrum esculentum* Moench. Because of the presence of heterostyly, plant breeders have had difficulty in their attempts to increase the yield of this economically important crop (Campbell, 1997; Wang et al., 2005), and interest in doing so appears to drive much research of this species. Researchers have examined proteins (Miljuš-Djukić et al., 2004), amplified fragment length polymorphism markers (Nagano et al., 2001; Yasui et al., 2004), random amplified polymorphic DNA (RAPD) markers (Aii et al., 1999), and genes involved in *SI* and style length (Matsui et al., 2003; Wang et al., 2005; Fesenko et al., 2006) and constructed a bacterial artificial chromosome (BAC) library (Nagano et al., 2005) to identify the putative supergene in both heterostylous buckwheat and a homostylous mutant of the species. These techniques have identified a number of genes involved in the morphological differences between heterostylous flowers, but the identity and location of the putative supergene remain elusive.

Researchers have continued to study *Primula* to identify the genetic components of the *S*-locus. Investigators have taken advantage of the numerous mutant lines developed both for heterostyly research and for ornamental breeding purposes (cf. Webster and Gilmartin, 2006; Li et al., 2008). Using these mutant lines, Li et al. (2008) identified floral development genes, one of which is linked to the *S*-locus, and Li et al. (2007) discovered two genes that cosegregate with the *S*-locus. Using RAPD and random fragment length polymorphism (RFLP) markers, Manfield et al. (2005) identified an 8.8-kb stretch of the genome that includes an SS specific RFLP marker. Barrett and Shore (2008) suggest that with the construction of a BAC library, the results of Li et al. (2007) and Manfield et al. (2005) can be used to identify the genes of the *S*-locus through positional cloning. McCubbin et al. (2006) took a different approach. These authors compared cDNA between the flowers of the two morphs and identified 11 classes of differentially expressed genes; however, the authors hypothesized that the genes are not included in the supergene as none were found to be linked to it. As with studies of *Turnera* and *Fagopyrum*,

investigators have yet to identify the supergene and its components in *Primula*.

Self- and intramorph-incompatibility—Darwin was aware of the impact self-fertilization can have on plants and therefore why a decreased amount of selfing would be beneficial, but he regarded the utility of intramorph-incompatibility as more obscure, asking “How can it be any advantage to a plant to be sterile with half its brethren, that is, with all individuals belonging to the same form?” (Darwin, 1877, p. 265). Later in the same section, he appears to reconcile the utility of intramorph-incompatibility as a component of heterostyly by explaining that “the great difference in the degree of sterility between the various heterostyled species when illegitimately fertilised, and between the two forms of the same species when similarly fertilised, harmonises well with the view that the result is an incidental one which follows from changes gradually effected in their reproductive systems, in order that sexual elements of the distinct forms should act perfectly on one another” (Darwin, 1877, p. 268). Although he was clear about the importance of SI in heterostylous species, he did not propose any physiological mechanism by which SI acts.

Since Darwin’s time, many researchers (e.g., Barlow, 1923; Dulberger, 1964, 1992; Shivanna et al., 1981; Stevens and Murray, 1982; Schou and Philipp, 1984; Barrett and Cruzan, 1994; McCubbin, 2008) have focused on identifying the components of heteromorphic SI. According to McCubbin (2008, p. 290), “Heteromorphic SI is a breeding system that combines genetically controlled floral polymorphism with diallelic, sporophytically controlled biochemical SI,” and it is almost exclusively found in heterostylous species. In contrast, homomorphic SI, the more common type of SI, is characterized by a lack of morphological differences between mating types as well as the presence of many alleles at the *S*-locus (McCubbin, 2008). Despite these differences, much of the research on heteromorphic SI appears to have taken a similar form to that on homomorphic SI; however, evidence, such as the sites and mechanisms of incompatibility (e.g., Schou and Philipp, 1984; Wedderburn and Richards, 1990; Dulberger, 1992), suggests that these two systems differ (Lewis, 1954; Brewbaker, 1959; Brewbaker and Majumder, 1961; Gibbs, 1986; Lloyd and Webb, 1992a; Barrett, 1993; Barrett and Cruzan, 1994; McCubbin, 2008).

