The Mendelian inheritance of gynomonoecy: insights from *Anacyclus* hybridizing species

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ABSTRACT

- **Premise of the study** Gynomonoecy is an infrequent sexual system in Angiosperms, although widely represented within the Asteraceae family. Currently, the hypothesis of two nuclear loci that control gynomonoecy is the most accepted. However, the genic interactions are still uncertain. *Anacyclus clavatus*, *A. homogamos* and *A. valentinus* differ in their sexual system and floral traits. Here, we investigate the inheritance of gynomonoecy in this model system to understand its prevalence in the family.
- **Methods** We selected six natural populations (two per species) for intra- and interspecific experimental crosses, and generated a total of 1123 individuals from F_1, F_2, and backcrosses for sexual system characterization. The frequency of gynomonoecy observed for each cross was tested to fit different possible hypotheses of genic interaction. Additionally, the breeding system and the degree of reproductive isolation between these species were assessed.
- **Key Results** Complementary epistasis, in which two dominant alleles are required for trait expression, explained the frequencies of gynomonoecy observed across all generations. The heterozygosity inferred in *A. valentinus*, as well as its lower and variable seed set, is congruent with its hybrid origin.
- **Conclusions** In our model system gynomonoecy is controlled by complementary epistasis of two genes. A common origin of this sexual system in Asteraceae, in which genic duplications, mutations and hybridization between lineages played a

key role, is hypothesized whereas independent evolutionary pathways and possibly diverse underlying genetic factors are suggested for gynomonoecy expression in other Angiosperm families.

Keywords: Anthemideae, Asteraceae, epistatic control, floral traits inheritance, genic interactions, hermaphroditic flowers, hybridization, plant sexual systems, unisexual flowers, Western Mediterranean.

INTRODUCTION

Among the sexual systems in plants, hermaphroditism is the most common, whereas dioecy, monoecy or gynomonoecy (i.e., the presence of female and hermaphrodite flowers in one individual) are much less frequent (Richards, 1997). Gynomonoecious species, however, are overrepresented within the Asteraceae (Yampolsky and Yampolsky, 1922; Torices et al., 2011). Gynomonoecious plants in this family comprise female peripheral ray flowers surrounding numerous hermaphroditic disk flowers, forming the typical radiate capitulum inflorescence (Torices and Anderberg, 2009). The occurrence of a ray (i.e., showy ligule) in female flowers suggests a functional link between gynomonoecy and radiate capitulum in this family and has led to the proposition that selection for this showy inflorescence might have led to subsequent reduction of stamens in these flowers to pay off the cost of the ligule production (Bawa and Beach, 1981).

The presence of radiate and non-radiate capitula within a genus (e.g., *Layia*, *Matricaria*, *Senecio*, *Tanacetum*, among others) or within a single species (e.g., *Bidens pilosa* L., *Senecio vulgaris* L.) suggests that variation in this floral trait may have a simple genetic basis. In *Senecio*, studies of hybrid species suggest that two major loci govern variation in ray flower expression (Abbott et al., 1992, 2009; Lowe and Abbott, 2000; Andersson, 2001; James and Abbott, 2005), contradicting the earlier hypothesis that the trait was controlled by a dominant allele at a single locus (Trow, 1912; Richards, 1975; Ingram and Taylor, 1982). The two loci model was also hypothesized to explain the presence of rayed and rayless species in other genera, such as in *Layia* (Ford and Gottlieb, 1990) and in *Dubautia*, *Madia*, and *Raillardiopsis* (Carr et al., 1996).

Whitkus et al. (2000) studied the expression of the female function in *Tetramolopium rockii* Sherff (Astereae) and found that at least two loci that interacted by complementary or alternatively recessive epistasis might be involved in the loss or gain of this function, although a more complex hypothesis with three or four genes could not be rejected. Some molecular genetic evidence supports the two loci model. The *cycloidea* family genes (CYC genes) that control floral symmetry also regulate the expression of Asteraceae ray flowers (Gillies et al., 2002; Broholm et al., 2008; Kim et al., 2008; Chapman and Abbott, 2010; Bello et al., 2017). Recently, Yang et al. (2019) suggested that the interaction between two transcription factors (i.e., *Cm*WUS and *Cm*CYC) regulate the reproductive organ development in *Chrysanthemum morifolium* (Ramat.) Hemsl. (Anthemideae), supporting the link between CYC genes in both ray and reproductive organs expression. Although it is clear that genes involved in gynomonoecy and those in ray expression are linked or might be similar, no mention was reported on the sexual systems observed in any of these previous study cases.

Within Asteraceae, the tribe Anthemideae includes several genera (*Anthemis*, *Cotula*, *Soliva*, and *Anacyclus*) in which some species are hermaphroditic (i.e., no female flowers are present) and capitula are non-radiate, while others are gynomonoecious (i.e., female flowers are present in the distal part of the capitula) and capitula may be radiate with showy ligules or non-radiate when ligules are inconspicuous or absent. Here we specifically focus on the inheritance of gynomonoecy, instead of the number and length of rays. Besides, we included for the first time in the experiments a discoid gynomonoecious species (i.e., *A. valentinus*), which turned out key to understand the whole system.

Anacyclus is a Mediterranean genus represented by eight species of mostly weedy annual herbs (Humphries, 1979; Vitales et al., 2018). Two of these species (*A. homogamos* (Maire) Humphries and *A. monanthos* Thell.) are hermaphroditic with discoid (i.e., non-radiate) capitula, and the remaining are gynomonoecious with radiate capitula, except *A. valentinus* whose capitula are discoid. The presence of non-rayed gynomonoecious species in *Anacyclus* was interpreted as a consequence of hybridization between one hermaphroditic (i.e., *A. homogamos*) and another gynomonoecious species (i.e., *A. clavatus* (Desf.) Pers. or *A. radiatus* Loisel.) (Humphries, 1979), although there are no molecular data that proves this hypothesis. Experimental crosses between all annual species pairs of *Anacyclus* are viable (Humphries, 1981) obtaining diploid artificial hybrids that showed intermediate floral traits (i.e., mainly the length and width of ligules). These intermediate phenotypes were also observed in current sympatric populations of *A. clavatus* and *A. valentinus* L. along their overlapping areas of distribution in the western Mediterranean (Humphries, 1979; Álvarez, 2019). Homoploid hybridization between these two species was suggested to explain current patterns of 45S rDNA site-number variation in wild populations (Rosato et al., 2017), as well as genome size variation patterns across similar geographic areas (Agudo et al., 2019).

Here, we investigate the gynomonoecy inheritance to understand the evolution of this sexual system in Asteraceae using the species *Anacyclus clavatus*, *A.homogamos,* and *A. valentinus* as model system. To achieve this goal, we first determined the breeding system and the degree of reproductive isolation in these three species. Frequencies of gynomonoecy were observed in the first and second generation hybrids including backcrosses for all species pairs. A joint analysis of the frequencies observed across all generations and field observations of the distribution of this sexual system in *Anacyclus*, suggested that complementary epistasis between two loci controls the gynomonoecious phenotype.

MATERIALS AND METHODS

Study system— This study was focused on three species within *Anacyclus* genus (*A. clavatus*, *A. homogamos*, and *A. valentinus*). These species grow in anthropogenic habitats as herbs in western Mediterranean (Humphries, 1979; Álvarez, 2019; Fig. 1). We have selected these species because they partially overlap their distribution areas and represent the sexual system and female floral traits diversity found in the genus (i.e., hermaphroditic, gynomonoecious with radiate capitula, and gynomonoecious with discoid capitula). The other hermaphroditic species in the genus, *A. monanthos*, is mostly restricted to Tunisia and does not overlap its range of distribution with that of *A. valentinus*. *Anacyclus clavatus* occupies the largest area, from coastal to inland regions (Fig. 1), even sometimes scattered in central and eastern Mediterranean; *A. homogamos* is mainly restricted to Middle and High Atlas in Morocco, although a few and scattered observations in coastal regions in Algeria, Morocco, and Spain were reported in the recent past; and *A. valentinus* is widely distributed in coastal regions, although may also be observed in Morocco and Iberian inland areas. Morphologically, they mainly differ by their sexual systems (i.e., hermaphroditic in *A. homogamos* vs. gynomonoecious in *A. clavatus* and *A. valentinus*), and by the ligule length and width in the corolla of the female flowers (i.e., 0.5-2.25 mm length in *A. valentinus* resulting in discoid capitula vs. 7.5-11.5 mm in *A. clavatus* that form radiate capitula). Capitula in *A. homogamos* are discoid, and all flowers are tubular. Two independent phylogenetic analyses based on nuclear and chloroplast markers (Oberprieler, 2004; Vitales et al., 2018) indicate that *A. clavatus* and *A. valentinus* are more closely related to each other than either is to *A. homogamos*.

Plant material— Seeds from six natural populations of *A. clavatus*, *A. homogamos*, and *A. valentinus* (two populations per species), were collected for sowing and cultivation in the Research Greenhouse of the Royal Botanic Garden-CSIC in Madrid (Fig.1; Appendix 1). The two sampling localities for each species were far enough apart (>50 km) such that they represent genetically distinct populations. Sampling and sowing was previously described in Torices et al. (2013). After germination, seedlings were grown individually in a mix of COMPO SANA® Universal Potting Soil (COMPO GbmH, München, Germany) and siliceous sand (3:1) until the first 4-6 leaves developed. After that, around 20-30 plants per population were planted in a similar soil mix for the experimental crosses and sexual system characterization. Out of these, 4-7 plants per population were selected as ovules donors and the remaining were designated as pollen donors.

