The Genetic Architecture of Stamen Whorl Variation in *Aquilegia*

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ABSTRACT

Variation in floral form can occur in many different aspects of morphology, including organ number and positioning, organ identity, and organ elaboration. Since the discovery of floral organ identity genes and the establishment of the ABC model in the early 90's, most attention has been paid to the interactions between the master control genes and their downstream genetic programs controlling organ size, color, fusion, symmetry, etc. Surprisingly, however, after 30 years of progress in understanding the genetic basis of flower morphogenesis, still very little is known about how variation can be generated at the most fundamental level – through the activity of floral meristem (FM) – despite the fact that meristem forms the foundation of development of any kinds in all plants. Here, **I will take the initiative to explore the natural variation in FM proliferation by investigating the genetic architecture underlying stamen whorl number variation**. Utilizing the existing variation in stamen whorl numbers among *Aquilegia* species, I will obtain a F2 population by crossing two sister species, *A. brevistyla* and *A. canadensis*. By integrating quantitative trait locus (QTL) analysis and RNA sequencing, I will generate **the first dataset** to uncover the genomic regions and loci that are contributing to stamen whorl variation. Results from this study will provide essential foundation for investigating further questions such as how changes in the timing of FM termination and male-female organ boundary are controlled at the evolutionary level.

Key Words: floral meristem proliferation; stamen whorl number; *Aquilegia*; QTL; RNAseq

BACKGROUND

Although we often focus on the allure of watching a flower bud unfolding and blooming, the earliest stages of development, when the flower is forming from a dome of cells, is equally compelling. Unlike animals, plants possess the ability to generate new organs throughout their entire lifespans due to the activity of stem cells located in the dome-shaped meristems. During the reproductive phase, the floral meristem (FM) will give rise to all the floral organ primordia in sequential whorls or spirals. Because the number of organs in a flower is finite, the activity of FM will be terminated at a certain time point, and all the stem cells will then lose their pluripotency and commit to specific fates. Therefore, during these earliest stages of flower development, variation in floral morphology can be generated by the FM from two main sources: 1) the initial size of the FM can strongly influence how many organ primordia initiate in each whorl, and 2) the duration of FM activity will determine how many whorls of primordia will be generated.

The identities of the organ primordia are determined by the A, B, and C classes of master genes [1]. Most flowers exhibit four types of floral organs: sepals, petals, stamens, and carpels, which are determined by A function alone, A+B, B+C, and C alone, respectively. The ABC program has been greatly improved and refined in the past two decades, but the general conservation for B and C class genes has been shown across various taxa [2]. Indeed, as the ultimate goal of a flower is to ensure the plant to reproduce successfully, the conservation of B and C class genes in part reflects the conservation of the functions of male and female organs in all flowering plants. The B-C boundary, where B class genes expression ends and only C class gene expression persists, is very critical because it defines the male-female organ boundary.

In some plants, such as model species *Arabidopsis thaliana* and *Antirrhinum majus*, the FM is terminated relatively quickly – only four whorls of organs will be produced, each whorl claiming one kind of identity, with sepals in the outer most whorl and carpels in the inner most whorl. In many other flowers, however, there are more than four whorls organs. This thus provides the flowers with important raw materials to generate morphological diversity and novelty. For instance, staminodes are organs that ancestrally expressed stamen identity, but became specialized over evolution to play other functions while the role of pollen production is fulfilled by remaining stamens in other whorls [3, 4]. Moreover, shifts in the B-C boundary can generate different male and female organ ratios, which is likely to play a significant

role in the evolution of different reproductive strategies in flowering plants [5, 6]. In the extreme cases, sliding the B-C boundary to make B gene expression disappear or completely cover the C domain is one mechanism to generate unisexual female or male flowers, respectively [7, 8].

To date, few key genes controlling floral meristem proliferation, termination, or male-female organ boundary have been identified in *A. thaliana.* For instance, the C class gene *AGAMOUS* (*AG*) is responsible for both determining organ identity and terminating floral meristem activity through the transcriptional factor *KNUCKLES* (*KNU*) [9, 10] and *SUPERMAN* plays a role in regulating the B-C boundary by restricting the expression domain of B class genes outside of the carpel whorl [11, 12]. However, a number of lines of evidence suggest that *AG-KNU* is not the only pathway in controlling the window of FM activity [e.g. 12], and how *SUP* establishes the male-female organ boundary is still unknown. In addition, hardly anything is known about other genetic interactions contributing to these processes, whether these pathways are conserved in other taxa, or how these processes vary at the evolutionary level. To that end, we need a system that possesses natural variation in FM proliferation and timing of FM termination so that we can implement a non-candidate gene approach to uncover novel loci that contribute to such variation.