Studies of heteromorphic SI from across the angiosperms, including species of Primulaceae (Golynskaya et al., 1976; Shivanna et al., 1981, 1983; Stevens and Murray, 1982; Wedderburn and Richards, 1990), Boraginaceae (Philipp and Schou, 1981; Schou and Philipp, 1983, 1984; Gibbs, 1986), Rubiaceae (Faivre, 2002; Massinga et al., 2005; Klein et al., 2009), Gentianaceae (Wolfe et al., 2009), Oxalidaceae (Wong et al., 1994), Amaryllidaceae (Dulberger, 1964), and Turneraceae (Tamari et al., 2001; Safavian and Shore, 2010), demonstrate that the breeding system originated multiple times (e.g., Darwin, 1877; Barrett, 1992; McDill et al., 2009; Weller, 2009). Furthermore, data from studies of these taxa provide evidence that self- and intramorph-incompatibility mechanisms should differ among heterostylous species (Schou and Philipp, 1984; Wedderburn and Richards, 1990; Dulberger, 1992; Lloyd and Webb 1992a). Some of this evidence involves the location of incompatibility in species with heteromorphic SI. The location can occur on the stigmatic surface, stigma, style, and/or ovary, and it can differ between or among morphs (Barlow, 1923; Brewbaker, 1959; Dulberger, 1964; Shivanna et al., 1981; Stevens and Murray, 1982; Schou and Philipp, 1984; Dulberger, 1992; Barrett and

Cruzan, 1994; McCubbin, 2008; Safavian and Shore, 2010). The SI interaction can be even more complex in tristylous taxa, as Barlow (1923, p. 135) noted, “...the two ♂s of one plant differ in their compatibility with the same ♀.” The occurrences of multiple sites of inhibition of pollen grains in different taxa and morphs indicate that generalizations concerning this feature of heteromorphic SI are premature. Lloyd and Webb (1992a, b) suggested that differences in the location of SI interactions provide evidence that SI evolved after the morphological aspects of heterostyly had been established (see Phylogenetic Distribution and Evolution of Heterostyly later) and that each morph (but not necessarily each species) acquired SI independently.

Because the incompatibility reaction can differ between or among morphs, with the site of SI being between the pollen grain and the stigmatic surface or the pollen tube and the stigma, style, or ovary, these data suggest that heteromorphic SI may not be exclusively sporophytic (Lewis and Jones, 1992; McCubbin, 2008). Lewis and Jones (1992), McCubbin (2008), and others have proposed that sporophytic SI operates in the SS morph, and either sporophytic or gametophytic SI functions in the LS morph. Furthermore, even though it may appear that the surface between the pollen and stigma is the site of incompatibility interactions for most SS plants, a closer examination may demonstrate that the primary site of interaction is between the pollen tube and the stigma, rather than the pollen grain and stigma (cf. Stevens and Murray, 1982).

To identify genes underlying heteromorphic SI and the heterostylous morphology, researchers can employ next-generation sequencing technology. Through comparisons of transcriptomes of floral organs at various stages of development, it will be possible to identify the genes that contribute to floral organ differences. McCubbin et al. (2006) employed this type of method, but next-generation sequencing technology can generate a much greater quantity of sequence data. Utilizing next-generation sequencing may not identify the location of the putative supergene, but it will allow for the identification of the genes involved in the heterostylous syndrome. It is important to remember that the supergene may not comprise the actual genes involved in the heterostylous syndrome, but rather the supergene could include transcription factors or other genetic components that would affect or trigger a cascade of developmental genes that would affect the physiology of the stigma, style, and/or pollen and/or increase or decrease organ length, cell division, and/or cell elongation.