Breeding system and inter-specific crosses— We defined the breeding system of each studied species based on the number of mature seeds per capitulum/inflorescence (seed set) produced by the plants selected as ovules donors after each pollen addition. Since in the Asteraceae each flower may produce only one seed, the total number of mature seeds and flowers were counted in each inflorescence to calculate the seed set rate for each type of pollen addition (i.e., number of mature seeds/number of flowers). Eight different types of pollination (one per inflorescence) were performed on each ovules donor: 1) no pollen addition to test spontaneous autogamy; 2) pollen addition from the same individual to test non-spontaneous autogamy, hereafter the self-compatibility test; 3) pollen addition from individuals of the same population as the ovules donor to test intra-population outcrossing; 4) pollen addition from individuals of a different population of the same species, to test inter-population outcrossing; 5-8) pollen addition from each of the four remaining populations of different species to test viability of inter-specific crosses. All manipulated capitula were bagged before anthesis until fruits were collected. A mix of pollen from different individuals was used for each experiment to ensure viability and to favor pollen saturation. Pollen was collected from style tips with tweezers and was mixed in 1.5 ml Eppendorf Tubes® (Eppendorf, Hamburg, Germany) for its immediate use. Pollen addition was made using a paintbrush for each treated capitulum, which started when the first stigmatic branches developed in the capitulum of the ovules donor, and finished at least a week after the last stigmatic branches were developed (2-4 weeks depending on the size of the capitulum). All experiments were performed in 2012. Fruits were collected at least 4 weeks after the manipulations were finished. All types of crosses were performed reciprocally and the seeds obtained here constituted the F_1 used in subsequent generations. Additionally, germination success, survival and flowering ability was also analyzed in each case.

The second hybrid generation— To explore the existence of post-zygotic barriers, a second generation of hybrids (F_2) and backcrosses (BCs) were obtained using the methods described above. In this case one population per species and 3-8 ovules donor plants were selected from each type of cross. Since selecting the same type of achenes for sowing maximizes matching phenology, here we used the winged achenes, whose seeds have faster germination times than the non-winged ones (Torices et al., 2013). Three types of pollen addition (one per capitulum) were performed on each hybrid individual: 1) pollen addition from individuals of the same population (F_2); 2) pollen addition from individuals of the population of one of the parents (BC); 3) pollen addition from individuals from the other parent population (BC). Additionally, pollen from F_1 hybrids was added to individuals of the parents' populations to test pollen viability of the hybrids. Due to space limitation in the greenhouse, the F_2 and BCs generated between *A. clavatus* and *A. homogamos* were performed in 2013, whereas those corresponding to *A. clavatus* and *A. valentinus*, as well as those between *A. homogamos* and *A. valentinus* were done in 2014. Environmental conditions (i.e., light cycle, watering regime, relative humidity, and substrate) were similar for all treatments each year. All types of crosses were performed reciprocally.

Sexual system characterization— Both wild and cultivated plants including parental lines and hybrid generations were used for sexual system characterization (i.e., gynomonoecy vs. hermaphroditism). One capitulum/inflorescence per individual was randomly selected for observations, which were made with the aid of SZX7 Olympus® binoculars (Olympus, Shinjuku, Tokyo, Japan).

Tested hypotheses of genic interactions for gynomonoecy expression— We first considered the hypothesis that gynomonoecy expression is cause by one single dominant locus *A*. Under this hypothesis, the gynomonoecious *A. clavatus* and *A. valentinus* would be *AA* homozygotes, and the hermaphroditic *A. homogamos* would be *aa*. If this were true, we would expect that the frequencies of gynomonoecy in an F_1 between a hermaphrodite and gynomonoecious individual should be similar. Other segregation patterns would require contributions from a second locus. We therefore tested the case with a dominant allele in the two gynomonoecious species *A. clavatus* and *A. valentinus* (*A* __), and the recessive one for the hermaphroditic *A. homogamos* (*aa* __). The main Mendelian genic interactions were tested for all F_1 hybrids between all species pairs: a) simple epistasis, in which one specific dominant allele is required for gene expression (*A*_); b) complementary epistasis, in which two dominant alleles are required (*A*_ *B*_); c) duplicate dominant epistasis, in which any of two dominant alleles is required (*A*_), (*B*_); and d) inhibitory epistasis, in which heterozygosis in one locus (*A*_) or, the recessive in the alternative one (*bb*) is required. After testing these hypotheses for all F_1 hybrids (see Results), we inferred homozygosis for both dominant alleles in *A. clavatus* (*AA BB*), at least in the first locus for *A. valentinus* (*AA* __), and homozygosis of the recessive allele in the first locus for *A. homogamos* (*aa* __). Therefore, we have only considered these allele combinations for the subsequent analyses in the F_2 and backcrosses in all cases. To easily assess the expected frequencies of gynomonoecy, all possible allelic combinations for the F_1, F_2 and backcrosses between the three species were represented (Appendices S1-S3; see Supplemental Data with this article). In order to explore alternative hypotheses that better explain the observations in specific cases, we also tested expected frequencies of gynomonoecy under complementary epistasis when the allelic combination *AA bb* was not present.

Statistical analyses— Experimental crosses were assessed by Generalized Linear Mixed Models (GLMMs). The effect of different crosses on the probabilities of setting a viable seed was investigated by fitting GLMMs via restricted maximum likelihood (Patterson and Thompson,

1971) with the SAS® 9.4 software (SAS Institute, Cary, North Carolina, USA) using the GLIMMIX procedure with the DIFF option in the LSMEANS statement. Satterthwaite's method was used to determine the approximate denominator degrees of freedom. The probability of producing a viable seed was modelled using a binary distribution with a logit function. All models included one fixed factor: the type of pollination (i.e., addition from the different pollen donors), and one random factor: the ovules donor plants. In order to assess whether the observed frequencies of gynomonoecy in F_1, F_2 and backcrosses fitted to the expected values under the different hypotheses of genic interaction, exact binomial tests were performed in each case. For each observed value, we adjusted the significance level for the different number of hypotheses tested using the Bonferroni procedure. All these tests were performed in R 3.5.2 (R Core Team, 2018).

RESULTS

Breeding system— Self-fertilization led to a lack of production of viable seeds or very limited production in the three studied species. When there was a production of viable seed by self-pollination, it was significantly lower than that corresponding to intra-population outcrosses (Appendix S4). Viable seeds from self-fertilization exhibit substantial germination (~75%, n = 60). From the seeds that germinated, a large portion survived (i.e., 76% surviving seedlings). From the surviving seedlings, 62% developed flowers with pollen failure being detected in 24% of the cases.

The probability of setting a viable seed in the intra-specific crosses were similar or higher than in the corresponding intra-population ones, except in *A. valentinus* that showed lower values and a remarkable variation (Fig. 2, Appendix S4). This pattern of variation was also observed for all *A. valentinus* maternal lines and for all inter-specific crosses using population W of *A. valentinus* as pollen source (Fig. 2). As a whole, similar probabilities were obtained in both *Anacyclus clavatus* and *A. homogamos* maternal lines, whereas in *A. valentinus* lower probabilities accompanied by a high variation were observed. A detailed analysis in *A. valentinus* by ovules donor (not shown) suggested maternal effects as the source of variation (i.e., fertility varied depending on the ovules donor rather than on population). The results of germination tests showed high success in all cases (>75%, n = 691) and were similar to those obtained in natural populations (Torices et al., 2013), 98% survived, of which 98.5% developed flowers, and only 0.7% did not produce pollen.

In all F_1 hybrid lines, the probability of setting a viable seed decreased significantly compared to their corresponding maternal lines after the inter-specific crosses in all cases (Fig. 3, Appendix S5). This decline in fertility was observed after both intra-population crosses (F_2) and backcrosses. However, a notable variation and higher probabilities was observed in those crosses in which *A. valentinus* was involved (Fig. 3). Besides, addition of pollen from F_1 hybrids on each species (i.e., non-hybrid ovules donors) showed lower probabilities compared with the intra-specific outcrosses, and was statistically significant in *A. clavatus* and *A. homogamos* but not in *A. valentinus* (Appendix S6). Results of the tests of germination, survival and flowering ability in the second generations for all crosses were similar to those of the corresponding F_1.

Gynomonoecy inheritance— The total of 288 F_1 hybrids generated between *A. clavatus* and *A. valentinus* (n = 31-34 per ovules donor) and between *A. clavatus* and *A. homogamos* (n = 25-37 per ovules donor) were all gynomonoecious (Appendix S7). However, the observed frequencies of gynomonoecy in F_1 hybrids between *A. valentinus* and *A. homogamos* (n = 136) were in all cases not significantly different from a ¾ ratio (0.7-0.84; n = 32-37 per ovules donor; Table 1).

Incongruent results were obtained in the *A. valentinus* ovules donor FF3077 depending on the pollen pool used for crossing (Appendix S8). The observed frequency of gynomonoecy when the pollen from *A. valentinus* × *A. homogamos* F_1 hybrids was used (94% gynomonoecious, n = 18) indicated *AA Bb* as the most probable allelic combination for this ovules donor. On the contrary, *AA BB* was inferred as the most probable combination because of the absolute frequency of gynomonoecy observed when pollen from *A. homogamos* × *A. valentinus* F_1 hybrids was used (100%, n = 19). To explain the fact that at least one hermaphroditic individual was observed after these crosses (97% gynomonoecious, n = 37) heterozygosity for the second locus in this ovules donor is required (*AA Bb*). A strong bias to allele *B* in the pollen pool may produce in the progeny a significant higher frequency of gynomonoecy than expected in equilibrium. To test for a possible bias to allele *B* in this case, we estimated the expected frequencies by excluding the *AA bb* allelic combination in the pollen pool (Table 2). Under this model, the most probable allelic combination for the ovules donor FF3077 was *AA Bb* in all cases, which is congruent with the complementary epistasis interaction.

DISCUSSION

The hypothesis of two loci that control gynomonoecy in Asteraceae is the currently accepted, although the genic interactions are still uncertain. By analyzing gynomonoecy inheritance across two hybrid generations between three inter-fertile *Anacyclus* species, our common garden study provides new insights on the genic interactions that rule the expression of this sexual system in Asteraceae. Despite limitations of sample size in the second hybrid generations due to post-zygotic barriers, overall there is a good support for the hypothesis presented here for the genetic basis of gynomonoecy in *Anacyclus*. Our results are congruent with previous studies indicating that gynomonoecy expression in Asteraceae is underlay by the epistatic interaction of at least two loci (Whitkus et al. 2000; Yang et al., 2019).

