Sexual system variation in legumes (Leguminosae): underpinning genomic study with new tools to describe inflorescence morphology

Short Title: Sexual system variation in legumes

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Abstract

Although the great majority of legume species are cosexual with hermaphrodite flowers, a variety of sexual systems are observed in the family, including monoecy, andromonoecy, androdioecy and dioecy. Such broad terms conceal much variation, details that may be of importance in understanding the evolutionary and ecological basis of reproductive systems. This variation is often inadequately described in taxonomic works which, through practical necessity, require brevity. Here, we provide a brief overview of the sexual systems of legumes and propose a simple notation for summarizing sexual variation in the form of an unequivocal formula: the inflorescence formula. We also suggest a protocol for the detailed description of sexual variation, which we hope will be useful in guiding future studies of sexual variation in plants. Thorough knowledge of sexual variation at the morphological level is an important prerequisite for molecular investigation of the evolutionary developmental biology of sex systems. Legumes provide a rich field for future evo-devo investigations in this area.

Introduction

In a previous volume in this series (Advances in Legume Systematics 13), a contribution (Egan and Vatanparast 2019) comprehensively reviewed the enormous advances in legume genomics until that time. Progress has continued unabated. Some of the most exciting developments are associated with understanding the genetic basis and evolution of key legume functional traits, such as nodulation (Griesmann et al. 2018), ushering in a new era of comparative legume genomics. Among other traits that could be advanced as candidates for detailed analysis are floral developmental traits which are of great interest to the systematist, but also to the ecologist and agriculturalist, as development controls breeding systems, fruit set and reproductive success. However, clear, concise and comparable descriptions are not always available for such traits, hampering advances in our knowledge of genomics, development and evolution of floral morphology.

Genomics and trait evolution

Before genomic resources were available for legumes, candidate gene approaches using developmental genes discovered in unrelated model organisms such as *Arabidopsis* Heynh. (Brassicaceae) and *Antirrhinum* L. (Plantaginaceae) had to be used (e.g. Raimundo et al. 2013). This approach was limited by the requirement for conserved developmental pathways and orthologous genes of similar function. Nevertheless, the candidate gene approach has sometimes worked well. An example is the discovery of the control mechanisms for floral symmetry in legumes by transfer of insights gained from transposon-tagged mutagenesis in the model organism *Antirrhinum majus* L. (the snapdragon). The discovery of a key role for the gene *CYCLOIDEA* (*CYC*) in controlling adaxial floral identity, and hence floral zygomorphy, in *Antirrhinum* allowed orthologues of *CYC* to be cloned from the legume family (Citerne et al. 2003). Subsequent work with in situ hybridization showed that expression patterns of the CYC orthologue *LegCYC1B* involved in the establishment of the dorsoventral axis of *Lupinus nanus* Douglas ex Benth. flowers putatively implicates a similar role for *CYC* in legume floral morphology (Citerne et al. 2006). A model could then be proposed and tested to explain the anomalous floral symmetry in the papilionoid legume *Cadia* Forssk. Instead of being an evolutionary reversal, the radial flowers of *Cadia* come from a gain-of-function change in expression of *LegCYC1B* that promotes dorsal identity in all petals, rather than only on the standard petal, the typical condition in the bilateral papilionoid flower (Citerne et al. 2006). Thus, instead of a single dorsal petal, the *Cadia* flower effectively has five "standard petals".

The problem is that many important legume traits, particularly those of tropical and woody

legumes, cannot be studied by forward genetic screens, as in those groups there are no convenient model organisms. A good model organism should have small size be early flowering, so they can be taken through their life cycle in the laboratory, as well as being suitable for mutagenesis screens and genetic transformation. Woody legumes are therefore challenging because they do not exhibit these traits. Furthermore, many of the morphological traits of interest have no equivalent in other families where more tractable genetic resources exist, such as *Arabidopsis*. Even model papilionoids such as *Lotus* L. and *Medicago* L. may have limited use when studying non-papilionoid legumes. In such cases, whole genome studies are the only practical line of investigation.

A good example is the variation of sexual systems in plants, where several control mechanisms have been worked out by genomic approaches and where every family appears to have a unique molecular mechanism (Diggle et al. 2011; Hobza et al. 2018), rendering the candidate gene approach hard to use (Cronk and Müller 2020). It is therefore possible that dioecy and monoecy in legumes are controlled by legume-specific or even clade-specific molecular mechanisms. This seems to be the case in other families for which molecular mechanisms have been worked out. For instance in *Diospyros lotus* L. (date plum; Ebenaceae) feminization is produced by the autosomal transcription factor *Male Growth Inhibitor* (*MeGI*), which can be toggled to male by a Y-chromosome specific suppressor, *Oppressor of MeGI* (*OGI*) (Akagi et al. 2014). OGI, present only in males, is a gene that produces suppressing small RNAs. In dioecious *Populus trichocarpa* Torr. and A.Gray ex Hook. and *P. tremuloides* Michx. (Salicaceae) there is a constitutive feminizing response regulator gene *popARR17*, which is suppressed to make males by a male-specific locus that produces small RNAs responsible for RNA-directed DNA methylation (RdDM) of *popARR17* (Müller et al., 2020). While the overall mechanism has some similarity between the two trees *Diospyros* and *Populus*, the genes and details of the pathway are completely different, as we would expect from dioecy having independent origins in these two cases. The same seems to be true of monoecy (Cronk 2021), the basis of which has been worked out in *Zea mays* L. (maize; Poaceae) and *Cucumis melo* L. (melon; Cucurbitaceae). In maize, spatial separation of the sexes is due to the interplay of masculinizing genes *TASSEL SEED 1&2* and the female-promoting gene *SILKLESS* (Li and Liu 2017). Melon has a superficially similar system, with masculinizing gene *WIP1* and female-promoting gene *ACS11* (Boualem et al. 2015). However, the nature of the genes themselves and the details of their action are different.