From the data collected from all of the heterostylous species examined, one important conclusion can be drawn: more species should be examined to understand the SI and molecular biology of heterostyly. SI differs among species of *Primula* as well as heterostylous species in other families and orders (e.g., Dulberger, 1964, 1992). Lloyd and Webb (1992a) have suggested that Boraginaceae and Plumbaginaceae are the families most likely to include species that have evolved heterostyly in a different manner than did species of *Primula*. Perhaps, given this hypothesis, researchers should undertake more intensive studies of members of these families. However, it is clear that any taxon would provide useful information because heterostyly is known to have originated independently in 28 families and multiple times within some. Therefore, each study increases our knowledge because an independent origin represents an independent data point from which to gain information related to the genes, gene expression, and physiological interactions involved in the morphology and SI of heterostyly.

PHYLOGENETIC DISTRIBUTION AND EVOLUTION OF HETEROSTYLY

“The family of the Rubiaceae contains not far short of as many heterostyled genera as all the other thirteen families together... Several closely allied genera in this family probably owe their heterostyled structure to descent in common; but as the genera characterised are distributed in no less than eight of the tribes into which this family has been divided by Bentham and Hooker, it is almost certain that several of them must have become heterostyled independently of one another. What there is in the constitution or structure of the members of this family which favours their becoming heterostyled, I cannot conjecture.” —Darwin, 1877 (p. 256)

As evidenced from this quoted passage, Darwin recognized that heterostyly originated multiple times throughout the angiosperms, and he hypothesized that the breeding system originated only once in some families, such as Menyanthaceae, and multiple times in others, including Rubiaceae. Within the last 25 years, advances in phylogenetics and evolutionary methodology have allowed for the development of testable hypotheses to examine the evolution of heterostyly (e.g., Kohn et al., 1996; Schoen et al., 1997; McDill et al., 2009), and the resulting phylogenies have reconstructed both simple and complex patterns for the evolution of the breeding system.

Darwin (1877) noted the occurrence of heterostyly in 14 families; today, 28 families are known to include heterostylous species (Barrett and Shore, 2008). In some of these, such as Lamiaceae (Barrett et al., 2000b) and Olacaceae (Adams, 1972; Tomlinson, 1974), heterostyly has been described in only a few members, but in other families, such as Rubiaceae (Ganders 1979a), Boraginaceae (Johnston, 1954), and Primulaceae (Richards, 2003; Mast et al., 2004, 2006), numerous species exhibit the heterostylous syndrome.

Heterostyly has only been reconstructed as ancestral in one family, Menyanthaceae (Tippery et al., 2008). In this family, heterostyly has broken down and been lost on numerous occasions, giving rise to species that are either homostylous or dioecious (Tippery et al., 2008). The breeding system has also been reconstructed as ancestral in some genera, such as *Turnera* (Truyens et al., 2005), *Primula* (Mast et al., 2006), and *Amsinckia* (cf. Schoen et al., 1997). In all of these genera, the breakdown and loss of heterostyly has occurred in multiple species, and this breakdown has resulted in the evolution of self-compatible, homostylous species. This breakdown is due to either recombination, as is the case in some species of *Primula* (Mast et al., 2004, 2006), or polyploidization, as is often observed in species of *Amsinckia* (Schoen et al., 1997) and *Turnera* (Truyens et al., 2005; Shore et al., 2006).

Investigators have reconstructed fewer phylogenies in which heterostyly is the ancestral condition than in which it is the derived state; multiple origins of the breeding system commonly occur in some groups. For example, in Lithospermeae, a tribe of Boraginaceae, heterostyly has evolved independently at least once in each of three genera, *Arnebia*, *Glandora* D. C. Thomas, Weigend, and Hilger, and *Lithodora* Griseb. (Thomas et al., 2008; Ferrero et al., 2009), and at least five times in *Lithospermum* (J. Cohen and J. Davis, unpublished manuscript). These results reflect only one tribe in the family and derived members of two other tribes exhibit heterostyly (Ray and Chisaki, 1957;

