Do *s* genes or deleterious recessives control late-acting self-incompatibility in *Handroanthus heptaphyllus* (Bignoniaceae)? A diallel study with four full sib progeny arrays

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- **Background and Aims** Genetically controlled self-incompatibility (SI) mechanisms constrain selfing and thus have contributed to the evolutionary diversity of flowering plants. In homomorphic gametophytic SI (GSI) and homomorphic sporophytic SI (SSI), genetic control is usually by a single multi-allelic locus S. Both GSI and SSI prevent self pollen tubes reaching the ovary and so are pre-zygotic in action. In contrast, in taxa with late-acting self-incompatibility (LSI), rejection is often post-zygotic, since self-pollen tubes grow to the ovary where fertilization may occur prior to floral abscission. Alternatively, lack of self fruit set could be due to early-acting inbreeding depression (EID). The aim of our study was to investigate mechanisms underlying lack of selfed fruit set in *Handroanthus heptaphyllus* in order to assess the likelihood of LSI versus EID.
- **Methods** We employed four full sib diallels to study the genetic control of LSI in *Handroanthus heptaphyllus* using a precociously flowering variant. We also used fluorescence microscopy to study the incidence of ovule penetration by pollen tubes in pistils that abscised following pollination or initiated fruits.
- **Key Results** All diallels showed reciprocally cross-incompatible full-sibs (RCI), reciprocally cross compatible full-sibs (RCC), and non-reciprocally compatible full-sibs (NRC) in almost equal proportions. There was no significant difference between the incidence of ovule penetrations in abscised pistils following self- and cross-incompatible pollinations, but those in successful cross pollinations were around twofold greater.
- **Conclusions** A genetic model postulating a single S locus with four *s* alleles, one of which, in the maternal parent, is dominant to the other three, will produce RCI, RCC and NRC situations each at 33 %, consistent with our

diallel results. We favour this simple genetic control over an early-acting inbreeding depression (EID) explanation since none of our pollinations, successful or unsuccessful, resulted in partial embryo development, as would be expected under a whole genome EID effect.

Key words: Full sib diallel crosses, *Handroanthus heptaphyllus* (Bignoniaceae), late-acting self-incompatibility.

INTRODUCTION

Self-incompatibility (SI), as found in many hermaphrodite-flowered (and some monoecious) angiosperm species, comprise genetic systems that recognize and constrain growth of self-pollen. Typically, homomorphic SI mechanisms have a Mendelian genetic control with (most commonly) a single multi-allelic locus denominated as S. In homomorphic gametophytic SI (GSI), the *s* allele located in the nucleus of the haploid pollen grain determines incompatibility in an interaction with the two *s* alleles in the pistil, whilst in homomorphic sporophytic SI (SSI) both *s* alleles in the diploid pollen parent determine compatibility (de Nettancourt, 1977; Franklin-Tong, 2008). Another SI mechanism, heteromorphic SI (HetSI), associated with heterostylous species, is based on a single diallelic locus in the widespread distylous condition and two such loci in the rare tristylous one (Ganders, 1979; Lewis and Jones, 1992). Recently, a fourth SI mechanism, (DSI), also based on a diallelic locus but not associated with floral heteromorphy, has been reported for three genera in the Oleaceae: *Phillyrea*, *Fraxinus* and *Olea* (Samitou-Laprade *et al.*, 2010, 2017; Vernet *et al.*, 2016). In all four SI mechanisms the incompatibility reaction is pre-zygotic, with self pollen grain germination on the stigma, or self pollen tube growth in the style impeded.

A fifth mechanism, late-acting SI (LSI), is often post-zygotic in action (Seavey and Bawa, 1986; Sage *et al.*, 1994; Gibbs, 2014). Typically in LSI species, self pollen tubes grow to the ovary with seemingly equal facility as cross pollen tubes, and in many studied cases ovules are penetrated and self-fertilization occurs before the selfed pistils are abscised. Self ovule penetration and fertilization are singular features for a SI mechanism and critics have argued that early acting inbreeding depression (EID), with deleterious recessives affecting selfed embryo development, is a more likely explanation for the failure of fruit set in selfed flowers of alleged LSI species (Klekowski, 1988; Krebs and Hancock, 1991; Nic Lughadha, 1998).