Although the availability of genomic resources, and in particular whole genome sequences, make such studies tractable, they do not necessarily make them easy. In the *Populus* example elucidation of the sex determination mechanism required study by genome-wide association (GWAS;

Geraldes et al. 2015), transcriptomics (Cronk, Soolanayakanahally and Braeutigam 2020), methylomics (Braeutigam et al. 2017), single-molecule long-read sequencing (Müller et al. 2020) and sRNA-seq (Müller et al. 2020). However, with ever-reducing costs and improved bioinformatic pipelines, many of these techniques are becoming more and more straightforward. Sex determination (in dioecy) is recognized to be a particularly problematic trait to investigate at the genomic level. Critical sequences are generally hemizygous (on Y or Z chromosomes), and they may be associated with repetitive and inverted sequences, long palindromes and segmental duplications. Some indication of the difficulties in genomic studies of sexual systems comes from the fact that although the first draft of the human genome was released in 2001 (Venter et al. 2001), sequencing and assembly of the Y chromosome was only completed in 2023 (Rhie et al. 2023). By contrast, the genes controlling monoecy do not have the problem of being situated in problematic regions of the genome, but they have other problems, as they do not segregate between individuals and are thus difficult to study genetically. Monoecious systems (including andromonoecy and gynomonoecy) are very common and are important as a step in the evolution of dioecy, yet they have been described as "surprisingly understudied" (Cronk 2021).

Legumes are a large family, and therefore it is no surprise that there are many instances of sexual system variation within the family, even though the bulk of the family is monomorphic for hermaphrodite flowers. Dioecy appears frequently, and even more common is andromonoecy, especially in the mimosoids (Caesalpinioideae, tribe Mimoseae; Bruneau et al. 2024). However, there are as yet no worked-out examples of the molecular basis for such variation. With the rapid accumulation of legume genomic resources, including many whole genomes, we believe the time is right for a concerted investigation of legume sexual system variation.

There are two complementary methods of explaining sexual system variation, and both are required for a full understanding. First, there is the mechanistic or "how question" that requires uncovering the molecular mechanism for sex determination or spatiotemporal variation in sex expression. Second, there is the theoretical or "why question" of how we can model the selective forces underlying the evolution of sex systems.

Developmental biology of sex system evolutionary transitions
The mechanistic approach not only involves a knowledge of the genes involved but also a knowledge of the determinants of spatial and temporal regulation of gene expression, for instance by phytohormones and phytohormone gradients across inflorescences. Hormones can be artificially

manipulated, as has been done in melon (*Cucumis melo*). Sex expression in melon is regulated by the ethylene pathway (Martínez and Jamilena 2021) and forced outgassing of ethylene by growing female melons under hypobaric conditions results in the formation of hermaphrodite flowers, as endogenous ethylene is involved in the development of female flowers (Byers et al. 1972).

The andromonoecious system that is present in many mimosoid legumes is promising for study as the hypothesis that floral morphogenesis is responding to hormonal gradients across the mimosoid capitulum can be tested. An analogy can be made with the Asteraceae capitulum, which is strictly acropetal in development. In the developing Asteraceae capitulum, there is a temporal variation of auxin concentration to which development responds, the patterning of the capitulum therefore being heterochronic. Initially, the young Asteraceae capitulum has a high concentration of auxin, but as the developing capitulum expands, the concentration of auxin decreases markedly (Zoulias et al. 2019). Thus, early developing organs (the often gynoecious ray florets) develop in a high auxin environment, whereas the inner florets (hermaphrodite disc florets) develop in a low auxin environment. Exogenous addition of auxin to the later-stage capitulum induces the reappearance of ray florets in the centre of the capitulum (Zoulias et al. 2019). Floral development in *Dichrostachys cinerea* (L.) Wight & Arn. (Fig. 1) indicates that similar mechanisms may be at play in mimosoid legumes, including *Neptunia* Lour. (Tucker 1988b). Inflorescences of that species include sterile flowers towards the base and fertile flowers towards the apex, with intermediate flowers in between both (Venkatesh 1951). Gradual variation in the development of fertile structures in the intermediate flowers along the inflorescence axis (Venkatesh 1951) suggests the existence of such a hormonal gradient controlling sexuality and the presence or absence of particular organs.

Tucker pointed out that in legumes loss of organs may result from two different ontogenetic pathways (Tucker 1988c). In the first pathway, primordia of the missing sexual organs may be entirely absent from inception, as in the petals of the papilionoid *Ateleia* DC. (Tucker 1990). In the second pathway, in contrast, organ primordia may form, but the organs may be suppressed after initiation (Tucker 1992b). In the case of many legume unisexual flowers, pistil or stamen developmental pathways are not completely deleted, but follow the second pathway, and are suppressed after organ formation. This is also the case with the petal suppression in *Amburana cearensis* (Allemão) A.C.Sm. (Leite et al. 2015). With sexual organ suppression, in male flowers a pistil forms and is suppressed later in ontogeny, leaving a small pistillode. In female flowers, the developmental suppression of stamens may happen at midstage producing staminodes, as in *Bauhinia* L. (Tucker 1988a). Occasionally, normal stamen development may be altered even later in development, producing sterile stamens which fail
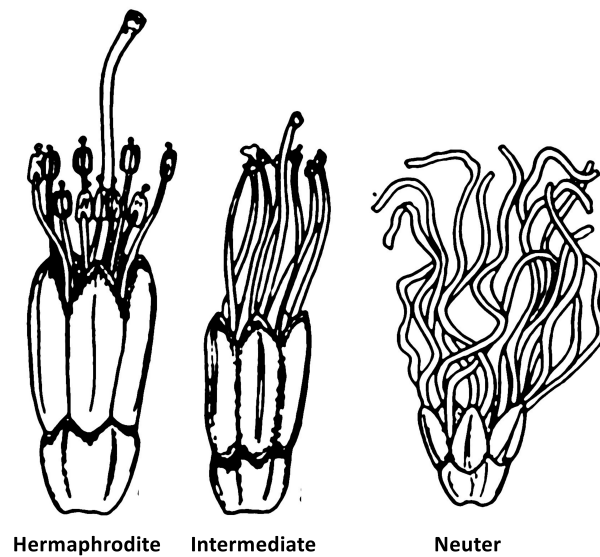
Hermaphrodite **Intermediate** **Neuter**