Ganders, 1979a; Casper, 1985; Al-Shehbaz, 1991; Schoen et al., 1997; Brys et al., 2008; Mansion et al., 2009; J. Cohen and J. Davis, unpublished manuscript). Therefore, it appears that heterostyly originated at least 10 times within Boraginaceae. This is the case in other groups as well. Heterostyly originated at least four times in Pontederiaceae (Kohn et al., 1996), five times in Lythraceae (Morris, 2007), 3–10 times in *Linum* (Armbruster et al., 2006; McDill et al., 2009), and twice in *Narcissus* L. (Amaryllidaceae) (Pérez and Arroyo, 2004; Graham and Barrett, 2004; Barrett and Harder, 2005; Pérez-Barrales et al., 2006), a genus with independent origins of distyly and tristily.

A survey of all of these phylogenetic studies provides evidence that many independent origins of heterostyly have been reconstructed, and these data contradict the claim that heterostyly does not evolve frequently (Kohn et al., 1996; McDill et al., 2009). This claim appears plausible given the occurrence of the breeding system across all angiosperms—heterostylous species occur in approximately 7.5% of angiosperm families (28 families with heterostylous members/ca. 375 families of angiosperms), and a much smaller percentage of angiosperm species. This small percentage of angiosperm species that exhibit heterostyly supports the hypothesis that species have difficulty acquiring heterostyly *de novo*; however, the repeated evolution of heterostyly in some groups provides evidence suggesting the contrary, that with adequate conditions (e.g., tubular corolla shape, variation in the positions of sexual organs, chromosomal synteny of genes controlling floral organogenesis and SI) the breeding system can originate multiple times. However, the order in which the breeding system develops—morphology before SI or SI before morphology—is a question that still must be addressed empirically, and phylogenies will help to do so.

The order of the evolutionary development of heterostyly has remained an issue for over 100 years, and it has yet to receive adequate investigation in most groups. Darwin proposed that the morphological differences between flowers of the morphs arose prior to heteromorphic SI, albeit with a suggestion that the ancestral species was at least partially self-incompatible. He suggested that “the first step toward a species becoming heterostyled is great variability in the length of the pistil and stamens, or of the pistil alone,” while on the next page he states, “The parent-species of our several existing heterostyled plants may have been, and probably were (judging from their present constitution) in some degree self-sterile...” (Darwin, 1877, pp. 261, 262). Darwin’s proposed evolutionary pathway fell out of favor for many years, with the opposing evolutionary order becoming more widely accepted. In 1979, Deborah and Brian Charlesworth formalized the prevailing view at the time: the evolutionary developmental of SI prior to the heterostylous morphology. Using an ancestral homostylous population, they postulated that any change in the height of the anthers or stigmas within an individual would result in less efficient pollen transfer and receipt, and therefore the fitness of the plant would suffer. Consequently, SI must arise prior to herkogamy. According to their model, because a sporophytic and diallelic SI system evolves in heterostylous species, illegitimate crosses will occur between half of the individuals in the population. To ensure effective cross-pollination and fertilization, Charlesworth and Charlesworth (1979) proposed that reciprocal herkogamy then evolves.

In 1992, David Lloyd and C. J. Webb proposed the alternate order of events: species acquire the heterostylous morphology prior to the development of SI. Lloyd and Webb’s model involves a population of approach herkogamous individuals that

is invaded by a reverse herkogamous mutant, and the mutant invader's allele for short style length is dominant. The invasion of the population by this reverse herkogamous individual leads to more precise pollen placement; therefore, the mutant phenotype enjoys increased fitness which, coupled with the dominance of the allele for shorter style length, allows the reverse herkogamous morphology to quickly spread throughout the population until equilibrium is reached. Lloyd and Webb (1992b) suggest that self- and intramorph-incompatibility subsequently evolves to avoid the impact of inbreeding depression. This evolutionary series is quite similar to one put forth by Anderson (1973). In the early 1970s, to propose that the morphological features of heterostyly arose prior to SI went against the dogma of the evolution of heterostyly (Lewis, 1954; Dowrick, 1956; Baker, 1966). Due to this ideology, Anderson had difficulty publishing his heterodox ideas on the subject (W. R. Anderson, University of Michigan, personal communication).