Gynomonoecious species are widespread in several Asteraceae tribes (Torices et al., 2011). By contrast, outside of the Asteraceae, gynomonoecy is found in only 3% of Angiosperm genera (Torices et al. 2011). In some of these cases, gynomonoecy is functional, and rather than female flowers with no trace of stamens as in Asteraceae, these species present sterile anthers or staminodes in their female flowers (Bernardello et al., 1999; Méndez & Munzinger, 2010; Mamut et al., 2014). In other cases, gynomonoecy occurs as a manifestation of polymorphic sexual systems in dioecious species (Koelewijn & Van Damme, 1996; Onodera et al., 2008; Casimiro-Soriguer et al., 2015). Finally, gynomonoecy is also found as occasional or rare within a species, which has been interpreted to be caused by both genetic and environmental factors (Walsh, 2005; Ghadge et al. 2014; Abdusalam et al., 2017).

Out of Asteraceae, the genetic control of gynomonoecy was secondarily studied within Caryophyllales, as part of gynodioecious-gynomonoecious species. In *Spinacia oleracea* L. (Amaranthaceae), Onodera et al. (2008) suggested that two main loci and an indeterminate number of modifier genes are involved in the sexual system expression. Similar results were shown for *Silene nutans* L. (Caryophyllaceae) by Garraud et al. (2011), in which the epistatic interactions between two cytoplasmic male sterility genes and four restorer genes determine the sexual expression, suggesting that different set of genes regulate gynomonoecy expression in Caryophyllales vs. Asteraceae. Therefore, while mutations ―probably related with organ identity genes— (Garraud et al. 2011; Ghadge et al. 2014) might have occurred independently along Angiosperm evolution leading to gynomonoecy as an occasional or secondary sexual system, the causes, mechanisms, and timing underlying gynomonoecy are likely to be other in Asteraceae, where gynomonoecy is the major sexual system in all species with radiate capitula.

In Asteraceae, in addition to the organ identity genes, those controlling floral symmetry (CYC-like genes) are also involved in sexual system expression (Yang et al. 2019). The diversification of CYC-like genes occurred in the Asteraceae (Bello et al. 2017) might have favored the acquisition of new functions for these genes in this family, such as the gynomonoecy expression linked to the radiate capitula. The hypothesis presented here is consistent with, and follows from, all these previous findings.

Considering a dominant allele for a first locus in the two gynomonoecious species *Anacyclus clavatus* and *A. valentinus* (*AA* __), and a recessive one for the hermaphroditic *A. homogamos* (*aa* __), all F_1s between the three species are expected to be gynomonoecious under any of the tested hypotheses, except under complementary epistasis between two loci when the ovules donor was recessive or heterozygous for the second locus (i.e., __*bb* and __*Bb*; Table 1, Appendix S7). In these cases, the expected frequencies for gynomonoecious F_1 offspring would vary from ½ to ¾ depending on the parental allelic combinations. This is what we found in the F_1 hybrids between *A. homogamos* and *A. valentinus*. Therefore, heterozygosis in the second locus is required for gynomonoecy expression in *A. valentinus* (*AA Bb*) whereas *A. clavatus* and *A. homogamos* should be dominant homozygous for the two loci (*AA BB*) and indifferent for the second one (*aa* __), respectively. Note that the occurrence of plants homozygous for the second locus (*BB* and *bb*) could not be discarded in populations of both *A. valentinus* and *A. homogamos*. In the latter, this would be irrelevant regarding the phenotype observed, because any individual would be hermaphroditic (*aa*__). However, in *A. valentinus* the heterozygosity for the second locus (*AA B_*) would produce gynomonoecy, whereas the recessive homozygous (*AA bb*) would produce hermaphroditism. According to this model, ¼ of *A. valentinus* individuals would be hermaphroditic in an ideal population in equilibrium and yet no hermaphroditic individuals were observed in any intra- and inter-population cross within *A. valentinus*.

A sampling bias in our study, both in pollen pool and ovule donors (i.e., 2 populations and 4-7 ovules donors and ~20 pollen donors per population), might explain the absence of hermaphrodites (*AA bb*) in all intra-specific crosses performed in *A. valentinus*. There is circumstantial evidence for the unnoticed natural occurrence of those hermaphrodites in *A. valentinus.* Scattered hermaphroditic individuals observed in coastal regions in Iberia and northern Africa (green triangles in Fig. 1) that were taxonomically interpreted as *A. homogamos*

(Humphries, 1979; Álvarez, 2019) could actually correspond to individuals of *A. valentinus* recessive for the second locus (*AA bb*). From an evolutionary standpoint, this is a more likely explanation than *A. homogamos* (*aa*__) occurring isolated and surrounded by *A. valentinus* populations. Notwithstanding, the very low frequency of hermaphrodites found along these regions (i.e., 4.6%, n = 260; Álvarez, 2019) suggests disequilibrium in the allelic combinations in natural populations. Moreover, this bias was also found in all *A. valentinus* intra-specific synthetic crosses, since there were no hermaphrodites observed. This fact indicates that such a bias is, at least in part, independent of environmental factors.

Allelic incompatibilities that manifest in hybrids (Bateson, 1909; Dobzhansky, 1936; Muller, 1942) tend to be purged over time in hybrid lineages. It is feasible that the scarcity of hermaphroditic individuals in *A. valentinus* is caused by selection against the *AA bb* genotype. This scenario together with the heterozygosis inferred here for *A. valentinus* (*AA Bb*) are congruent with the hypothesis of a hybrid origin for this species (Humphries, 1979). On the other hand, lethal nuclear-cytoplasmic interactions (Levin 2003) would lead to a high variation in fertility, and overall lower levels, in hybrid species. This is in agreement with the patterns of variation found in all *A. valentinus* intra-specific crosses, compared to those of *A. clavatus* and *A. homogamos*, in which fertility was higher with lower variation (Fig. 2). Likewise, the fact that fertility observed in offspring from all inter-specific crosses involving *A. valentinus* was more variable than in those between *A. clavatus* and *A. homogamos* (Fig. 2) is consistent with the high levels of variation expected in reproductive isolation in hybrids (Cutter, 2012).

The complex scenario for the evolution of *Anacyclus* that involve hybridization has shed light on the evolution of gynomonoecy in Asteraceae. In *Anacyclus*, an ancestral source of genetic variation is required to explain the presence of heterozygosis in at least two species of the genus (i.e., *A. homogamos* and *A. valentinus*) that are phylogenetically distant (Vitales et al., 2018; Fig. 4). The sister species to the *Anacyclus* clade, *Heliocauta atlantica* (Litard. & Maire) Humphries, is hermaphroditic (*aa*__; __ *bb*), suggesting the occurrence of a single mutation, or alternatively one event of hybridization to generate the heterozygotes required for the gynomonoecious lineages (*A_B_*). Interestingly, in the closely related families to Asteraceae such as Calyceraceae, Goodeniaceae, and Menyanthaceae, hermaphroditism is prevalent as in most of the angiosperm families. Therefore, it is possible that the origin of gynomonoecy in Asteraceae was promoted by similar allelic variation at the early stages of diversification in this family.

CONCLUSIONS

The fact that all species in our model system are self-incompatible and inter-fertile, although partially limited by reproductive post-zygotic barriers, allowed us to perform synthetic crosses for an adequate interpretation of the inheritance of the sexual system. Another crucial factor for allowing the conclusions is the allelic combinations of *Anacyclus valentinus*, which – together with the significant variation in reproductive isolation and success-- supports the hypothesis of its hybrid origin. The frequency of gynomonoecy observed across different crossings indicates a Mendelian inheritance for this trait and a complementary epistasis between two loci as the simplest possible model of genic interaction.

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AUTHOR CONTRIBUTIONS

IA and RT conceived the research, ABA, AH, IA, and RT designed and executed the experiments, ABA, IA, and RT provided plant material, IA and RT analyzed the data, IA wrote the drafts, ABA, AH, and RT provided critical comments on drafts. All authors read and approved the final version of the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information section at the end of the article:

Appendix S1. Inferred allelic combinations for *Anacyclus clavatus* (*AA BB*) and *A. valentinus* (*AA*__), their F_1 hybrids, F_2s and backcrosses (BCs) for the two loci hypothesis of the gynomonoecy expression.

Appendix S2. Inferred allelic combinations for *Anacyclus clavatus* (*AA BB*) and *A. homogamos* (*aa*__), their F_1 hybrids, F_2s and backcrosses (BCs) for the two loci hypothesis of the gynomonoecy expression.

Appendix S3. Inferred allelic combinations for *Anacyclus homogamos* (*aa*__), and *A. valentinus* (*AA*__), their F_1 hybrids, F_2s and backcrosses (BCs) for the two loci hypothesis of the gynomonoecy expression.

Appendix S4. Effects of different pollination experiment on the probability of setting a viable seed in the three studied species.

Appendix S5. Effects of intra-population crosses and backcrosses on the probability of setting a viable seed in the six types of hybrid lines generated.

Appendix S6. Least-square means (± SE) of the probability of setting a viable seed by non-hybrid lines of *Anacyclus clavatus*, *A. homogamos* and *A. valentinus* treated with pollen from their corresponding F_1 hybrids.

Appendix S7. Observed and expected frequencies of gynomonoecy in F_1 hybrids between *A. clavatus* and *A. valentinus*, and between *A. clavatus* and *A. homogamos* under the different hypotheses of genic interaction tested.

Appendix S8. Observed and expected frequencies of gynomonoecy for complementary epistasis in all F_2s and backcrosses analyzed.