Figure 1. Floral morphs of *Dichrostachys cinerea* after Venkatesh (Venkatesh 1951). The inflorescence formula (see text) is: n34 i11 h117>. The flower numbers in the formula are average counts from 4 inflorescences.

to form pollen, as in certain of the diversified stamens of *Senna* Mill. (Tucker 2003). This pattern of suppression allows the prediction of separate, late-expressed stamen- and pistil-suppressing genes. In andromonoecy, for instance, a pistil-suppressing gene is likely to be under hormonal or other spatiotemporal control. As andromonoecy appears to have had multiple evolutionary origins, it is an open question whether the same gene has been recruited numerous times or whether different genes may have been recruited independently. The discovery of such a gene or genes would be of great interest for the evolutionary study of plant sexual systems.

Evolutionary biology of sex system transitions

Theoretical approaches require the formulation of models to explain the selective advantages of evolutionary shifts from the hermaphrodite norm. The widespread occurrence of the hermaphrodite flower throughout the angiosperms may in part be explained by developmental canalization, as the conserved four whorl (sepal-petal-stamen-pistil) structure in eudicots (including legumes) appears remarkably stable. However, there is a theoretical reason why bisexual flowers might be selected for. The allocation theory (Charlesworth and Morgan 1991) suggests that a single bisexual flower is efficient in that it invests in non-sexual, but essential, structures like sepals and petals that benefit

both male and female function together, without having to duplicate them separately for each sex. A combination of developmental canalization and allocation efficiency together may therefore provide a strong barrier to the evolution of non-hermaphrodite flowers. It is thus reasonable to assume that in cases where transitions have happened, there has been a selective pressure, and the modeling of this selective pressure has produced an extensive literature, largely built on two influential papers from the 1970s (Charlesworth and Charlesworth 1978a; Charlesworth and Charlesworth 1978b).

In the case of dioecy, outbreeding (increased offspring quality) is an obvious driver, but this is less convincing in monoecious systems. A classic study (Primack and Lloyd 1980) of andromonoecy in *Leptospermum scoparium* J.R.Forst. & G.Forst. (Myrtaceae) ruled out andromonoecy as simply an outbreeding mechanism, instead suggesting two hypotheses. First, that there might be an advantage for pollen to be presented in more flowers than just seed-bearing flowers (i.e. the pollen donation hypothesis: that additional pollen is advantageous), and secondly, that an individual plant could adjust its reproductive output to its physiological conditions by reducing seed-bearing flowers (i.e. the reallocation hypothesis: reducing investment in seed production to invest in other fitness traits). More recently a third hypothesis, that of increasing female function, has been added (Vallejo-Marin and Rausher 2007a; 2007b). The latter may work, for instance, by increasing pollinator attractiveness and hence seed set, or by allowing reallocation of resources to seed production. These hypotheses are not necessarily mutually exclusive. The further application in monoecious species of resource allocation analyses of the sort that have been applied in dioecious and hermaphrodite species (Allen and Antos 1988; Deplph 1990; Ehrlén 1993) would be of considerable interest here.

Sexual variation in legumes: a preliminary survey

Legume flowers may be hermaphrodite, male, female or neuter. Neuter flowers, although having no direct sexual role, are part of the synorganization of the inflorescence and therefore have a likely functional role in inflorescence reproductive ecology by increasing the attractiveness of the inflorescence to pollinators. The concept of synorganization, which we define here as *"the provision of a novel or more efficient function by different plant organs working in concert"*, is an important principle in floral evolution (Endress 2016), but it also applies to inflorescence evolution when flowers are heteromorphic in an inflorescence, such as ray and disc florets in Asteraceae. Neuter flowers (as in the mimosoid *Dichrostachys* (DC.) Wight & Arn., *Neptunia* and *Parkia* R.Br.) are of special interest as in

addition to the suppression of anthers and pistils they may have greatly enlarged, flattened and coloured staminodes (Tucker 1988b; Venkatesh 1951).

Table 1. Some genera of interest, taken from general references (Renner 2014; Yampolsky and Yampolsky 1922), and specific references as given in table. Species numbers are taken from Plants of the World Online (POWO 2023). A more complete list for Caesalpinioid legumes is given as supplementary data.