Because in most cases of homomorphic SI only one locus controls the self-incompatibility reaction, full-sibship progeny from a cross between two plants will show segregation for alleles with up to four intra-incompatible, inter-compatible groups. Consequently, diallel crosses using full sib

progeny have been used to determine the genetic control, and thus the type of SI mechanism, in GSI (Ross, 1978; Boyle, 1997; Talavera *et al.*, 2001) and SSI (Stevens and Kay, 1988; Kowyama *et al.*, 1994; Goodwillie, 1997; Hiscock, 2000) species, although interpretation of the diallel results for SSI species can be complicated by dominance-recessive interactions between *s* alleles in the pollen and pistil; additionally, instances of non-reciprocal compatibility between full-sib pairs may occur. Grasses (Poaceae) with GSI have two loci (S and Z) so that diallels can present up to 16 compatibility groups, and again instances of non-reciprocal compatibility may occur (Hayman, 1956; Murray, 1974). A few taxa have been reported to have more than two S loci, and in such cases the interpretation of full sib diallels is complicated (Lundquist *et al.*, 1973; Larsen, 1977). Species in some 21 families with GSI and six families with SSI have had genetic control of SI confirmed by diallel studies (Gibbs, 2014).

A few studies have employed full sib diallel with LSI species in an attempt to discover whether major gene control may be involved in this mechanism as with conventional SI. However, only two diallel based studies, using *Theobroma cacao* and *Asclepias exaltata*, have presented results that clearly implicate major gene control of LSI.

In the Malvaceae *sensu lato*, in subfamilies Bombacoideae and Sterculioideae (Bayer *et al.*, 1999), or these treated as the distinct families: Bombacaceae, Byttneriaceae and Sterculiaceae (Cheek, 2007), LSI has been reported in species of diverse genera (cited in Gibbs, 2014). In *Theobroma cacao* Knight and Rogers (1955), working with trees cultivated in Ghana, investigated genetic control of SI using three full sib diallels. They proposed a single locus model with five alleles in their plants, with a hierarchy of dominance $S_1 > S_2 = S_3 > S_4 > S_5$, and their postulated genotypes of the parental plants were $S_{1.5}$, $S_{3.5}$ and $S_{2.4}$. This incompatibility model, with its elements of sporophytic control by the diploid pollen parent, resembles SSI.

Cope (1962) in a study of post-pollination events in the pistils of *T. cacao* found that although self pollen tubes penetrate most ovules, some ovules do not show gamete fusion, i.e., following ovule penetration the male gamete nuclei are discharged into a synergid cell but they do not fuse with the

egg or central cell nuclei. The commonest proportion of such "non-fusion" ovules was 25 %, but less frequently, depending on the tree, 50 %, and rarely 100 % of the ovules showed this lack of syngamy. In keeping with his observations, Cope (1962) had to modify the Knight and Rogers (1955) genetic model somewhat, proposing the action of two accessory loci, but he accepted the major gene control with dominance effects. Recent molecular studies with *T. cacao* have also largely accepted the Knight and Rogers/Cope hypotheses for major gene control of LSI (Yamada *et al.*, 2010; Royaert *et al.*, 2011; Da Silva *et al.*, 2016; Lanaud *et al.*, 2017). Both Da Silva *et al.* (2016), and Lanaud *et al.* (2017), found compatibility markers associated with chromosomes 1 and 4 on which they identified various candidate genes.

Genetic control of LSI in *Asclepias exaltata* (Apocynaceae) was investigated by Lipow and Wyatt (2000) using four full sib progeny diallel crosses. In all diallels, the majority of crosses (63 %) were incompatible. In two diallels, four intra-incompatible and inter-compatible groups were discernible as would be expected with a single locus model for SI. The third diallel results deviated somewhat from expected groupings; however, by postulating a modifier gene that weakened the action of one of the *s* alleles, almost all cases of reciprocal incompatibility could be explained under a single locus model. This study did not find evidence for dominance-recessive effects between incompatibility alleles, or of non-reciprocal compatibility between full-sibs, as might be expected under homomorphic SSI. However, in the single locus control of LSI proposed by Lipow and Wyatt (2000) one shared allele is sufficient for incompatibility - a situation found in SSI and not in GSI; but the authors noted that where the haploid genotype of pollen tubes determines SI (i.e. gametophytic control) then a cross between two full-sibs that share one *s* allele in common would result in 1:1 fertilized:non-fertilized ovules. If more than 50 % of ovules need to be fertilized to prevent abscission of the pistil, then a gametophytic mechanism could explain the observed diallel results. Whether the proposed single locus control of LSI in *A. exaltata* resembled conventional GSI or SSI was therefore left unresolved.