Phylogenetic analyses provide the ability to investigate the order of the evolution of the components of heterostyly, but to do so these studies must include both major features of the breeding system—type of herkogamy and SI. Observing the type of herkogamy is the easier task, as this can be done with pressed herbarium specimens. The more time intensive data collection, determining the presence of self-compatibility or self-incompatibility, involves crossing studies, such as those of Stebbins (1950) and Grant (1971, 1981). Kohn et al. (1996) and Pérez-Barrales et al. (2006) have undertaken phylogenetic studies utilizing both kinds of data, and the results from these studies differ. In Pontederiaceae, Kohn et al. (1996) reconstructed the tristylous morphology having evolved in species that are self-compatible, but in *Narcissus* (Pérez-Barrales et al., 2006), self-incompatibility is the ancestral condition with the reciprocal herkogamous morphology subsequently evolving. Given the limited number of critical studies, it cannot be determined which evolutionary developmental series, if either, predominates within angiosperms. However, dogma appears to have shifted in the order of the evolutionary development of heterostyly, with more recent literature (e.g., Faivre, 2000; Barrett et al., 2000b; Barrett and Shore, 2008) favoring morphological features evolving prior to SI rather than subsequently.

DEVELOPMENT OF HETEROSTYLOUS FLOWERS

“But by the time the flowers [of the long-styled morph of *Linum perenne*] have expanded, the five stigmas twist round so as to face the circumference, owing to the torsion of that part of the style which lies beneath the stigma.” Darwin, 1877 (pp. 94, 95)

Many studies have focused on the morph-specific differences of the mature flowers of heterostylous species (e.g., Darwin, 1877; Ganders, 1979c; Dulberger, 1992; Wolfe et al., 2009), but fewer investigations have examined the developmental differences between or among these morphs. Floral development in heterostylous species often follows intricate patterns because two or three different developmental pathways occur in a species, one in each morph. Darwin (1877) only touched on the topic of floral development in *Different Forms of Flowers*, noting late-stage floral developmental differences between the two morphs of species of *Linum*. Recently, Armbruster et al. (2006) have treated this subject in more detail. During the last 100 years, fewer than 20 studies have critically examined the floral

developmental patterns in heterostylous species. These studies can complement investigations into the morphological, ecological, phylogenetic, and molecular aspects of heterostyly.

Floral developmental patterns differ between distylous and tristylous species because distylous species require organs positioned at two, rather than three, heights. Because only two organ heights must be attained, the flowers of distylous species generally follow less intricate developmental patterns than those of tristylous species; therefore, floral developmental patterns in distylous species will be discussed prior to those in tristylous species.

In distylous species, the anthers of the two morphs can attain their distinct heights through differences in filament length and/or point of attachment of the filaments to the corolla. Regardless of the organs involved in the positioning of the anthers in each morph, the two heights are attained by different uniform growth rates, with these organ(s) in the LS morph having a slower growth rate than those in the SS morph (Riveros et al., 1987; Richards and Barrett, 1992; Richards and Koptur, 1993; Faivre, 2000; Hernández and Ornelas, 2007; Cohen et al., 2008). Anther height differences between morphs that are primarily due to the point of adnation of the filaments to the corolla tube occur in *Guettarda scabra* Lam. (Rubiaceae) (Richards and Koptur, 1993), *Bouvardia ternifolia* Schlttdl. (Rubiaceae) (Faivre, 2000), *Primula vulgaris* Huds. (Webster and Gilmartin, 2006), *Quinchamalium chilense* Molina (Santalaceae) (Riveros et al., 1987), and species of *Lithospermum* (Cohen et al., 2008). In *Lithospermum*, the lengths of the epidermal cells of the corolla tube do not differ between the two morphs, and the contribution of filament length to anther height is negligible. Therefore, differential cell division, either above or below the point of attachment of the filaments to the corolla tube, determines anther height in each morph (Cohen et al., 2008). Differences in the heights of the anthers between morphs due to a combination of filament length and point of adnation of the filaments to the corolla tube occurs in *Hedyotis salzmanii* (DC.) Steud. (Rubiaceae) (Riveros et al., 1995), *Palicourea padifolia* (Hernández and Ornelas, 2007), and some species of *Psychotria* L. (Rubiaceae) (Faivre, 2000).