Genus	Species	Sex type	References
Acacia	1084	Hermaphrodite, andromonoecious	(Sedgley, Harbard, Smith, Wickneswari and Griffin 1992)
Adenanthera	12	Hermaphrodite, andromonoecious	(Borges, Machado and Lopes 2017)
Albizzia	99	Hermaphrodite, andromonoecious	(Wyatt and Lipow 2021)
Anthonotha	17	Andromonoecious	(Beavon and Chapman 2011)
Apuleia	1	Andromonoecious	(Falcao et al. 2020; Zimmerman, Prenner and Bruneau 2013)
Ateleia	27	Dioecious	(Tucker 1990)
Bauhinia	193	Hermaphrodite, andromonoecious, dioecious, androdioecious, monoecious	(Torres-Colin, de Stefano and Can 2009; Tucker 1988a)
Caesalpinia	10	Andromonoecious	(Bullock 1985)
Calliandra	149	Andromonoecious	(Prenner 2004)
Cenostigma [Caesalpinia]	15	Andromonoecious	(Bullock 1985)
Ceratonia	2	Dioecious, hermaphrodite	(Arista, Ortiz and Talavera 1999; Tucker 1992a)
Chamaecrista	367	Subgynodioecy (rare male steriles)	(Williams and Fenster 1998)
Coulteria [Caesalpinia]	10	Andromonoecious	(Bullock 1985)
Dichrostachys	16	Hermaphrodite + neuter	(Venkatesh 1951)

Erythrostemon	33	Hermaphrodite, andromonoecious	(Calvino and Galetto 2010; Gibbs, Lewis and Lughadha 1999; Lewis and Gibbs 1999)
Gleditschia	13	Androdioecious, functionally dioecious	(Cerino, Castro, Richard, Exner and Pensiero 2018; Ruiz et al. 2022)
Gymnocladus	6	Dioecious, androdioecious	(Choudhury, Khan and Dayanandan 2014; Zaya and Howe 2009)
Heteroflorum	1	Dioecious	(Sousa 2005)
Libidibia [*Caesalpinia*]	7	Andromonoecious	(Bullock 1985)
Mimosa	600	Hermaphrodite, andromonoecious	(Wyatt and Lipow 2021)
Neptunia	11	Andromonoecious + neuter	(Tucker 1988b)
Parkia	38	Andromonoecious + neuter	(Pedersoli and Teixeira 2016)
Pithecellobium	23	Hermaphrodite, andromonoecious	(Bawa and Beach 1981)
Stryphnodendron	28	Andromonoecious	(Pedersoli and Teixeira 2016)
Vachellia	157	Andromonoecious	(Baranelli, Cocucci and Anton 1995; Marquez, Carbone, Aguilar and Ashworth 2019)
Vatairea	8	Andromonoecious	(Costa and da Silva 2015)
Zapoteca [*Calliandra*]	23	Andromonoecious	(Bullock 1985; Hernandez 1989)

The different sexual floral morphs found in plants may be present in individuals and populations according to various patterns. In legumes specifically, most species bear flowers which are uniformly hermaphrodite, but andromonoecy is common, and other types are occasionally found. We have not attempted a complete survey of the family here, which would be a challenging task and would probably require concerted investigation by a coordinated team of researchers. However, we have compiled a list of examples of genera departing from strict hermaphroditism, taken from the readily available taxonomic, ecological and morphological literature (Table 1; Supplementary Table S1). As can be seen from Table 1, andromonoecy is the most common such change, with numerous

examples of dioecy. Interestingly, in the context of the Caesalpinioideae surveyed here (Supplementary Table S1), dioecy occurs sparsely across the base of the caesalpinioid phylogeny backbone (Fig. 2), while andromonoecy is particularly common in the mimosoid clade and in closely related genera previously thought to be transitional between the caesalpinioid and mimosoid flower morphologies (e.g., *Dimorphandra* Schott and *Pentaclethra* Benth.; De Barros et al. 2017). This particular phylogenetic distribution of andromonoecy indicates that the andromonoecious morphospace was first evolutionarily explored by different lineages and then fixed as a constituent trait of the mimosoid inflorescence. Elucidation of this question and how it relates to alternative models of evolution (the pollen donation hypothesis in this case) ultimately relies on a detailed account of sexual variation. Unfortunately, the sexual system of many genera and species is not clearly described in taxonomic accounts, particularly when variations, such as andromonoecy in the mimosoids, are seen as commonplace.

A particular problem is the widespread use of the ambiguous term "polygamy" without qualification, particularly in older botanical works. This term was coined by Linnaeus (Linnaeus 1751) and has been used in several senses. In the broad sense, it means any and all combinations of hermaphrodite and unisexual flowers, so it may refer to many types of monoecy and dioecy (see Table 2). In the *Philosophia Botanica* he defines it as "*quaeque hermaphroditis & femineis aut masculis simul, POLYGAMA dicitur*" [Each [species] with hermaphroditic and female or male [flowers] together, is called POLYGAMOUS] (Linnaeus, 1751). One of the examples he gives is a legume, *Gleditsia* L., of which he writes "*Polygami ex Hermaphrodita & Masculo in eadem planta: Femina in distincta*" [Polygamous from Hermaphrodite & Male on the same plant: Female on a separate one] However, in the same work, Linnaeus gives seven other, very different, ways of being polygamous. This is a general term, and more specific ways of describing sexual variation would be advantageous.

A formula for summarizing sexual variation of inflorescences

Aiming to facilitate the description of sexual systems in a clear and straightforward manner, here we introduce a simple notation for collecting basic information within and between inflorescences and individuals. The formula offers a convenient way of collecting information in the field or herbarium using certain standard categories. As such it may act as an *aide memoire* for the collection of standardized information. The notation is given in Table 3. The inflorescence formula described here is only intended to describe the distribution of floral polymorphism within and between inflorescences.

It is not intended to describe inflorescence architecture (branching pattern) which can be very complex and is best represented diagrammatically.

Table 2. Notation used in inflorescence formula. Note that a basic formula may use just flower types and separators; more precise formulas may use numerics, phenology or proximo-distal notation. The summary plant mating category (Amc, Dc etc, see Table 3) does not need to be given with the formula but may be give for convenience.