The family with the largest cohort of LSI species is the Bignoniaceae. Most of the around 62 species of this family that have been studied for their reproductive biology do not produce fruits on

selfing, and of the 37 species that have been studied for post-pollination events, all show self pollen tube growth to the ovules prior to pistil abscission and so indicate LSI (Bittencourt, 2017). No bignoniaceous species with conventional homomorphic SI have been reported. Various studies (Gibbs and Bianchi, 1993; Bittencourt and Semir, 2005; Bittencourt and Morães, 2010; Bittencourt, 2017) have shown the LSI phenomenon in species of the genera *Tabebuia* and *Handroanthus* (previously treated in *Tabebuia*).

As noted by Talavera *et al.* (2001), most studies of the genetic control of SI employing diallels have been with herbaceous species; tree species present particular problems to undertake the multiple pollinations required for full diallels, not least the time required to raise tree saplings to flowering, and managing to accumulate sufficient flowers to achieve adequate if not full diallel crosses. Unsurprisingly, tree species for which SI diallel studies have been undertaken to determine genetic control of SI, e.g. *Prunus avium* (Crane and Brown, 1937), *Theobroma cacao* (Knight and Rogers, 1955), *Corylus avellana* (Thompson, 1979), have been with economically important taxa for which plants with known SC or SI were available in cultivation.

Our aim was to investigate the genetic control of LSI in the bignoneaceous tree species *Handroanthus heptaphyllus*, with the objective of distinguishing between LSI and EID as drivers of unsuccessful selfed fruit-set. We studied genetic control of LSI in *Handroanthus heptaphyllus* using diallels for four full sib progeny arrays that originated from a natural precocious flowering variant of this species. Our expectation was that the pattern of compatibilities revealed by these diallel crosses, and associated studies on ovule penetrations and seed set in cross- and selfed pistils, would provide insights into: (1) whether genetic control of LSI in this species is due to major gene action as found in conventional homomorphic SI mechanisms; (2) whether our results provided any support for the role of early acting inbreeding depression effects providing an alternative explanation for the LSI syndrome in this species.

MATERIALS AND METHODS

Origin of full-sibs for diallel crosses

Handroanthus heptaphyllus (ipê, lapacho rosado) are trees of 10-30 m that occur from Central and SE Brazil, Paraguay, to NE Argentina. Due to their mass flowering they are widely cultivated as street ornamentals. The full sib progenies studied here were generated from seed of three out of six seed lots provided in October 2010 by Dr Gabriela Facciuto of the Instituto de Floricultura (IF) of the Instituto Nacional de Tecnología Agropecuaria (INTA), located at Castelar, Buenos Aires province, Argentina. A germplasm collection of native *Tabebuia*/*Handroanthus* species was established at the IF with plants grown from seed collected in various provinces, and with the focus of studies on plants that showed ornamental horticultural promise. The six seed lots came from crosses between plants showing precocious flowering (PF) within 12-18 months after sowing (Facciuto, 2007). These PF plants occurred among treelets raised at INTA with seed originating from three fruit capsules collected from a tree growing in Misiones province. The area was a private estate, with some trees as survivors in a field from cut-over woodland. Only one other conspecific was observed in the locality, and so this would be a likely pollen parent for the three fruits. For our full sib diallel crosses we chose three seed lots that gave good germination (average germination was 47.3 % ± 22.6) and so provided the opportunity to have diallel numbers of 25 or more full-sibs (Supplementary data Table S1). These lots were C179, C180 and C190: the first two were from fruits derived from two crosses between IF-INTA Castelar plants numbered pk2-8 x pk2-23 (i.e. two seed lots from fruits of two crosses between the same parents) and the third from a cross between pk2-8 x pk3. Thus, C179 and C180 are in effect extensions of a common full-sibship, though arising from different fruit, and all three seed lots had one parent, pk2-8, in common.

Diallel plants were cultivated in a glasshouse of the Facultad de Ciencias Agrarias, Universidad Nacional de Rosario (UNR), at the Campo Experimental Villarino, Zavalla (Santa Fe). Initially, seed were sown on vermiculite in Ziploc plastic bags and young plantlets successively transplanted to trays, then at two months to individual 12 cm pots, and at six months to pots of 4L

capacity. We used a compost comprising a 1:1:1 mix of river 'resaca', crushed pine needles and soil as in Facciuto (2007). The glasshouse was protected from excess heat during spring-summer with a green anti-UV sunshade net roof (diurnal temperatures of 12-35° C). At 12 months some plants of the diallels C179 and C180 began to flower, as did those of C190 at 15 months (Fig. 1A, B). At this time, full sib progenies C179, C180 and C190 comprised 26, 41 and 22 individuals respectively.