Gynoecium development between the two morphs follows more diverse developmental patterns than does androecium development. Researchers have described four developmental pathways of gynoecium development in distylous species. In two species of Rubiaceae, *Hedyotis salzmanii* (Riveros et al., 1995) and *Bouvardia ternifolia* (Faivre, 2000), different rates of stilar growth early in development have been reported between the two morphs. Different developmental patterns have been described in other species of Rubiaceae. For example, two species of *Psychotria* develop structural differences early in ontogeny that continue throughout development (Faivre, 2000); therefore, even though the gynoecia of both morphs grow at the same rate, early developmental differences in initial organ size result in the distinct stigma heights in each morph. In other taxa, such as *Guettarda scabra* (Richards and Koptur, 1993), *Faramea suerrensis* Donn.Sm. (Richards and Barrett, 1992), *Palicourea padifolia* (Hernández and Ornelas, 2007), *Primula vulgaris* (cf. Webster and Gilmartin, 2006), *Quinchamalium chilense* (Riveros et al., 1987), and species of *Lithospermum* (Cohen et al., 2008), no differences in initial growth rate exist between the two morphs. In these species, the style of the LS morph grows at a constant rate, and the shorter style of the SS morph develops either via a decreased rate of late-stage stilar growth, as is the case in *Guettarda scabra* (Richards and

Koptur, 1993), or through cessation of styler elongation, as occurs in species of *Lithospermum* (Cohen et al., 2008). Because researchers have examined the developmental patterns of only a small number of distylous species, other floral developmental pathways may also occur.

Investigators have studied the floral developmental patterns of members of three of the six families known to include tristylous representatives—Pontederiaceae, Oxalidaceae, and Lythraceae (Richards and Barrett, 1984, 1987, 1992; Mal, 1998), and the developmental sequence can differ within morphs, between morphs, and among taxa. Patterns of stamen development in tristylous species of Pontederiaceae differ from those observed in tristylous members of Oxalidaceae and Lythraceae (Richards and Barrett, 1992). In species of Pontederiaceae, the stamens of the three morphs tend to develop through the two androecial whorls growing at different rates; while the stamens of the three morphs of species of Oxalidaceae and Lythraceae that have been studied develop via variation in the timing of stamen primordium initiation and subsequent size differentiation (Richards and Barrett, 1992). Mal (1998) described different rates of filament growth among the three morphs of *Lythrum salicaria*, and he concluded that these growth rates contribute to the different heights of the two whorls of anthers in each morph. However, Mal also recognized that early developmental differences in timing and organ size may play a role in stamen development.

Gynoecia of the three morphs of tristylous species tend to develop following a less intricate pattern than stamens of tristylous species. In general, the stigma heights in each morph of the tristylous species of Pontederiaceae, Lythraceae, and Oxalidaceae that have been examined develop via different growth rates, with one exception (Richards and Barrett, 1984, 1987, 1992; Mal, 1998). In *Pontederia cordata* L. (Pontederiaceae), Richards and Barrett (1987, 1992) observed that the rate of growth of the gynoecium of the SS morph greatly slows or stops much earlier in development than that of either the MS or LS gynoecium. Therefore, the stigma of the SS morph attains its height much earlier in development than that of either the MS or the LS morph (Richards and Barrett, 1992).