NOTATION	EXPLANATION
Flower types	
h	Hermaphrodite (bisexual) flower
m	male flower
f	female flower
n	neuter flower
i	intermediate flower type (position in formula relative to other flowers types indicates nature of intermediacy, e.g. mif = intermediate between males and females)
Separators	
+	separates different individuals in population, where gender differences segregate in population
/	separates different inflorescences on a plant, where inflorescence polymorphism within an individual exists
Position within inflorescence (proximal-distal)	
>	distal (apical)
>>	more distal, most distal
*	scattered in inflorescence
Phenology	
^	indicates type of flower opening first
Flower number and rarity	
numeric value	number of flowers of given type in inflorescence, e.g. 10-18f 4-8m
–	range
()	rare type, e.g. mf(h), male and female flowers (with rare hermaphrodites). See discussion under pleogamy.

The flower types are obvious (m, f, h etc.), and particular attention should be paid to the separators (/ = different inflorescences; + = different plants). If no separator is used, all inflorescences are of the same type. For example, strict dioecy will be m+f, while monoecy with male and female flowers in separate inflorescences will be m/f. A basic use of the formula only requires floral types and separators. However, the position of floral morphs within an inflorescence, the order of anthesis and the number or rarity of floral morphs can also be indicated by additional symbols. The formula allows for the use of quantitative information (number of flowers of each morph). We suggest that typical numbers only are given here as detailed statistical information on flower number variation can be given separately if needed for a particular study. If quantitative information is not available, or not germane to a particular study, the inflorescence formula can be used without numbers, to indicate the distribution of floral morphs within and between inflorescences and within and between individuals.
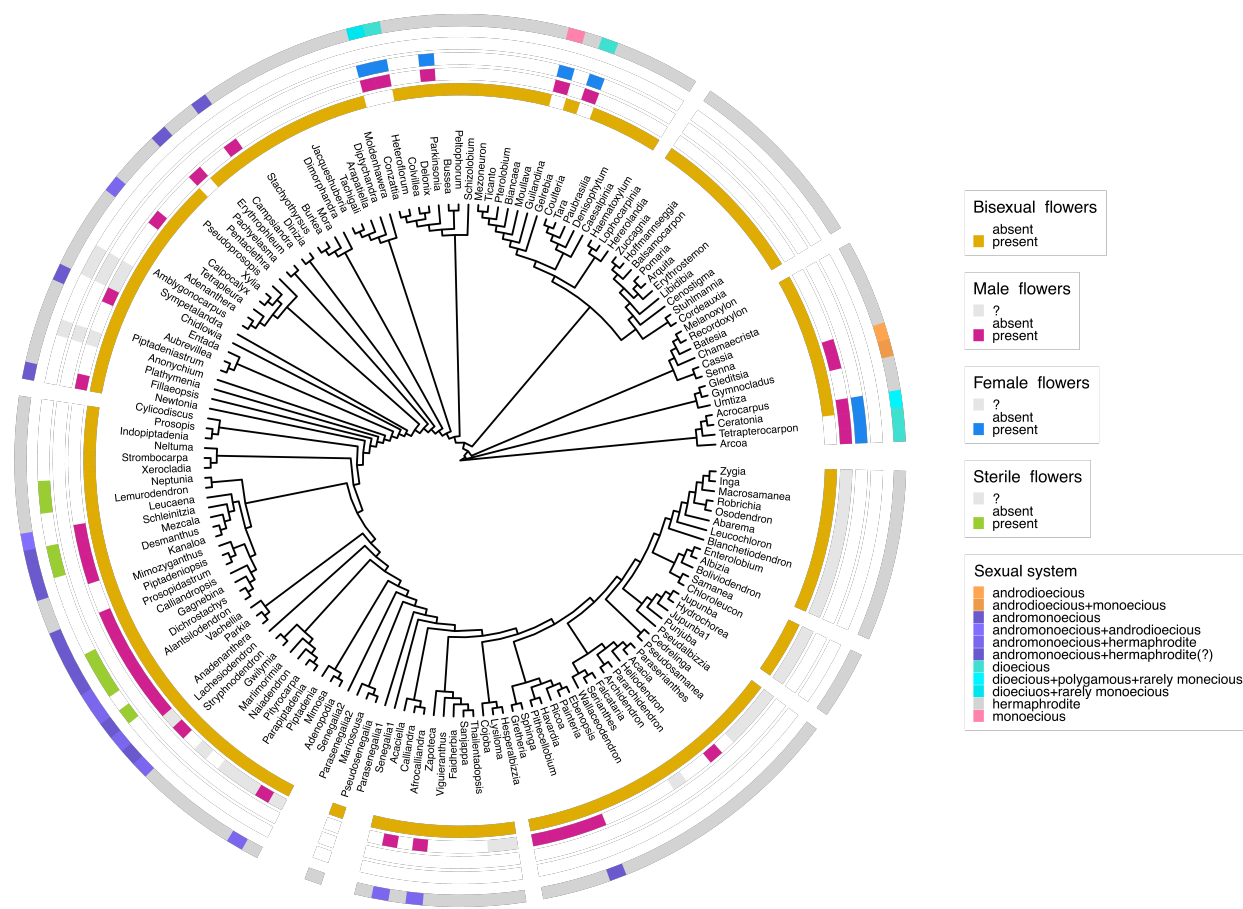

Figure 2. Phylogeny of the caesalpinioids showing the variation of sexual systems (explained in the key at right). The five concentric rings represent (outer to inner) the presence or absence (respectively) of (1) bisexual flowers, (2) male flowers, (3) female flowers, (4) neuter flowers, and (innermost ring) the sexual system. Note that the ambiguous term 'polygamy' is used when this is recorded in the literature with no further details. See discussion in text.