Pollinations between plants in the full sib diallel crosses C179, C180 and C190

As with many Bignoniaceae, *Handroanthus* flowers have bifid tactile stigmas which promptly close on receiving conspecific pollen and do not reopen (Milet-Pinheiro *et al.*, 2009). This greatly facilitated hand self and cross pollinations since it was unnecessary to bag individual flowers. Pollinations were effected on day one of anthesis by depositing a pollen load on the stigma with fine forceps, and flowers were tagged with date and pollen donor. We made at least one self pollination to confirm self-incompatibility, and depending on the availability of flowers, we attempted to make reciprocal crosses between as many full-sibs as possible in the diallels, with repeat crosses when possible.

When at least one pollination resulted in fruit set, we have taken this to indicate cross compatibility regardless, in some cases, of additional pollinations that resulted in pistil abscission. Given limited flower production by these small treelets, and the fact that one flower could provide pollen for at least four pollinations but receive pollen from only one donor, we inevitably accumulated some unreciprocated pollinations. To maximise possible pollinations we occasionally used pollen that had been stored in a refrigerator at 4° C under dry conditions (pollen placed in Ependorff tubes and stored over silica gel) for up to one month. The numbers of flowers produced by these small treelets obviously limited the number of pollinations we could make in any season, and we continued to make yearly pollinations with these three diallels over five flowering seasons (April - November) in the years 2012-2014 for C190 and 2013/2014 and 2017/2018 for C179 and C180 (three and four years of study, respectively). In 2019, with all diallel arrays somewhat diminished due to death of plants, we

made some pollinations between surviving plants from C179 and C180, i.e. the two full sib diallels that share the same parents to generate a fourth diallel designated as Cfusion.

All pistils that abscised following hand pollinations due to self- and cross-incompatibility were individually fixed in modified FAA (Lersten and Curtis, 1988) and stored for future study of pollen germination and pollen tube growth under fluorescence microscopy (Martin, 1959) using a Nikon, Eclipse E200 microscope. With the aim of prolonging flowering we also harvested between 12-25 days immature but developing fruits from compatible pollinations, and these were also fixed in FAA to assess young seed development. Towards the end of the flowering season in the third year of study, 2014, and again in 2018, we allowed some capsules (six and six respectively) resulting from cross-pollinations to mature before harvesting so as to assess seed set and germinability. Seeds were sown on vermiculite in ziploc bags as before, and the number of healthy seedlings noted.

Statistical analyses

Quantitative variables were statistically analysed using *InfoStat* software version 2013 (Di Rienzo *et al.*, 2011) or the Data Analysis tool in Microsoft Excel. The normality of the empirical distribution of all variables was assessed by the Shapiro-Wilk test. Homogeneity of variance was assessed by the Levene test setting alpha 0.05. For pistil abscission we used Student *t*-test to compare days to abscission within each diallel, for self-pistils vs. cross-pistils that abscised in a narrow range of days after pollination (DAP), and also to compare abscised self-pistils vs. unsuccessful crosses overall (n = 945 pistils). The comparison of days to abscission between diallels C179 vs. C180 vs. C190 for total abscised pistils was assessed by Kruskal-Wallis test (n = 82 plants). For ovule penetrations we compared estimates of the incidence of ovule penetration in our fixed pistils in order to detect any differences between abscised self- vs. abscised cross- pistils after pollination in diallel family C190 using Chi square (X^2) (n = 3,074 ovules). With immature harvested capsules, the number of young seeds with embryos per pistil was analysed by ANOVA in order to compare ovule penetration for compatible crosses between diallels C179, C180 and C190 (n = 42). A comparison of the number of penetrated ovules in abscised pistils (self- and failed cross- pollinations) vs. ovule

penetration in immature capsules of successful crosses was analysed using Student *t*-test (n = 67 pistils-capsules). Seed set (the number of normal, malformed and total seeds) per mature capsule between reciprocally cross compatible (RCC) vs. not reciprocally compatible sibs (NRC) in the three diallel families (n = 12 capsules), and the germination rate in the three IF-INTA progenitors vs. that in seeds from mature capsules from cross-compatible sibs (n = 8), were assessed by Student *t*-test, with percentages normalized with the arcsin conversion. Obviously, crosses that were reciprocally incompatible (RCI) did not set seed. In order to compare different cross types, we used the ANOVA statistical analysis of cross effort and success rates in our results.