***Aquilegia* as a model system**

Aquilegia, as one of the few plant systems that are both amenable to genetic manipulation and possess multiple whorls of stamens, is ideal for investigating the above mentioned questions. *Aquilegia* belongs to the order Ranunculales, an early diverging lineage of the eudicot clade [13]. There are approximately 70 *Aquilegia* species, resulting from a relatively recent adaptive radiation, and consequently have low sequence variation and a high degree of fertility among species. *Aquilegia x coerulea* ("Origami") is a promising model system with a fully sequenced genome ($2n = 14$*; ca.* 400 Mbp) and an established virus-induced gene silencing protocol for gene knock-down studies [13]. Interesting morphological characters of *Aquilegia* flowers, such as the elaborated petal spur and the novel floral organ, staminodia, are also remarkable systems for studying how complex floral organ shapes are built and how novel floral organ identities are evolved (Fig. 1).

RESEARCH AIM

In this study, I will explore the natural variation in FM proliferation by investigating the genetic architecture underlying stamen whorl number variation between sister species in *Aquilegia*. Unlike a candidate gene approach, my combination of quantitative trait locus (QTL) mapping and RNA sequencing (RNAseq) will identify genomic regions associated with the phenotype without making *a priori* assumptions about the loci involved. Such approach has been extensively used for uncovering loci controlling inflorescence meristem maturation rate and size, and shoot apical meristem size in several agricultural crops [14, 15, 16], but natural variation generated at the FM level has never been explored.

RESEARCH METHODS

All *Aquilegia* species have multiple whorls of stamens (Fig. 1), and the number of stamen whorls varies both within a single plant and between species. Very often, the terminal flower of an individual inflorescence will exhibit a higher number of stamen whorls than the late-emerging lateral flowers. Although the genetic basis is unclear, this phenomenon is frequently observed in different floral organs in many plant taxa, and is generally considered a variation attributed to aging [17]. On the other hand, the number of stamen whorls in the terminal flower can vary between species, and this variance can be much larger than the variance observed within a single plant.

Currently, the Kramer Lab is working with close collaborators in the Hodges Lab (UCSB) to generate a F2 population derived from crossing *A. brevistyla* and *A. canadensis*, two sister species that display variation in a number of morphological traits. This cross is also ideal for investigating my question of interest because *A. brevistyla* is a smaller flower with fewer whorls of stamens compared to *A. canadensis* (Fig. 1E, F). Twenty F1 individuals have bloomed, each producing about 20 flowers. These flowers were

then self-fertilized to generate the seeds for F2 population, which will take about eight months in total to flower.

Objective 1: Obtain phenotypic distribution of parental and F2 generation

To analyze the phenotypic distribution, I will count stamen whorl numbers of the first five flowers (i.e. terminal flower plus four lateral flowers; Fig. 1D) of each individual to obtain a mean stamen whorl number (μ-st) and the standard error for the mean (SE-st) for parental and F2 generations. I will also use the distribution of these two parameters in the parental generation to calculate the broad-sense heritability (H^2), which gives an estimate of how much of the phenotypic variation is due to genetic variation. H^2 of SE-st will also inform me about whether SE-st should be considered as a phenotypic parameter for QTL mapping: if SE-st appears to be relatively heritable ($H^2 > 0.5$), I will also look for the potential QTL contributing to stamen whorl variation *within* an individual, and it will then be very intriguing to see whether QTL of this trait overlap with the QTL controlling between individual/species variation.