Within mimosoids, the inflorescences of the *Abarema-Albizia* Alliance are often florally dimorphic but without difference in sex (sensu Barneby and Grimes 1996), i.e. having hermaphrodite flowers in two distinct forms, but not generally differing in sex. These may be distinguished by Greek letters α and β as below. An exception is *Pseudosamanea guachapele* (Kunth) Harms in which the terminal flower is functionally male (due to a reduced or rudimentary ovary). Usually, the difference between the two floral types is abrupt, but occasionally it is gradual as in *Jupunba langsdorffii* (Benth.) M.V.B.Soares, M.P.Morim & Iganci [= *Abarema langsdorfii* (Benth.) Barneby & Grimes] in which case the flowers are given as hα-β with no separate numbers for the α and β types. The genus *Leucochloron* was founded on all homomorphic species, but the genus *Chloroleucon* contains both homomorphic (*C. guantanamense* (Britton) Britton & Rose, *C. dumosum* (Benth.) G.P.Lewis, *C. tenuiflorum* (Benth.) Barneby & Grimes, *C. chacoense* (Burkart) Barneby & Grimes) and dimorphic species (*C. foliolosum* (Benth.) G.P.Lewis, *C. mangense* (Jacq.) Britton & Rose, *C. acacioides* (Ducke) Barneby & Grimes, *C. tortum* (Mart.) Pittier).

Examples of applying the inflorescence formula for species with dimorphic flowers but without sexual variation are as follows:

Pseudosamanea guachapele 20-33h 1m>

Pseudosamanea cubana 20-25hα 1hβ>

Chloroleucon guantanamense 15-20h

Chloroleucon mangense 15-30hα 1-3hβ>

Abarema langsdorfii 12-27hα-β>

Abarema levelii (Cowan) Barneby & Grimes 6-13hα 1hβ>

Samanea saman (Jacq.) Merr. 15-21hα 1hβ>

Albizia julibrissin Durazzini 12-19hα 1hβ>

The type of heteromorphism seen in these plants was called "heteromorphic 2" by Ringelberg et al. (2022). In that study, "heteromorphic 1" was defined as having basal flowers of the inflorescence with showy staminodia, while "heteromorphic 2" has the central flower (or flowers) enlarged/sessile versus the peripheral (often pedicellate) flowers. Examples of "heteromorphic 1" species given by Ringelberg et al. (2022) are *Dichrostachys cinerea* (L.) Wight & Arn. and *Parkia bahiae* H.C.Hopkins, and examples of "heteromorphic 2" species are *Hydrochorea corymbosa* (Rich.) Barneby & J.W.Grimes and *Albizia* spp.

It should be noted that in the terminology of Barneby and Grimes (1996), the term primary inflorescence is used for reproductive branch systems, while "inflorescence units" refer to units with a common peduncle. Our "inflorescence" is the "inflorescence unit" of Barneby and Grimes (i.e. the actual raceme, spike or capitulum). However, the inflorescence formula as described here can be used to describe compound inflorescences (sensu Weberling 1989) of repeating units by describing the basic unit in square brackets and then using a multiplication sign to indicate the compounding. So for instance the tropical ginger *Alpinia monopleura* K.Schum. has large inflorescences which are highly modular, huge racemes with about 150 short lateral cincinni in five ranks (S. Senjaya, pers. comm.). Each cincinnus has about 5 flowers (male and hermaphrodite, with the hermaphrodite opening first). So, the inflorescence formula for *A. monopleura* can be written as: [H1^, M3-4>] x c.150. This notation is also potentially relevant to the 'pseudo-racemes' of the Papilionoideae (Tucker 1987) and double-racemes of many Mimoseae.

An interesting case arises when rare, or apparently aberrant, floral morphs are noticed within an inflorescence. If these are teratomorphic or unusual, they should not be allowed to indicate separate sex systems, but it may be worth indicating them in the formula. The use of brackets () for rare floral morphs allows this. The occurrence of unusual floral morphs is called pleogamy, defined as "the rare occurrence of unexpected floral forms in plants of a given mating system" (Cronk 2021). An example might be the occurrence of the occasional hermaphrodite flower in an otherwise strictly dioecious species. The symbols used in the inflorescence formula are mainly those found on a standard keyboard for ease of typing. The formula clarifies and extends the very broad use of the "-oecy terminology" (Table 2) which when used alone can hide some important variations. For example, monoecious plants may have male and female flowers in the same inflorescence or in separate male and female inflorescences. Furthermore, polygamodioecy (mixed bisexual and unisexual flowers, varying across individuals) covers a variety of different possibilities. It covers individuals in a species that have male flowers only vs male and hermaphrodite flowers, or female flowers only vs female and hermaphrodite flowers, and numerous other possibilities which the catch-all term polygamodioecy does not distinguish. Of course, the formula still only provides a rough summary of the total variation of interest, and suggestions for a much more detailed descriptive protocol follow in the next section, for use when fuller studies are being conducted.