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TABLES

Table 1. Observed and expected frequencies of gynomonoecy in F_1 hybrids between *A. homogamos* and *A. valentinus* under the different hypotheses of genic interaction tested: ^{a}simple epistasis (A_); ^{b}complementary epistasis (A_ B_); ^{c}duplicate dominant epistasis (A_, B_); ^{d}inhibitory epistasis (A_, _bb). n = number of individuals observed; Obs. = number of gynomonoecious individuals observed; Obs. freq. = frequency of gynomonoecy observed. *P*-value of the exact binomial test is showed between parentheses. The significance level was adjusted to 0.017 (0.05 / 3) to correct for multiple testing in each data set (row). Rejected hypotheses are underlined.

Ovules donor	Pollen origin	n	Obs.	Obs. freq.	Expected frequencies for possible allelic combination in ovules donor and hypotheses tested			Possible allelic combinations in pollen pool
A. valentinus	*A. homogamos*				*AA BB*	*AA Bb*	*AA bb*	
F151	Population At	18	14	0.78	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (1.0)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b} (0.031)	*aa BB, aa Bb, aa bb*
F151	Population Z	19	12	0.63	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b}(0.286)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b} (0.359)	
F151	Pooled	37	26	0.70	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.568)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b}(0.020)	

W575	Population At	13	9	0.69	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b}(0.748)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b}(0.267)
W575	Population Z	19	18	0.95	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.060)	$1^{\underline{a,c,d}}$ (<0.001); $0.5^{\underline{b}}$ (<0.001)
W575	Pooled	32	27	0.84	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b}(0.301)	$1^{\underline{a,c,d}}$ (<0.001); $0.5^{\underline{b}}$ (<0.001)
A. homogamos	*A. valentinus*				*aa BB*	*aa Bb*	*aa bb*
At492	Population F	17	12	0.71	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.779)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b} (0.144)
At492	Population W	18	13	0.72	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.787)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b}(0.096)
At492	Pooled	35	25	0.71	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.696)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b}(0.017)
							AA BB, AA Bb, AA bb
Z420	Population F	18	16	0.89	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.274)	$1^{\underline{a,c,d}}$ (<0.001); $0.5^{\underline{b}}$ (0.001)
Z420	Population W	14	10	0.71	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.760)	$1^{\underline{a,c,d}}$ (<0.001); 0.5^{b} (0.180)
Z420	Pooled	32	26	0.81	$1^{\underline{a,b,c,d}}$ (<0.001)	$1^{\underline{a,c,d}}$ (<0.001); 0.75^{b} (0.541)	$1^{\underline{a,c,d}}$ (<0.001); $0.5^{\underline{b}}$ (<0.001)

Table 2. Observed and expected frequencies of gynomonoecy in crosses of *A. valentinus* ovules donor FF3077 with F_1 hybrids between this species and *A. clavatus*, and *A. homogamos* under different hypothesis: [a]complementary epistasis; [b]idem but in absence of *AA bb* allelic combination. n = number of individuals observed; Obs. = number of gynomonoecious individuals observed; Obs. freq. = frequency of gynomonoecious individuals observed. *P*-value of the exact binomial test is showed between parentheses. The significance level was adjusted to 0.0125 (0.05 / 4) to correct for multiple testing in each data set (row). Rejected hypotheses are underlined.

Ovules donor	Pollen origin	n	Obs.	Obs. freq.	Expected frequencies for possible allelic combination in ovules donor and hypotheses tested			Possible allelic combinations in pollen pool
A. valentinus					*AA BB*	*AA Bb*	*AA bb*	
FF3077	F_1 produced by *A. clavatus* × *A. valentinus*	15	15	1	$1^{a,b,c}$ (1.0)	0.83^{a} (0.091); 1^{b} (1.0)	$0.67^{\underline{\mathbf{a}}}$ (0.004)	*AA BB, AA Bb*
FF3077	F_1 produced by *A. valentinus* × *A. clavatus*	13	13	1	$1^{a,b,c}$ (1.0)	0.83^{a} (0.144); 1^{b} (1.0)	$0.67^{\underline{\mathbf{a}}}$ (0.007)	
FF3077	Pooled	28	28	1	$1^{a,b,c}$ (1.0)	$0.83^{\underline{\mathbf{a}}}$ (0.010); 1^{b} (1.0)	$0.67^{\underline{\mathbf{a}}}$ (<0.001)	
FF3077	F_1 produced by *A. valentinus* × *A. homogamos*	18	17	0.94	$1^{\underline{\mathbf{a,b}}}$ (<0.001)	0.75^{a} (0.059); 0.86^{b} (0.498)	$0.5^{\underline{\mathbf{a}}}$ (<0.001)	*Aa BB, Aa Bb, Aa bb*

FF3077	F_1 produced by *A. homogamos* × *A. valentinus*	19	19	1	$1^{a,b}$ (1.0)	$0.75^{\underline{a}}$ (0.007); 0.86^{b} (0.097)	$0.5^{\underline{a}}$ (<0.001)
FF3077	Pooled	37	36	0.97	$1^{\underline{a,b}}$ (<0.001)	$0.75^{\underline{a}}$ (<0.001); 0.86^{b} (0.054)	$0.5^{\underline{a}}$ (<0.001)

APPENDICES

Appendix 1. *Anacyclus* species included in this study indicating the code of the populations selected for the experiments, the origin and voucher information such country, locality, latitude and longitude, altitude in meters above sea level (m), date of collection, collector's number in italics, and the herbarium where the voucher was deposited.

Species	Population code	Origin and voucher information
A. clavatus	B	Spain: Carchuna, 36º 41' 49" N 3º 27' 33" W, 13 m, 27.04.2011, *Agudo 1*, MA
	V	Spain: Miraflores de la Sierra, 40º 47' 36.45" N 3º 43' 46.97" W, 883 m, 22.10.2011, *Álvarez 2173*, MA

A. homogamos	Z	Morocco: Asni, 31º 15' 4.5" N 7º 58' 40.18" W, 1160 m, 24.05.2010, *Álvarez 2115*, MA
	At	Morocco: Imouzzer, 31º 19' 55" N 7º 24' 32" W, 2224 m, 13.06.2009, *Gonzalo 1275*, MA
A. valentinus	W	Spain: Castelló d'Empuries, 42º 15' 47.2" N 3º 7' 45.5" E, 0 m, 29.06.2009, *Álvarez 2059*, MA
	F	Spain: Iznate, 36º 46' 35" N 4º 10' 45.2" W, 285 m, 30.03.2011, *Álvarez 2137*, MA

