# Sexual system variation in the legumes (Fabaceae): prospects for morphological, evolutionary and genomic study

**Short Title**: Sexual system variation in the legumes

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## Abstract

Although the great majority of legume species are cosexual with hemaphrodite 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 an 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 detailed description of sexual variation, which we hope will be useful in guiding future studies of sexual variation in plants. Detailed knowledge of sexual variation at the morphololological 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.

**Keywords** plant sex, plant gender, Fabaceae, floral formula

## Introduction: genomics and trait evolution

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 up 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 advances as candidates for detailed analysis are floral developmental traits. These are of great interest to the systematist, but also to the ecologist and agriculturalist as they control breeding systems, fruit set and reproductive success.

Before genomic resources were available for legumes, candidate gene approaches using developmental genes discovered in unrelated model organisms had to be used. 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 discovered via transposon tagged mutagenesis in the model organism *Antirrhinum majus* L. (the 'snapdragon'; Scrophulariaceae). 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, Luo, Pennington, Coen and Cronk 2003). Subsequent work showed that expression patterns of "*CYC1B*" in *Lupinus* L. implicated a similar role for *CYC* in legume floral morphology. A model could then be proposed and tested to explain the anomalous floral symmetry in the papilionoid legume *Cadia* Forssk. A gain of function change in expression in *Cadia* was confirmed, and this provides an elegant explanation for the change in floral symmetry (Citerne, Pennington and Cronk 2006).

The problem is that many important legume traits, particularly those of tropical and woody legumes, cannot be studied by forward genetic screens in convenient model organisms, and have no equivalents in other families where more tractable genetic resources exist, such as *Arabidopsis* (Brassicaceae). 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 example appears to be unique, rendering the candidate gene approach useless (Cronk & Müller 2020). 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, Henry, Tao and Comai, 2014). OGI, present only in males, is a gene that produces suppressing small RNAs. In dioecious *Populus trichcarpa* Torr. and A.Gray ex Hook. and *P. tremuloides* Michx. (Salicaceae) there is a constitutive feminizing response regulator gene *ARR17*, which is suppressed to make males by a male specific locus that produces small RNAs responsible for RNA-directed DNA methylation (RdDM) of *ARR17* (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 is 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 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 is completely 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 (Geraldes et al. 2015), transcriptomics (Cronk, Soolanayakanahally and Brautigam 2020), methylomics (Brautigam et al. 2017), single molecule long read sequencing (Müller et al. 2020) and sRNA-seq (Müller et al. 2020). However, with 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 this may be gained from the fact that although the first draft of the human genome was released in 2000, sequencing and assembly of the Y chromosome was only completed in 2023 (Rhie et al. 2023). These considerations do not apply to the control of monoecy. 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 this clade, 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. 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 of the molecular mechanism for sex determination or spatio-temporal variation in sex expression. Secondly 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 hormones and hormone gradients across inflorescences. Hormones can be artificially manipulated. 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, Baker, Sell, Herner and Dilley 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 captitulum has a high concentration of auxin but as the developing capitulum expands the concentration of auxin decreases markedly (Zoulias, Duttke, Garces, Spencer and Kim 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. 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.

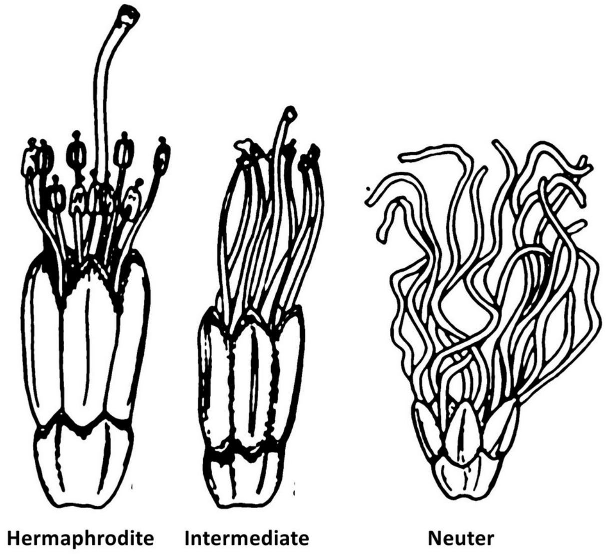

Figure 1. Floral morphs of *Dichrostachys cinerea* after Venkatesh (Venkatesh 1951). The inflorescence formula (see text) is: n34 i11 h117>. The flower numbers in the formula are average counts from 4 inflorescences.