Given below are hypothetical examples (used jointly with the -oecy terminology).
(1) Monoecious, 10 flowers on male inflorescences, 5 flowers on female inflorescences

Mc: 10m/5f

(2) Monoecious, 30 flowers per inflorescence, 10 female, 20 male, male distal

      Mc: 10f 20m>

(3) Dioecious, male plants with 20 flowers per inflorescence, female plants with 10 flowers per inflorescence

      Dc: 20m + 10f

(4) Andromonoecious, 50 flowers, 49 male, 1 hermaphrodite (distal)

      AMc: 49m 1h>

(5) Andromonoecious, ca. 100 flowers, 40 to 50 neuter at the base, 5 to 10 intermediate medial, 40 male medial to apical, 10 to 15 hermaphrodite at the apex

      AMc: 40–50n 5–10i> 40m>> 10–15h>>>

(6) Gynomonoecious, 10 flowers on female inflorescence, 10 to 20 flowers on hermaphrodite inflorescence. Hermaphrodite flowers open first

      GMc: 10f/10–20h^

(7) Andromonoecious, 25 flowers, 22 male, 3 hermaphrodite (distal), organized in a compound raceme with 30 to 40 partial inflorescences

      AMc: [22m 3h>] x 30–40

      Finally, it should be pointed out that our inflorescence formula has echoes of Barneby's "leaf formula", which describes the complex pinnation of legume compound leaves as follows: "a leaf-formula expressed as iii–vii/9–16 indicates that in the material studied, the largest leaf of all specimens consisted of three to seven pairs of pinnae and the longest pinna of nine to 16 pairs of leaflets" (Barneby and Grimes 1996). The aim is the same: to reduce variation to a simple form that is easily understandable, unambiguous and directly comparable.

Table 3. The -oecy terminology for major plant mating systems. These are the traditionally-used descriptor for plant sex variation but they can conceal important variation. The abbreviations may be used with the inflorescence formula.

Name	Abbr.	Notes	Notation in formula
Hermaphroditism	H	All flowers bisexual	h
Dioecy	Dc	female and male flowers on different individuals	m + f
Monoecy	Mc	female and male flowers on same individual	mf or m/f
Andromonoecy	AMc	male and hermaphrodite flowers	mh or m/h or h/mh or m/mh or m/h/mh

Gynomonoecy	GMc	female and hermaphrodite flowers	fh or f/h or h/fh or f/fh or f/h/fh
Trimonoecy	TMc	male, female and hermaphrodite flowers	mfh or m/f/h or mf/h etc
Androdioecy	ADc	male individuals and cosexual individuals	m + h or m+mf
Gynodioecy	GDc	female individuals and hermaphrodite individuals	f + h
Trioecy	Tc	Male, female and cosexual individuals	m + f + h or m + f + mf
Polygamodioecy	PDc	mixture of monoclinous and diclinous flowers, varying across individuals	m + mh or m + fh etc

A suggested protocol to describe sexual variation in plants with selected examples in legumes

Understanding the evolution of plant sex requires first a solid background in trait variation. However, as seen above, morphological descriptions usually include vague terms (e.g. "polygamous"; see above) or do not accurately describe variation in reproductive structures (e.g. secondary sexual characteristics, such as differences in perianth size associated with flower sex) and across flowering time (e.g. male flowers opening first). Thus, we provide here a protocol to describe the information needed to propose and verify hypotheses aiming to explain sexual transitions in plants. Although the full protocol is demanding and it may not be possible to collect all these data in a single study, particularly if only herbarium material is available, it is given as a framework rather than a requirement.

The protocol comprises three main types of variables: morphological, chronological and resource allocation. Morphological and resource allocation variables describe trait variation, and whether there is more investment in particular sexual morphs. For example, some species in *Hydrochorea* Barneby & J.W.Grimes and *Punjuba* Britton & Rose have andromonoecious inflorescences in which the apical hermaphrodite flower usually includes a nectariferous disk and is larger than the other (mostly male) flowers (Barneby and Grimes 1996; Iganci, Soares and Morim 2024). Chronological variables capture variations in flowering time between sexual morphs and are particularly important to investigate ecological or developmental mechanisms behind sexual variation. For instance, while both male and hermaphrodite flowers usually open synchronously in mimosoids,

one morph may reach anthesis first in most other legumes, as their flowers often initiate and develop acropetally (Tucker 2003).

Morphology

(1) Description of inflorescence (type, dimensions, shape, e.g., contracted, lax)

(2) Types of flowers in inflorescence (hermaphrodite, male, female, neuter, intermediate). The variation of flower types in the inflorescences is important to discriminate processes of sexual determination. Attention should also be given to the presence or absence of intermediate morphs, which are rarely recorded but may give clues about developmental processes (see discussion above for *Dichrostachys cinerea*).

(3) Approximate number of flowers in inflorescences

(4) Approximate proportions of different flower types

(5) Spatial distribution of flower types in the inflorescence (apical, basal, or dispersed). Spatial variation may indicate the role of gradients in shaping the different sexual morphs (see discussion above).

(6) Development of the inflorescence (i.e. sequence of flower development and anthesis: acropetal, basipetal, amphipetal, centripetal)

(7) Description of each individual flower type (size, shape of whorls, etc). Morphological differences in flower types may be linked to eco-evolutionary pressures (Huang et al. 2006; Humeau, Pailler and Thompson 2003; Yakimowski, Glaettli and Barrett 2011).

(8) Presence, size and shape of vestigial or sterile reproductive organs (staminodes, pistillodes). Despite not participating in the production of gametophytes, vestigial and sterile reproductive organs may still participate in reproduction, e.g. staminodes attracting pollinators in mimosoid legumes and other plants (Nicholls and Dorken 2012). Staminodes and pistillodes may be very small, and in these cases, just recording their presence (or absence) may be sufficient.