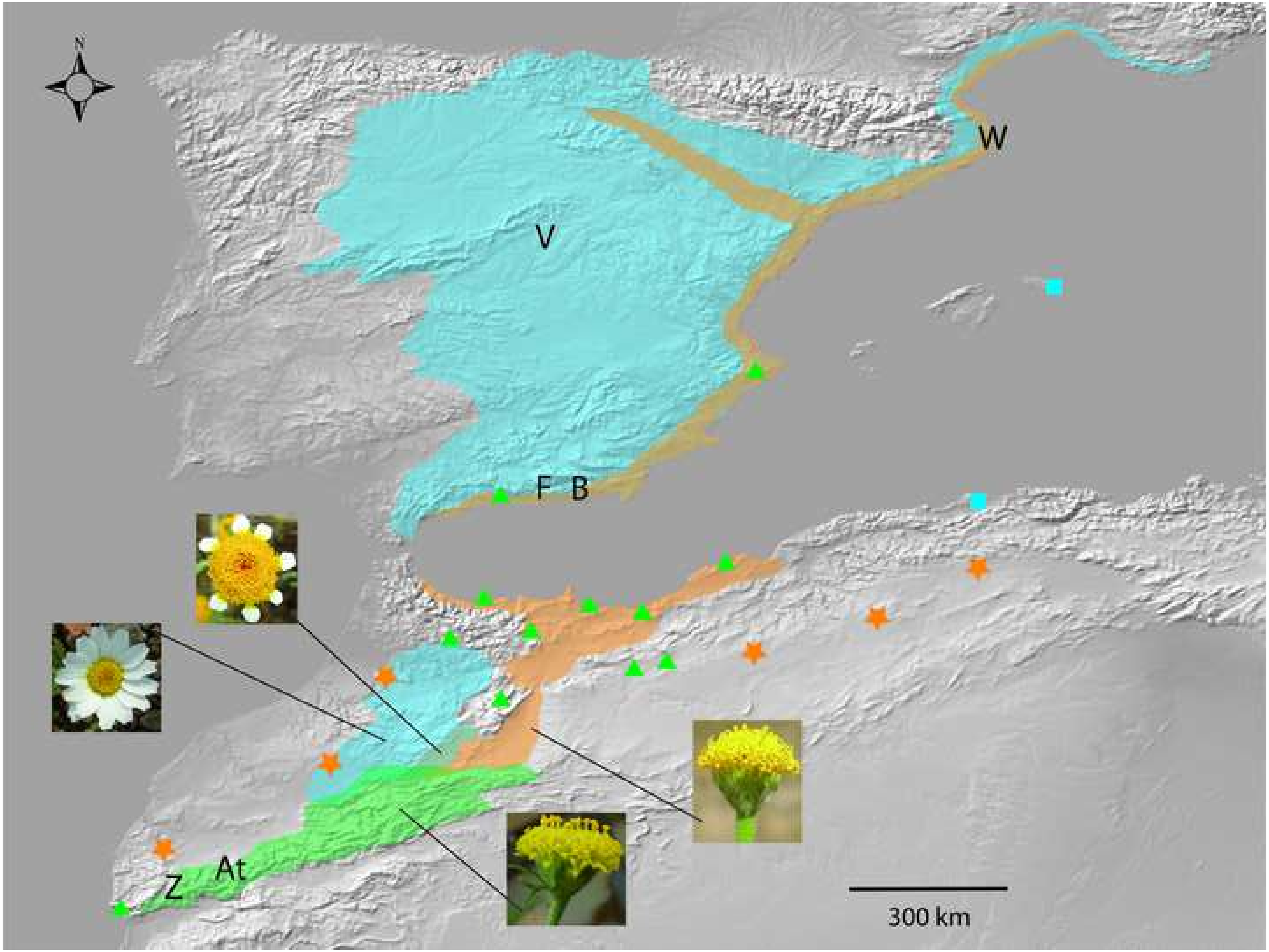
FIGURE LEGENDS

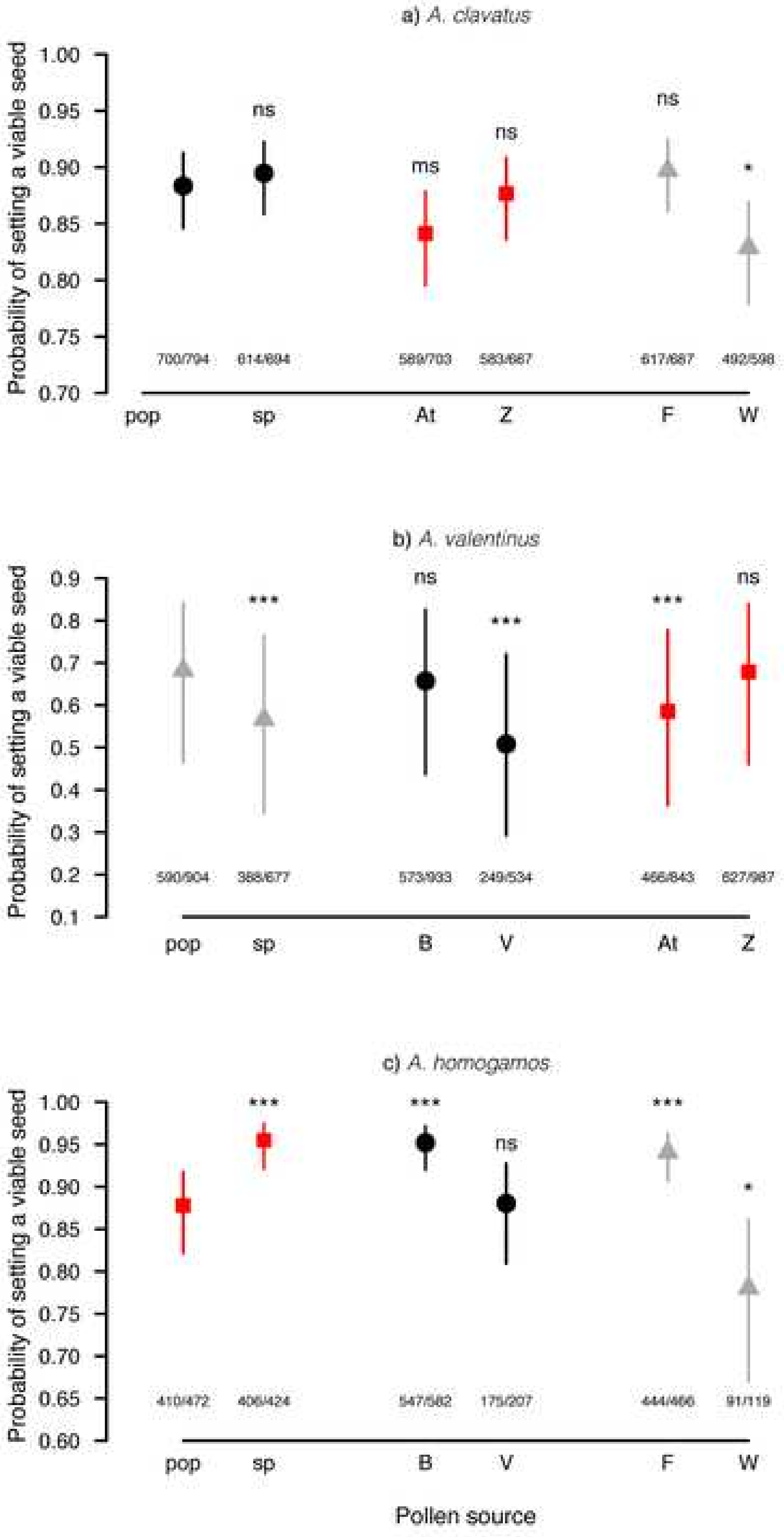
Figure 1. Map of distribution of the three studied species showing their overlapping areas: *Anacyclus clavatus* (blue), *A. homogamos* (green) and *A. valentinus* (orange). Isolated populations are represented by blue squares in *A. clavatus*, by green triangles in *A. homogamos*, and by orange stars in *A. valentinus*. Pictures of floral phenotypes of the three species and one intermediate phenotype found in an overlapping area are also shown. The approximate location of the populations used in the experiments are shown by letters (At and Z for *A. homogamos*; B and V for *A. clavatus*; F and W for *A. valentinus*).

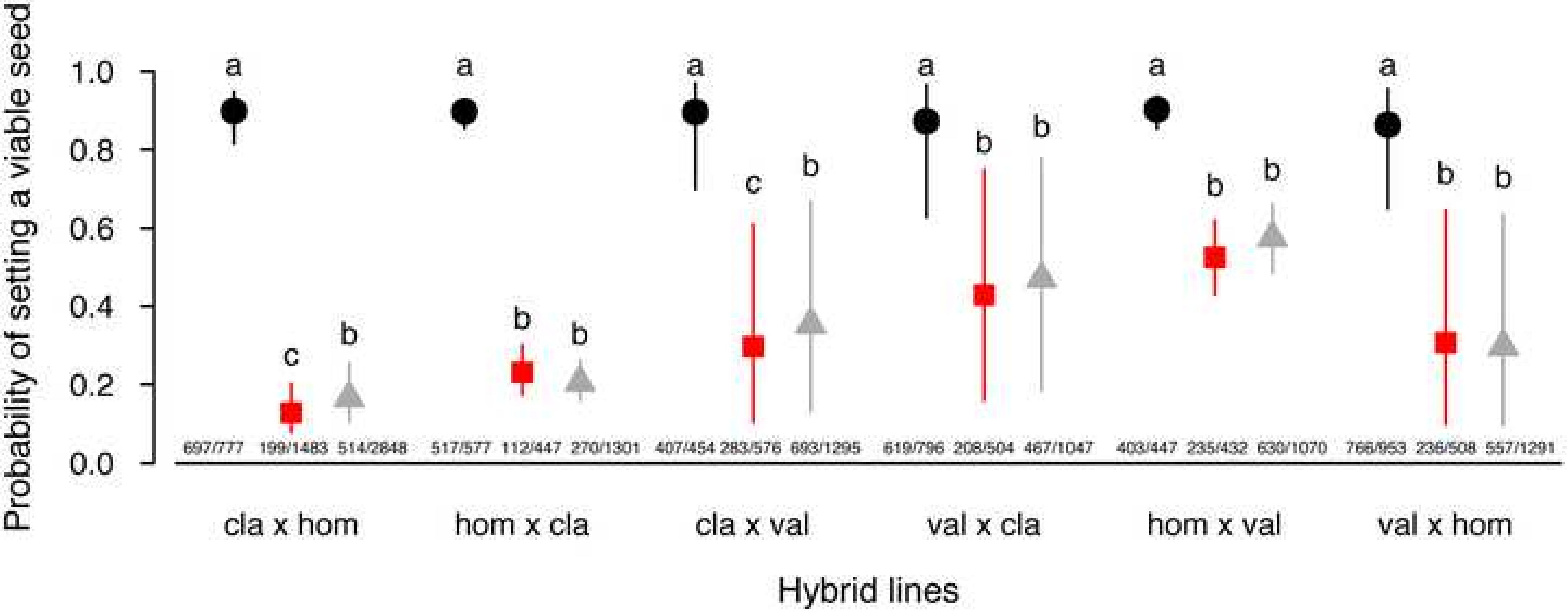
Figure 2. Least-square means (± 95% CI) of the probability of setting a viable seed by *Anacyclus clavatus* (a), *A. valentinus* (b), and *A. homogamos* (c). Treatments are pollen addition from different sources: pollen from individuals of the same population of which the ovules donor is from (pop); pollen from individuals of the same species but different population of which the ovules donor is from (sp); pollen from individuals of *A. clavatus* populations (B) and (V) both in black circles; pollen from individuals of *A. homogamos* populations (At) and (Z) both in red squares; and pollen from individuals of *A. valentinus* populations (F) and (W) both in grey triangles. Statistical comparisons with the corresponding intra-population outcross (pop) are showed (ns, $P > 0.01$; ms, $P < 0.1$; *, $P < 0.05$: **, $P < 0.01$; ***, $P < 0.001$). Sample size for each group is indicated above x-axis (no. of seeds / no. of pollinated flowers).

Figure 3. Least-square means (± 95% CI) of the probability of setting a viable seed by the F_1 hybrids when fertilized with pollen from the same F_1 line (F_2: red squares) and from non-hybrid lines (BCs: grey triangles) produced by the different type of crosses: *Anacyclus clavatus* × *A. homogamos* (cla × hom); *A. homogamos* × *A. clavatus* (hom × cla); *A. clavatus* × *A. valentinus* (cla × val); *A. valentinus* × *A. clavatus* (val × cla); *A. homogamos* × *A. valentinus* (hom × val); and *A. valentinus* × *A. homogamos* (val × hom). Data observed on the corresponding intra-specific crosses for each case were included (black circles). Different letters above each group indicate means statistically different between groups within each hybrid line ($P < 0.05$). Sample size for each group is indicated above x-axis (no. of seeds / no. of pollinated flowers).

Figure 4.- Phylogenetic relationships for all *Anacyclus* species and its outgroup (*Heliocauta atlantica*) following Vitales et al. 2018. Gynomonoecy (blue squares) and hermaphroditism (black squares) were represented for each species, as well as the possible inferred allelic combinations in each case.