Objective 2: Genotype F2 generation and identify QTL

Because the *Aquilegia* reference genome has been sequenced and assembled, it allows me to genotype the F2 individuals through whole-genome sequencing. Previous research in Hodges and Kramer Labs has conducted whole-genome sequencing of the *A. brevistyla* and *A. canadensis* parents to high coverage (*ca.* 40x) so we have large number of markers identified to distinguish the parents' genomes. I will conduct low coverage (*ca.* 2x) whole-genome sequencing for 300 F2, which will be sufficient to identify recombination breakpoints in the genome and assign genome ancestry, a method adapted from multiplexed shotgun genotyping [18]. Subsequently, I will associate genotypes with phenotypes and look for genomic markers that have the highest logarithm of the odds (LOD) score. The significance threshold of the LOD scores will be determined by performing a permutation test. Consequently, these analyses will inform me about the QTL(s) underlying stamen whorl number variation between these two *Aquilegia* species.

Objective 3: RNAseq of *A. brevistyla* and *A. canadensis* young inflorescences

QTL mapping will inform me about whether stamen whorl number is a trait controlled by few loci with large effects or many loci and each with relatively minor effect. Among all the loci resulting from the mapping, I will then narrow down the list of candidate genes by integrating RNAseq data from young inflorescences, because I am most interested in the processes that take place during the earliest stages of flower development. Specifically, I will sequence very young inflorescences of both *A. brevistyla* and *A. canadensis*. Young inflorescences harbor flower buds that are at the earliest developmental stages (Fig. 1G), which is ideal for my analysis.

For each species, I will collect six young inflorescences to ensure that there are enough biological replicates of the floral buds at the earliest developmental stages and send the samples for paired-end sequencing via Illumina HiSeq 2000 to generate 100 bp reads, which will then be aligned to the Origami genome. Due to the high quality genome of Origami, a sequencing coverage greater than five can yield accepted results for this alignment.

Objective 4: Identify candidate genes that contribute to floral meristem proliferation

I will overlap the QTL mapping results with the RNAseq data from both parental species to generate a list of genes of interest (Fig. 1H). I will consider candidates that are expressed in both *A. brevistyla* and *A. canadensis* young inflorescences, as well as genes that are differentially expressed between the species. This study will generate the first list of genes that are important for controlling the natural variation in stamen whorl numbers, providing an excellent starting point for dissecting the genetic basis controlling the natural variation in floral meristem proliferation and male-female organ boundaries.

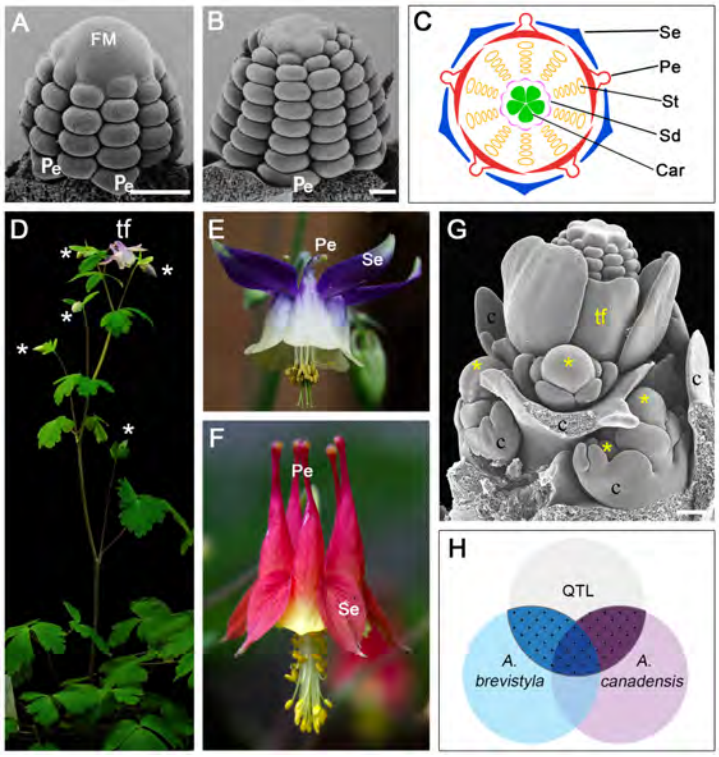

Figure 1. *Aquilegia* is an ideal system to investigate nature variation in FM proliferation. (A, B) Termination of the FM occurs after multiple whorls of organ primordia have initiated; adapted from [19] (C) Floral diagram of a typical *Aquilegia* flower. Se: sepals; Pe: petals; St: stamens; Sd: staminodia; Car: carpels; tf: terminal flowers; c: cauline leaves. (D) An individual of *A. brevistyla* with inflorescence bearing terminal flower and lateral flowers (asterisks). (E) *A. brevistyla.* (F) *A. canadensis.* (G) A young *Aquilegia* inflorescence bearing FMs of various stages; adapted from [20] (H) Diagram of data processing method in Obj. 4. Colored circles represent loci derived from QTL mapping (grey), RNAseq of *A. brevistyla* (blue) and *A. canadensis* (purple) inflorescences. Dotted area represents the list of genes of interest. Scale bars = 100 µm.