Tucker pointed out that in legumes loss of organs may result in two different ways. Primordia may be absent, as in the petals of the papilionoid *Ateleia* DC. (Tucker 1990), or alternatively 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 are suppressed after organ formation. In male flowers a pistil forms and is suppressed later in ontogeny, leaving a small pistillode. In female flowers the developmental suppression may happen even later in development, producing stamens, but which fail to form pollen (Tucker 2003). This pattern 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 multiple 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 evolutionary transitions

Theoretical approaches require the formulation of models to explain the selective advantages of evolutionary departures 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. 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 sepal and petal 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 therefore 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 building on influential papers from the 1970s (Charlesworth and Charlesworth 1978; Charlesworth and Charlesworth 1978).

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.

## 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* Lour. 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).

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 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).

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 | (Williams and Fenster 1998) |

| | | | |
|---|---|---|---|
| | | steriles) | |
| *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) |

As can be seen, andromonoecy is the commonest such departure, 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 formula for summarizing sexual variation of inflorescences

Aiming to facilitate the description of sexual systems in a clear and straightforward manner, here we offer 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 2.

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, position of floral morphs within an inflorescence, the order of anthesis and number or rarity of floral morphs can also be indicated by additional symbols. The formula allows for the use of quantitative information (numbers 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.

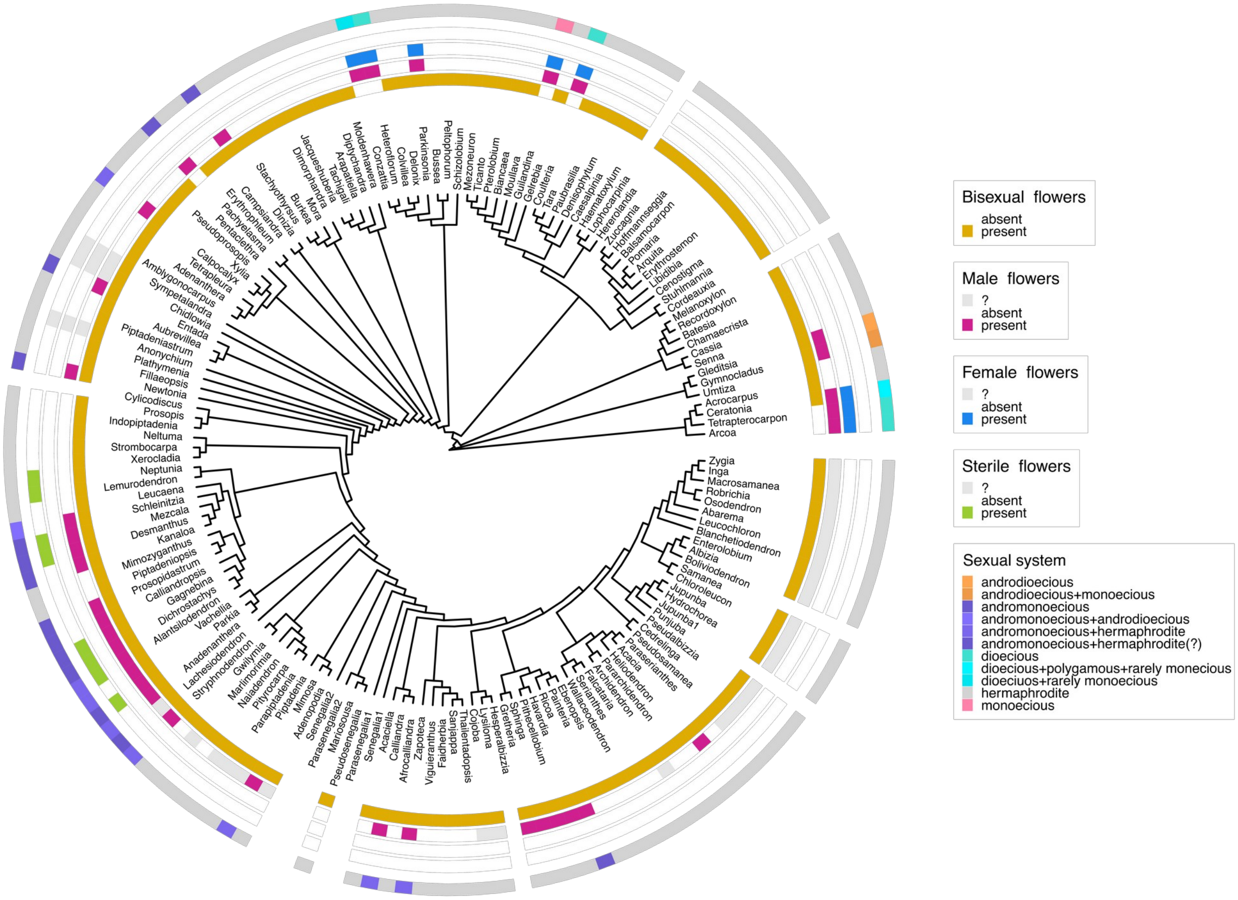

Figure 2. Phylogeny of the caesalpinioids showing the variation of sexual systems (explained in the key at right). The five concentric rings represent (outer to inner) the presence or absence (respectively) of (1) bisexual flowers, (2) male flowers, (3) female flowers, (4) neuter flowers, and (innermost 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.

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 those found on a standard keyboard for ease of typing. The formula clarifies and extends the very broad use of the “-oecy terminology” (Table 3) which when used alone can hide some important variation. 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 total variation of interest, and suggestions for a much more detailed descriptive protocol follows 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, 40–50 neuter at the base, 5–10 intermediate medial, 40 male medial to apical, 10–15 hermaphrodite at the apex

AMc: 40–50n 5–10i> 40m>> 10–15h>>>

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

GMc: 10f/ 10–20h^