N
W
V
F
B
At
Z
300 km


a) *A. clavatus*
Probability of setting a viable seed
1.00
0.95
0.90
0.85
0.80
0.75
0.70
ns
ms
ns
ns
*
700/794
614/694
589/703
583/667
617/687
492/598
pop
sp
At
Z
F
W
b) *A. valentinus*
Probability of setting a viable seed
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
***
ns
***
***
ns
590/904
388/677
573/933
249/534
466/843
627/987
pop
sp
B
V
At
Z
c) *A. homogamos*
Probability of setting a viable seed
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
***
***
ns
***
*
410/472
406/424
547/582
175/207
444/466
91/119
pop
sp
B
V
F
W
Pollen source


Probability of setting a viable seed
1.0
0.8
0.6
0.4
0.2
0.0
a
c
b
a
b
b
a
c
b
a
b
b
a
b
b
a
b
b
697/777
199/1483
514/2848
517/577
112/447
270/1301
407/454
283/576
693/1295
619/796
208/504
467/1047
403/447
235/432
630/1070
766/953
236/508
557/1291
cla x hom
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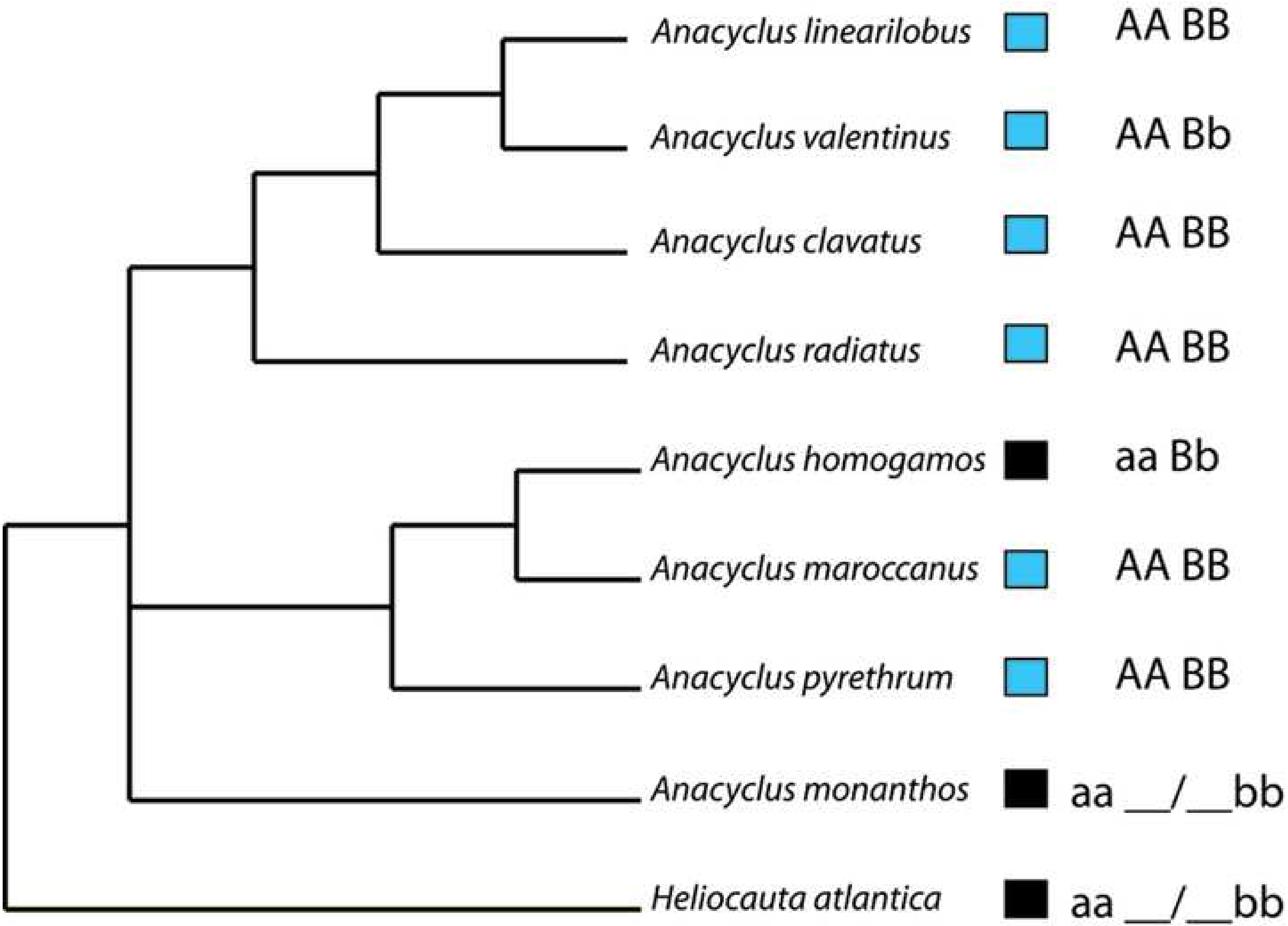
cla x val
val x cla
hom x val
val x hom
Hybrid lines


Anacyclus linearilobus
AA BB
Anacyclus valentinus
AA Bb
Anacyclus clavatus
AA BB
Anacyclus radiatus
AA BB
Anacyclus homogamos
aa Bb
Anacyclus maroccanus
AA BB
Anacyclus pyrethrum
AA BB
Anacyclus monanthos
aa __/__bb
Heliocauta atlantica
aa __/__bb

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Appendix S1. Inferred allelic combinations for *Anacyclus clavatus* (*AA BB*) and *A. valentinus* (*AA*__), their F_1 hybrids, F_2s and backcrosses (BCs) for the two loci hypothesis of the gynomonoecy expression. Boxes include combinations for each type of ovules donor.

		A B	A b	
A. clavatus × *A. valentinus*				
AA BB × AA BB		AA BB		
AA BB × AA Bb	A B	AA BB	AA Bb	F_1
AA BB × AA bb	A B		AA Bb	
F_1 × F_1				
AA BB × AA BB	A B	AA BB		
AA BB × AA Bb	A B	AA BB	AA Bb	
AA Bb × AA BB	A B	AA BB	AA Bb	F_2
AA Bb × AA Bb	A B	AA BB	AA Bb	
	A b	AA Bb	AA bb	
F_1 × *A. clavatus*				
AA BB × AA BB	A B	AA BB		BCs to *A. clavatus*
AA Bb × AA BB	A B	AA BB	AA Bb	
F_1 × *A. valentinus*				
AA BB × AA BB		AA BB		
AA BB × AA Bb	A B	AA BB	AA Bb	
AA BB × AA bb	A B		AA Bb	BCs to *A. valentinus*
AA Bb × AA BB	A B	AA BB	AA Bb	
AA Bb × AA Bb	A B	AA BB	AA Bb	
	A b	AA Bb	AA bb	
AA Bb × AA bb	A b	AA Bb	AA bb	

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Appendix S2. Inferred allelic combinations for *Anacyclus clavatus* (*AA BB*) and *A. homogamos* (*aa__*), their F_1 hybrids, F_2s and backcrosses (BCs) for the two loci hypothesis of the gynomonoecy expression. Boxes include combinations for each type of ovules donor.

A. clavatus × *A. homogamos*						
		A B	A b	a B	a b	
AA BB × aa BB	A B			Aa BB		
AA BB × aa Bb	A B			Aa BB	Aa Bb	F_1
AA BB × aa bb	A B				Aa Bb	
F_1 × F_1						
		A B	A b	a B	a b	
Aa BB × Aa BB	A B	AA BB		Aa BB		
	a B	Aa BB		aa BB		
Aa BB × Aa Bb	A B	AA BB	AA Bb	Aa BB	Aa Bb	
	a B	Aa BB	Aa Bb	aa BB	aa Bb	
Aa Bb × Aa BB	A B	AA BB	AA Bb	Aa BB	Aa Bb	F_2
	a B	Aa BB	Aa Bb	aa BB	aa Bb	
Aa Bb × Aa Bb	A B	AA BB	AA Bb	Aa BB	Aa Bb	
	A b	AA Bb	AA bb	Aa Bb	Aa bb	
	a B	Aa BB	Aa Bb	aa BB	aa Bb	
	a b	Aa Bb	Aa bb	aa Bb	aa bb	
F_1 × *A. clavatus*						
		A B	A b	a B	a b	
Aa BB × AA BB	A B	AA BB		Aa BB		BCs to *A. clavatus*
Aa Bb × AA BB	A B	AA BB	AA Bb	Aa BB	Aa Bb	
F_1 × *A. homogamos*						
		A B	A b	a B	a b	
Aa BB × aa BB	a B	Aa BB		aa BB		
Aa BB × aa Bb	a B	Aa BB		aa BB		
	a b	Aa Bb		aa Bb		
Aa BB × aa bb	a b	Aa Bb		aa Bb		BCs to *A. homogamos*
Aa Bb × aa BB	a B	Aa BB	Aa Bb	aa BB	aa Bb	
Aa Bb × aa Bb	a B	Aa BB	Aa Bb	aa BB	aa Bb	
	a b	Aa Bb	Aa bb	aa Bb	aa bb	
Aa Bb × aa bb	a b	Aa Bb	Aa bb	aa Bb	aa bb	

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Appendix S3. Inferred allelic combinations for *Anacyclus homogamos* (*aa*__), and *A. valentinus* (*AA*__), their F_1 hybrids, F_2s and backcrosses (BCs) for the two loci hypothesis of the gynomonoecy expression. Boxes include combinations for each type of ovules donor.