We have a greater understanding of the floral developmental patterns of heterostylous species today than we did in Darwin's time, but there is still a great deal to learn from studies of floral development. Future studies should focus on heterostylous species from different branches of the angiosperm phylogeny, such as members of Menyanthaceae, Lamiaceae, Erythroxylaceae, Amaryllidaceae, and Polygonaceae, because studies of these taxa would complement the current body of research. In addition, investigators should examine the floral developmental patterns of homostylous relatives of heterostylous species; these taxa may provide insight into the ancestral floral developmental pattern(s) of heterostylous species. Faivre (2000) took this approach and compared approach herkogamous and heterostylous species of *Psychotria*. She found that a slight modification in the developmental pattern of an approach herkogamous species could result in a flower with the same morphology as either the LS or the SS morph. In *Lithospermum*, Cohen et al. (2008) examined an approach herkogamous species, a reverse herkogamous species, and three heterostylous species. The authors discovered that floral developmental patterns in the approach and reverse herkogamous species were similar, respectively, to the patterns in the LS and SS morphs of the heterostylous species. More comparative floral developmental studies can increase our knowledge of the manners in which various floral

organs attain consistently different positions in the morphs of heterostylous species.

CONCLUSIONS AND FUTURE DIRECTIONS

Since the publication of *Different Forms of Flowers*, the plethora of studies related to heterostyly have added considerably to our understanding of the multiple aspects of the breeding system. Investigators have tested many of the hypotheses Darwin proposed, and because of these studies, many additional hypotheses have been advanced. Through the combination of modern techniques, such as next-generation sequencing technologies and phylogeny reconstruction tools, with classical methods, such as microscopy and crossing studies, researchers can continue to investigate heterostyly in a holistic manner.

I propose four areas of research that may help provide answers to questions Darwin investigated regarding heterostyly. These are:

(1) Examining the interconnection between SI and morphology, particularly micromorphology. Research has provided evidence for a connection between these components, but the extent of this connection remains unclear. Our attempts to understand the interrelationship between these traits, particularly between pollen grains and the stigma, will allow for the determination of the function of these features: which are adaptive and which are solely consequences of development.

(2) As molecular genetic data become available, investigators can undertake a variety of comparative studies. Intraspecific studies can investigate whether the genes that control the different components of the heterostylous syndrome differ between or among the flowers of the morphs, while interspecific studies can involve the exploration distantly related taxa to determine whether orthologues or different genes control the development and physiology of the morph-specific floral characteristics.

(3) Researchers should continue to study the evolution of heterostyly through phylogenetic reconstructions, and they should strive to include both major components of heterostyly—type of herkogamy and SI—in analyses as this strategy can help determine the order in which these characters originated.

(4) Heterostyly presents a unique opportunity to investigate developmental patterns and constraints. In order for a heterostylous species to be successful, the organs must grow to specific positions in each morph, and how these organs do so tends to differ among species. Therefore, floral developmental studies can identify different patterns in which the heterostylous morphology arises as well as provide clues to the evolutionary origin and pollination ecology of species. Furthermore, researchers can couple developmental data with molecular and/or phylogenetic studies to examine the interrelationship between floral development, molecular biology, and evolution.

As more data become available, we can synthesize all of this information, and this will allow for the establishment of rules that govern the evolution and development of heterostyly. In addition, these data will help provide an understanding of the "constitution or structure" by which heterostyly has repeatedly originated in some taxa, such as Rubiaceae, Linaceae, and Boraginaceae, but only rarely or ever in other groups, including Gentianaceae, Ericaceae, and Caryophyllaceae. Darwin (1877, p. 28) stated, "...[heterostyly is] a case to which no parallel exists in the vegetable or, indeed, in the animal kingdom," and, due to this remarkable case, it continues to fascinate botanists, geneticists, ecologists, and evolutionary biologists.

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