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 of the evolution of plant sex requires first a solid background on trait variation. However, as seen above, morphological descriptions usually include vague terms (e.g. “polygamous”) or do not accurately describe variation on reproductive strutures (e.g. secondary sexual characteristics, such as differences in 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 information needed to propose and verify hypotheses aiming to explain sexual transitions in plants. The full protocol is demanding and it may not be possible to collect all these data in a single study. It is provided 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 if 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 develop synchronously in mimosoids, one morph may arise first in most other legumes, as their flowers initiate and develop acropetally (Tucker 2003).

### *Morphology*

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

(2) Types of flowers in inflorescence (hermaphrodite, male, female, neuter, intermediate). The variation of flowers types in the inflorescences is important to discriminate process of sexual determination. Attention should also be given to the presence or absence of intermediate morphs, which are rarely recorded but may be cues about development 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 inflorescence (i.e. sequence of flower opening: 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, Tang, Sun and Lu 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 variation is present. The concept of functional gender was developed by Lloyd (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 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). 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 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) Numbers of inflorescences produced by plants relative to plant size as measured by standard metrics (such as diameter at breast height, dbh, for trees). Although difficult to measure and highly variable, this helps to provide measure of the total reproductive investment by individuals.

(4) Average dry weight of flowers of different types at anthesis (e.g. weigh 20 flowers). Measuring the weight of morphs separately is particular important 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.

*Chronology*

(1) Hermaphrodite flowers: whether protogynous or protandrous.

(2) Duration of functional anthesis 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) ate inflorescence anthesis, i.e.>75% of flowers post anthesis

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 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, Zhu, Qiu, Fan and Yuan 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 kmer analysis 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, Ioannidis, Bazakos and Kalantidis, 2023). However the ultimate test for any gene implicated in 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). Howevever, 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).

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, an inflorescence formula, which could be integrated into taxonomic accounts and, when coupled with recent phylogenetic advances,  will enable us to identify 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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## Supplementary data

**Table S1**. A preliminary list of legume sex systems that depart from strict hermaphroditism in the Caesalpinioideae (legume_sex_overview.xslx). TO BE MADE AVAILABLE.

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