A. homogamos × *A.valentinus*		A B	A b	a B	a b	
aa BB × AA BB	a B	Aa BB				
aa BB × AA Bb	a B	Aa BB	Aa Bb			
aa BB × AA bb	a B		Aa Bb			
aa Bb × AA BB	A B			Aa BB	Aa Bb	
aa Bb × AA Bb	a B	Aa BB	Aa Bb			
	a b	Aa Bb	Aa bb			
aa Bb × AA bb	a B		Aa Bb			F_1
	a b		Aa bb			
aa bb × AA BB	a b	Aa Bb				
aa bb × AA Bb	a b	Aa Bb	Aa bb			
aa bb × AA bb	a b		Aa bb			
F_1 × F_1						
		A B	A b	a B	a b	
Aa BB × Aa BB	A B	AA BB		Aa BB		
	a B	Aa BB		aa BB		
Aa BB × Aa Bb	A B	AA BB	AA Bb	Aa BB	Aa Bb	
	a B	Aa BB	Aa Bb	aa BB	aa Bb	
Aa BB × Aa bb	A B		AA Bb		Aa Bb	
	a B		Aa Bb		aa Bb	
Aa Bb × Aa BB	A B	AA BB	AA Bb	Aa BB	Aa Bb	
	a B	Aa BB	Aa Bb	aa BB	aa Bb	
Aa Bb × Aa Bb	A B	AA BB	AA Bb	Aa BB	Aa Bb	
	A b	AA Bb	AA bb	Aa Bb	Aa bb	
	a B	Aa BB	Aa Bb	aa BB	aa Bb	F_2
	a b	Aa Bb	Aa bb	aa Bb	aa bb	
Aa Bb × Aa bb	A b	AA Bb	AA bb	Aa Bb	Aa bb	
	a b	Aa Bb	Aa bb	aa Bb	aa bb	
Aa bb × Aa BB	A b	AA Bb		Aa Bb		
	a b	Aa Bb		aa Bb		
Aa bb × Aa Bb	A b	AA Bb	AA bb	Aa Bb	Aa bb	
	a b	Aa Bb	Aa bb	aa Bb	aa bb	
Aa bb × Aa bb	A b		AA bb		Aa bb	
	a b		Aa bb		aa bb	
F_1 × *A. homogamos*						
		A B	A b	a B	a b	

Aa BB × aa BB	a B	Aa BB		aa BB		
Aa BB × aa Bb	a B	Aa BB		aa BB		
	a b	Aa Bb		aa Bb		
Aa BB × aa bb	a b	Aa Bb		aa Bb		
Aa Bb × aa BB	a B	Aa BB	Aa Bb	aa BB	aa Bb	
Aa Bb × aa Bb	a B	Aa BB	Aa Bb	aa BB	aa Bb	BCs to *A. homogamos*
	a b	Aa Bb	Aa bb	aa Bb	aa bb	
Aa Bb × aa bb	a b	Aa Bb	Aa bb	aa Bb	aa bb	
Aa bb × aa BB	a B		Aa Bb		aa Bb	
Aa bb × aa Bb	a B		Aa Bb		aa Bb	
	a b		Aa bb		aa bb	
Aa bb × aa bb	a b		Aa bb		aa bb	
F_1 × *A. valentinus*						
		A B	A b	a B	a b	
Aa BB × AA BB	A B	AA BB		Aa BB		
Aa BB × AA Bb	A B	AA BB		Aa BB		
	A b	AA Bb		Aa Bb		
Aa BB × AA bb	A b	AA Bb		Aa Bb		
Aa Bb × AA BB	A B	AA BB	AA Bb	Aa BB	Aa Bb	
Aa Bb × AA Bb	A B	AA BB	AA Bb	Aa BB	Aa Bb	
	A b	AA Bb	AA bb	Aa Bb	Aa bb	BCs to *A. valentinus*
Aa Bb × AA bb	A b	AA Bb	AA bb	Aa Bb	Aa bb	
Aa bb × AA BB	A B		AA Bb		Aa Bb	
Aa bb × AA Bb	A b	AA Bb	AA bb			
	a b	Aa Bb	Aa bb			
Aa bb × AA bb	A b		AA bb		Aa bb	

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Appendix S4. Effects of different pollination experiment on the probability of setting a viable seed in the three studied species. No viable seeds were observed in any non-spontaneous autogamy treatment for *A. valentinus*. Data represent the Wald-type F-statistic with the degrees of freedom as sub-index for fixed factors, and the estimate for covariance parameter and its standard error for the random factor: Plant. Significant p-values are in bold.

	A. clavatus		*A. valentinus*		*A. homogamos*	
Non-spontaneous autogamy vs. intra-population outcrosses	*F*	*P*	*F*	*P*	*F*	*P*
Pollination experiment	$165.7_{1,\ 1588}$	**<0.0001**	-	-	$169.34_{1,\ 966}$	**<0.0001**
Plant (Estimate ± SE)	0.108 ± 0.122		-		0.395 ± 0.457	
Sample size	1590		1919		968	
Spontaneous autogamy vs. intra-population outcrosses						
Pollination experiment	$451.79_{1,\ 1493}$	**<0.0001**	$184.39_{1,\ 1773}$	**<0.0001**	$411.51_{1,\ 1026}$	**<0.0001**
Plant (Estimate ± SE)	0.292 ± 0.281		1.422 ± 1.193		0	
Sample size	1495		1775		1028	
Non-spontaneous vs. spontaneous autogamy						
Pollination experiment	$9.01_{1,\ 1495}$	**0.0027**	-	-	$9.29_{1,\ 1050}$	**0.0024**
Plant (Estimate ± SE)	1.620 ± 1.751		-		5.522 ± 6.6	
Sample size	1497		1886		1052	
Intra-specific vs. intra-population outcrosses						
Pollination experiment	$0.03_{1,\ 675.6}$	0.855	$18.4_{1,\ 1579}$	**<0.0001**	$8.98_{1,\ 894}$	**0.0028**
Plant (Estimate ± SE)	0.025 ± 0.045		0.771 ± 0.642		0.482 ± 0.536	
Sample size	1488		1581		896	
Inter-specific crosses vs. intra-population outcrosses						
Pollination experiment	$2.55_{1,\ 3447}$	0.1104	$9.9_{1,\ 4199}$	**0.0017**	$8.34_{1,\ 1846}$	**0.0039**
Plant (Estimate ± SE)	0.138 ± 0.122		1.42 ± 1.167		0.236 ± 0.256	

Sample size	3449	4201	1848

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Appendix S5. Effects of intra-population crosses and backcrosses on the probability of setting a viable seed in the six types of hybrid lines generated. Control represents the inter-specific crosses of the ovule donors in each case. Data represent the Wald-type χ^2 test for the fixed factor, and the estimate for covariance parameter and its standard deviation for the random factor: Plant. Significant p-values are in bold.

Type of cross	Pollination experiment			Plant
	n	χ^2	*P*	*Estimate ± SD*
A. clavatus × *A. homogamos*	5108	86.1	**<0.0001**	0.44 ± 0.66
A. homogamos × *A. clavatus*	3879	184.6	**<0.0001**	0.09 ± 0.31
A. clavatus × *A. valentinus*	2325	15.0	**0.0005**	1.28 ± 1.13
A. valentinus × *A. clavatus*	2347	6.63	**0.0364**	1.48 ± 1.21
A. homogamos × *A. valentinus*	1949	48.2	**<0.0001**	0.09 ± 0.30
A. valentinus × *A. homogamos*	2752	8.14	**0.0171**	1.52 ± 1.23

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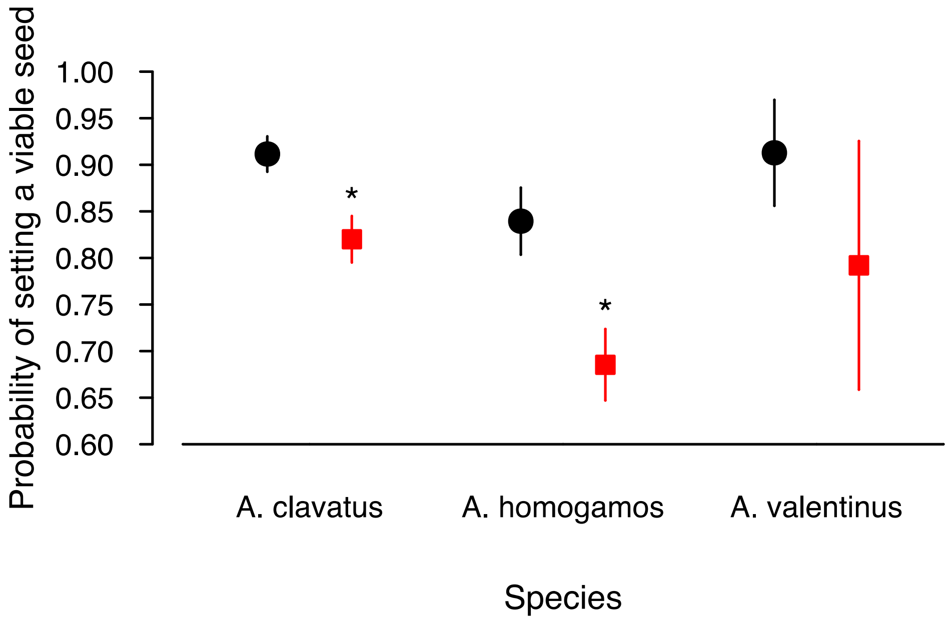
Appendix S6. Least-square means (± SE) of the probability of setting a viable seed by non-hybrid lines of *Anacyclus clavatus, A. homogamos* and *A. valentinus* treated with pollen from their corresponding F_1 hybrids (red squares). Data for each intra-specific cross is showed (black circles). Only significant differences with the corresponding intra-population outcross are indicated (*P < 0.05).



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Appendix S7. Observed and expected frequencies of gynomonoecy in F_1 hybrids between *A. clavatus* and *A. valentinus*, and between *A. clavatus* and *A. homogamos* under the different hypotheses of genic interaction tested: [a]simple epistasis (*A_*); [b]complementary epistasis (*A_ B_*); [c]duplicate dominant epistasis (*A_*, *B_*); [d]inhibitory epistasis (*A_*, *_bb*). *P*-value of the exact binomial test is showed between parentheses. The significance level was adjusted to 0.017 (0.05 / 3) to correct for multiple testing in each data set (row). Rejected hypotheses are underlined. n = number of individuals observed; Obs. = number of gynomonoecious individuals observed; Obs. freq. = frequency of gynomonoecious individuals observed.