(9) Variation in functional gender between individuals, when present. The concept of functional gender was developed by Lloyd (1980). In cases of strict hermaphroditism and limited floral polymorphism, the functional gender of all individuals is 0.5; i.e. there is exactly equal average chance of an individual contributing to the next generation via the male line (sperm) as via the female line (eggs). However, when there are varying numbers of male and female flowers, the functional gender of plants, or inflorescences, may vary. Gender is then estimated using the following equation: $G = f/[f+(m*E)]$ where G denotes functional femaleness varying between 0 and 1, f is the number of

functionally female flowers, and m the number of functionally male flowers (hermaphrodite flowers contribute to both). The equivalence factor (E) denotes the relative male and female fitness in the population (an estimate of the relative number of functionally female vs male flowers overall). The same method can be used to calculate the functional gender of inflorescences within a plant if these vary significantly in the proportions of floral morphs. This method can also be modified to calculate the functional gender of individual flowers if there is significant variation in the relative numbers or fertility of stamens and carpels between flowers.

Resource allocation

(1) Dry weight of inflorescence, as an indication of reproductive investment

(2) Dry weight of the inflorescence axis only (flowers and pedicels removed). This helps to determine resource allocation to the flowers specifically, relative to the supporting structures of the inflorescence.

(3) Reproductive allocation. The number of, or total dry weight of, inflorescences produced by plants, relative to plant size or performance as measured by standard metrics (such as "basal area" from diameter at breast height, dbh, for trees). Although this is difficult to measure (especially in trees) and may be highly variable, it helps to provide a measure of an individual's total reproductive investment. One study used as an index, reproductive dry weight divided by leaf dry weight: g/g leaf (Allen and Antos 1988). It should be noted that there are different "currencies" in which reproductive allocation can be measured besides biomass (Ashman 1994), and energy can be used instead of biomass via calorimetry.

(4) Average dry weight of flowers of different types at anthesis (e.g. weight of 20 flowers). Measuring the weight of morphs separately is particularly salient for cases in which flowers of different types occur in the same inflorescence, as it gives an indication of the relative resource investment in different morphs.

(5) Allocation to fruit: numbers and size (dry weight of fruit); proportion of functionally female flowers developing fruit.

Chronology

(1) Hermaphrodite flowers: whether protogynous or protandrous.

(2) Duration of functional post-anthesis period of single flower (male + female phase).

(3) Functional duration of inflorescence (time over which flowers at anthesis, i.e. open flowers, are seen).

(4) Variation in functional gender (see above) over inflorescence duration (e.g. number of functional male vs hermaphrodite flowers) at:

      (a) early inflorescence anthesis, i.e. <25% of flowers opened (i.e. post-anthesis)

      (b) mid inflorescence anthesis, i.e. 25-75% of flowers post-anthesis

      (c) late inflorescence anthesis, i.e. >75% of flowers post-anthesis

(5) Timing of fruit development and dispersal.

Anthesis may be synchronous across the whole inflorescence (Tucker 2003). To quote Tucker: "Mimosoid flowers .... share an unusual developmental feature: synchronous development of the flowers in any one inflorescence. As in racemes of the other subfamilies, these undergo acropetal, successive order of flower initiation, but each floral bud pauses after its initiation until all are initiated in that inflorescence … As a result, all flowers will be at the same stage of development in an individual inflorescence" (Tucker 2003). This is highly significant, as when it applies to andromonoecious species, such synchronous flowering means that the ratio of hermaphrodite to male flowers (functional gender) will not change during flowering.

Prospects for future morphological, genomic and evo-devo studies of sexual variation in legumes

With the growth of whole genome sequencing in legumes, genome-wide investigation of traits, including sexual system traits, is likely to become much easier. There is a good prospect of fast progress towards solving the molecular basis of andromonoecy using diverse methods. First, comparative transcriptomics can be employed to reveal different gene regulatory networks (GRNs) involved in the formation of hermaphrodite and male flowers in the same inflorescence (Rocheta et al. 2014). Secondly, natural variation (related species with and without andromonoecy) can be used with expression profiling in a similar way, as has been employed to investigate differences in leaf architecture in *Solanum* using self-organizing map (SOM) analysis of GRNs (Ichihashi et al. 2014). Finally, there is considerable scope for experimental manipulation of inflorescences to perturb sex expression by application of hormones or hormone inhibitors, organ tissue culture or wounding. Such traditional experimental manipulations, that go back to the early developmental literature (Heslop-Harrison 1957), could be informative, especially if combined with transcriptomics (Wu, et al. 2022).

The genera *Neptunia* and *Mimosa* L. are potential sources of material for study, both with a high degree of interspecific variation in their sexual systems. For dioecious species, the same considerations apply but with the added advantage that the sex-determining region (SDR) segregates in a population so additional techniques like GWAS and k-mer analyses can be applied (Vekemans et al. 2021). A good candidate for such studies is the carob bean (*Ceratonia siliqua* L.) for which whole genome resources are emerging (Bibi et al. 2023). However, the ultimate test for any gene implicated in the control of floral sex is genetic manipulation, for instance using CRISPR-cas9 (Müller et al. 2020). No legume system can be regarded as easily transformable and almost all studies have involved agriculturally important papilionoid species (Choudhury and Rajam 2021). However, innovative techniques such as root transformation/regeneration by cut-dip-budding may eventually solve this. Cut-dip-budding was originally developed for *Robinia* L. transformation (Han, Keathley, Davis and Gordon 1993) before being more widely applied (Cao et al. 2023). Above all, to understand the evolution of sex system variation in legumes, we need better and more systematically collected morphological information. Here we have provided two resources aiming to facilitate trait data description. The first is an inflorescence formula, which could be integrated into taxonomic accounts and, when coupled with recent phylogenetic advances, will assist us in identifying clade-specific patterns of sexual variation. The second resource, a descriptive protocol, extends this package by providing descriptors that aggregate the information needed to answer "how" and "why" questions related to the development and evolution of plant sexual systems. There has never been a better time to investigate this.

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