BUDGET

Except for DNA preparation for F2 individuals, all sample preparation and sequencing will be conducted at Harvard University Bauer Core Facility, and budget is calculated according to the fees listed in facility.

Description	Cost
Training fee	$248
Library preparation for 12 inflorescence samples	$10 x 12 = $120
Whole-genome sequencing of 300 F2 individuals: Illumina HiSeq Paired-end 3 lanes	$1972 x 3 = $5916
RNAseq for inflorescences (shared lane with other users)	$500

I will have $4000 student research fund for this project after my PhD qualification exam in April, 2017. The research award from BSA will help me to cover part of the sequencing cost as well.

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BIOGRAPHICAL SKETCH

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sample address. sample email; sample phone number

The current research proposal is an important component of my graduate thesis project, in which I will combine a candidate gene approach (not described here) with evolutionary genomic and genetic studies to analyze how the variation in floral meristem proliferation and the establishment of female-male organ boundary are controlled in an evolutionary context. I am very familiar with various techniques in molecular and plant development studies, including light and scanning electron microscopy, *in situ* hybridization, fine tissue dissection, and image analysis. Before graduate school, I completely a dual Master's Degree in Evolutionary Biology from two different European university, and received rigorous trainings in genetic and genomic data analysis, which provided me with strong support of conducting the proposed project in this application.

- **EDUCATION**

Harvard University	PhD student	June 2015–Present
Uppsala University	M.S. in Evolutionary Biology	2012–2014
University of Montpellier II	M.S. in Evolutionary Biology	2012–2014
Hokkaido University	Exchange student	2010-2011
Sichuan University	B.S. in Biology	2008–2012

- **PUBLICATIONS**

Min, Y., & Kramer, E. M. (2016). The *Aquilegia JAGGED* homolog promotes proliferation of adaxial cell types in both leaves and stems. *New Phytologist*. DOI: 10.1111/nph.14282.

Wang, H., Talavera, M., **Min, Y.**, Flaven, E., & Imbert, E. (2016). Neutral processes contribute to patterns of spatial variation for flower color in the Mediterranean *Iris lutescens* (Iridaceae). *Annals of Botany*, *117*(6), 995-1007.

Huang, S., ... **Min, Y.**, ... & Yu, J. (2013). Draft genome of the kiwifruit *Actinidia chinensis*. *Nature Communications*, 4:2640. DOI: 10.1038/ncomms3640.

- **PRESENTATIONS & POSTERS**

Wang, H., Talavera, M., **Min, Y.**, Flaven, E., & Imbert, E. (2016). Neutral processes contribute to patterns of spatial variation for flower colour in the Mediterranean *Iris lutescens* (Iridaceae). Abstract and poster presentation for *IBS Special Meeting* (Beijing, China).

Min, Y., & Kramer, E. M. (2015). *JAGGED* regulates lateral organ development and leaf adaxial identity in *Aquilegia.* Abstract and poster presentation for *Inaugural Meeting of Pan-American Society for Evolutionary Developmental Biology* (Berkeley, USA).

Ickert-Bond, S., Sousa, A., **Min, Y.**, Leitch, I. J., Pellicer, J. (2014) The evolution of genome size in the gymnosperm genus *Ephedra*: flow cytometry and new chromosome counts support high levels of polyploidy. Abstract and poster presentation for *Botany 2014* (*Boise,* USA).

- **TEACHING & ADVINSING**

Teaching fellow, OEB 50 – Genetics and Genomics (2016 Fall, Harvard University, Q evaluation: 4.9/5.0, received Distinction in Teaching Award)

Mentored one undergraduate student (Harvard College, '16) for senior honor's thesis