Ovules donor	Pollen origin	n	Obs.	Obs. freq.	Expected frequencies for possible allelic combination in ovules donor and hypotheses tested			Possible allelic combinations in pollen pool
A. clavatus	*A. valentinus*				*AA BB*	*AA Bb*	*AA bb*	
B23	Population F	15	15	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.031)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	*AA BB*, *AA Bb*, *AA bb*
B23	Population W	19	19	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.007)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
B23	Pooled	34	34	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (<0.001)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
V50	Population F	19	19	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.006)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
V50	Population W	14	14	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.028)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
V50	Pooled	33	33	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (<0.001)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
	A. homogamos							
B23	Population At	20	20	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.007)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	*aa BB*, *aa Bb*, *aa bb*
B177	Population Z	12	12	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.046)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
B186	Population Z	16	16	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.017)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
V50	Population At	18	18	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.011)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
V50	Population Z	19	19	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.007)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
V50	Pooled	37	37	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (<0.001)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	

A. valentinus	*A. clavatus*							
F151	Population B	17	17	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$n (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.011)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
F151	Population V	15	15	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.031)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
F151	Pooled	32	32	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (<0.001)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
								AA BB, *AA Bb*, *AA bb*
W575	Population B	19	19	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.007)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
W575	Population V	12	12	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.046)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
W575	Pooled	31	31	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (<0.001)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
A. homogamos	*A. clavatus*				*aa BB*	*aa Bb*	*aa bb*	
At492	Population B	19	19	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.007)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
At492	Population V	6	6	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.347)	$1^{a,c,d}$ (1.0); 0.5^{b} (0.031)	
At492	Pooled	25	25	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.002)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
Z420	Population B	15	15	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.031)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	*AA BB*, *AA Bb*, *AA bb*
Z420	Population V	18	18	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (0.011)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
Z420	Pooled	33	33	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); $0.75^{\underline{\mathbf{b}}}$ (<0.001)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	
Z747	Population B	15	15	1	$1^{a,b,c,d}$ (1.0)	$1^{a,c,d}$ (1.0); 0.75^{b} (0.031)	$1^{a,c,d}$ (1.0); $0.5^{\underline{\mathbf{b}}}$ (<0.001)	

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Appendix S8. Observed and expected frequencies of gynomonoecy for complementary epistasis in all F_2s and backcrosses analysed. *P*-value of the exact binomial test is showed between parentheses. The significance level was adjusted to 0.025 and 0.017 to correct for multiple testing when the same observed distribution was tested simultaneously against two or three different expected frequencies, respectively. *P*-values for rejected hypotheses are in bold. n = number of individuals observed; Obs. = number of gynomonoecious individuals observed; Obs. freq. = frequency of gynomonoecious individuals observed.

Ovules donor	Pollen origin	n	Obs.	Obs. freq.	Expected frequencies for the inferred allelic combination in ovules donor and hypotheses tested		Possible allelic combinations in pollen pool
A. clavatus					*AA BB*		
BB1115	F_1 produced by *A. clavatus* × *A. homogamos*	15	15	1	1 (1.0)		*Aa BB, Aa Bb*
BB1115	F_1 produced by *A. homogamos* × *A. clavatus*	12	12	1	1 (1.0)		
BB1115	Pooled	27	27	1	1 (1.0)		
BB1292	F_1 produced by *A. clavatus* × *A. homogamos*	10	10	1	1 (1.0)		
BB1292	F_1 produced by *A. homogamos* × *A. clavatus*	13	13	1	1 (1.0)		
BB1292	Pooled	23	23	1	1 (1.0)		
BB2799	F_1 produced by *A. clavatus* × *A. valentinus*	16	16	1	1 (1.0)		*AA BB, AA Bb*
BB2799	F_1 produced by *A. valentinus* × *A. clavatus*	16	16	1	1 (1.0)		
BB2799	Pooled	32	32	1			
F_1 between *A. clavatus* and *A. homogamos*	*A. clavatus*				*Aa BB*	*Aa Bb*	
BZ1119	Population B	10	10	1	1 (1.0)	1 (1.0)	*AA BB*
BZ1358	Population B	10	10	1	1 (1.0)	1 (1.0)	
ZB1249	Population B	10	10	1	1 (1.0)	1 (1.0)	
ZB1250	Population B	12	12	1	1 (1.0)	1 (1.0)	

		A. homogamos							
	BZ1119	Population Z	13	7	0.54	0.5 (1.0)	0.37 (0.253)		*aa BB, aa Bb, aa bb*
	BZ1358	Population Z	13	4	0.31	0.5 (0.267)	0.37 (0.778)		
	ZB1249	Population Z	14	4	0.29	0.5 (0.180)	0.37 (0.592)		
	ZB1250	Population Z	18	5	0.28	0.5 (0.096)	0.37 (0.475)		
	BZ1119	F₁ produced by *A. clavatus* × *A. homogamos*	12	9	0.75	0.75 (1.0)	0.62 (0.554)		*Aa BB, Aa Bb*
	BZ1358	F₁ produced by *A. clavatus* × *A. homogamos*	10	5	0.50	0.75 (0.134)	0.62 (0.519)		
	ZB1249	F₁ produced by *A. homogamos* × *A. clavatus*	12	8	0.67	0.75 (0.510)	0.62 (1.0)		
	ZB1250	F₁ produced by *A. homogamos* × *A. clavatus*	11	9	0.82	0.75 (1.0)	0.62 (0.225)		
F₁ between *A. clavatus* and *A. valentinus*		*A. clavatus*				*AA BB*	*AA Bb*		
	BF2767	Population B	10	10	1	1 (1.0)	1 (1.0)		*AA BB*
	FB2733	Population B	17	17	1	1(1.0)	1 (1.0)		
		A. valentinus							
	BF2767	Population F	11	11	1	1 (1.0)	0.75 (0.077)		*AA BB, AA Bb, AA bb*
	FB2733	Population F	21	21	1	1 (1.0)	0.75 **(0.004)**		
	BF2767	F₁ produced by *A. clavatus* × *A. valentinus*	15	15	1	1 (1.0)	0.83 (0.091)		*AA BB, AA Bb*
	FB2733	F₁ produced by *A. valentinus* × *A. clavatus*	14	14	1	1 (1.0)	0.83 (0.148)		
A. homogamos						*aa BB*	*aa Bb*	*aa bb*	
	ZZ1690	F₁ produced by *A. clavatus* × *A. homogamos*	18	10	0.56	0.5 (0.814)	0.42 (0.340)	0.33 (0.048)	*Aa BB, Aa Bb*
	ZZ1690	F₁ produced by *A. homogamos* × *A. clavatus*	14	6	0.43	0.5 (0.790)	0.42 (1.0)	0.33 (0.412)	
	ZZ1690	Pooled	32	16	0.50	0.5 (1.0)	0.42 (0.375)	0.33 (0.058)	
	ZZ1691	F₁ produced by *A. clavatus* × *A. homogamos*	13	5	0.38	0.5 (0.581)	0.42 (1.0)	0.33 (0.769)	
	ZZ1691	F₁ produced by *A. homogamos* × *A. clavatus*	11	6	0.55	0.5 (1.0)	0.42 (0.543)	0.33 (0.195)	
	ZZ1691	Pooled	24	11	0.46	0.5 (0.839)	0.42 (0.837)	0.33(0.196)	
	ZZ2772	F₁ produced by *A. homogamos* × *A. valentinus*	15	6	0.40	0.5 (0.607)	0.37 (0.795)	0.25 (0.228)	*Aa BB, Aa Bb, Aa bb*
	ZZ2772	F₁ produced by *A. valentinus* × *A. homogamos*	13	1	0.08	0.5 **(0.003)**	0.37 (0.040)	0.25 (0.207)	
	ZZ2772	Pooled	28	7	0.25	0.5 **(0.013)**	0.37 (0.241)	0.25 (1.0)	
F₁ between *A. homogamos* and		*A. homogamos*				*Aa BB*	*Aa Bb*	*Aa bb*	

A. valentinus									
	ZF2780	Population Z	13	4	0.31	0.5 (0.267)	0.37 (0.778)	0.25 (0.748)	*aa BB, aa Bb, aa bb*
	FZ2675	Population Z	9	2	0.22	0.5 (0.178)	0.37 (0.500)	0.25 (1.0)	
		A. valentinus							
	ZF2780	Population F	12	11	0.92	1 (<**0.001**)	0.75 (0.316)	0.5 (**0.006**)	*AA BB, AA Bb, AA bb*
	FZ2675	Population F	14	10	0.71	1 (<**0.001**)	0.75 (0.760)	0.5 (0.180)	
	ZF2780	F_1 produced by *A. homogamos* × *A. valentinus*	18	9	0.5	0.75 (0.025)	0.56 (0.641)	0.37 (0.329)	*Aa BB, Aa Bb, Aa bb*
	FZ2675	F_1 produced by *A. valentinus* × *A. clavatus*	19	13	0.68	0.75 (0.595)	0.56 (0.357)	0.37 (**0.007**)	
A. valentinus						*AA BB*	*AA Bb*	*AA bb*	
	FF3077	F_1 produced by *A. clavatus* × *A. valentinus*	15	15	1	1 (1.0)	0.83 (0.091)	0.67 (**0.004**)	*AA BB, AA Bb*
	FF3077	F_1 produced by *A. valentinus* × *A. clavatus*	13	13	1	1 (1.0)	0.83 (0.144)	0.67 (**0.007**)	
	FF3077	Pooled	28	28	1	1 (1.0)	0.83 (**0.010**)	0.67 (<**0.001**)	
	FF3077	F_1 produced by *A. valentinus* × *A. homogamos*	18	17	0.94	1 (<**0.001**)	0.75 (0.059)	0.5 (<**0.001**)	*Aa BB, Aa Bb, Aa bb*
	FF3077	F_1 produced by *A. homogamos* × *A. valentinus*	19	19	1	1 (1.0)	0.75 (**0.007**)	0.5 (<**0.001**)	
	FF3077	Pooled	37	36	0.97	1 (<**0.001**)	0.75 (<**0.001**)	0.5 (<**0.001**)	