With the lack of complete diallel arrays due to limitations of the biological material (tree species with limited flowers available for crosses in any one season), our diallel arrays are missing cross attempts such that it is difficult to reliably rearrange them into mating groups. For this reason, we have presented analysis of the frequency of occurrence of different mating outcomes (RCI, RCC, NRC) and used these as a basis for proposing a model for the genetic basis of SI in this species.

RESULTS

Pistil abscission

Pistils of all self pollinated flowers, and a proportion of crossed flowers - those of inter-incompatible full-sibs - abscised between 2-12 days after pollination (DAP). Compatible pollinations were signalled by rapid increase in the size of the pistil and so could be easily distinguished from pistils of incompatible pollinations (Fig. 1B). Abscised pistils after self- and failed cross-pollinations averaged 6-9 mm ± s.e. 0.8 in length at 11-12 DAP (n = 152) whereas developing fruits from successful crosses in diallels C179, C180, C190 for the same period averaged 7.8 cm ± s.e. 2.5 (n = 21).

Mean number of days to abscission was not significant for selfs vs. crosses within diallels, (C179: t = 0.83, df 317, p > 0.05; C180: t = 0.63, df 434, p > 0.05; C190: t = 0.78, df 188, p > 0.05) or between selfs and crosses overall (t = 2.91, df 943, p > 0.001). And the comparative analysis of days

to abscission between diallels C179 vs. C180 vs. C190 for total abscised pistils was not statistically different ($H_c = 29.92 < X^2_{30,0.05}$, $p = 0.4657$).

Ovule penetrations in abscised pistils and harvested, immature capsules

We based our expectation of timing of pollen tube growth, ovule penetration, and abscission of selfed or incompatible crosses on previous work. In all LSI Bignoniaceous species that have been studied for post-pollination events (e.g. Gibbs and Bianchi, 1993; Bittencourt, 2017; see Gibbs, 2014 for survey and images), self-pollen tubes grow to the ovary as rapidly as cross and penetrate some or all ovules. Penetration is followed by double fertilization, with a resting zygote (i.e. no ensuing cell divisions) and endosperm initiation that proceeds until pistil abscission, usually at 5-10 days. In our *H. heptaphyllus* plants, following self- and cross-incompatible pollinations, pollen tube growth to the ovary and penetration of some ovules, was similar to that observed in other bignon species (Fig. 2A-E).

In order to determine the range of ovule penetrations possible for crosses in the present study, the number of ovules per flower was counted in six pistils, two from each diallel, and was found to vary from 151 - 253 with a mean of 199.66 ± 37.36. Pollen tube ovule penetrations for abscised pistils following selfing or incompatible full-sib crosses were assessed for diallel C190 by counting ovules with pollen tube 'tails' at the micropyle under fluorescence microscopy. For successful cross pollinations, with harvested immature capsules, ovule penetrations by pollen tubes were no longer clearly discernible and, as a proxy, we estimated ovule penetration as the number of young seeds with embryos, expressed as percentages of the mean ovule number per pistil as 200. There was no significant difference between ovule penetrations for selfs and incompatible crosses, or as young seeds with embryos in successful crosses between diallels (C179 = 89.40 %, C180 = 74.75 % and C190 = 74.25 %, $F_{2, 39} = 0.135$, $p > 0.05$; $n = 42$ capsules). But the difference between these two treatments was significant (Fig. 3), and showed that pistils of successful pollinations had around twice as many penetrated ovules as those from selfs and unsuccessful crosses. Moreover, since it is likely

that some penetrated ovules did not survive to form young seeds with embryos, the values for penetrated ovules in compatible pollinations are probably under-estimates of ovule penetrations.

Seed set in mature capsules from compatible crosses

The results of cross-pollinations for each diallel (C179, C180, C190, Cfusion) showed two compatibility types between full siblings: reciprocal cross-compatible (RCC) and non-reciprocal compatible (NRC). In both cases, capsules were mature at around two months. In these mature capsules there were no or very few unfertilized ovules but it was possible to distinguish normal seeds from aborted ones. After discounting any malformed (shrivelled) seeds - which were present in only some of these capsules - the mean value of normal seeds per capsule for RCC was 138.8 ± 68.7 and for NRC 83.9 ± 53.9. This difference was not significant (t = 1.489, df 10, p > 0.05) [see Supplementary data Table **S**2].

Germination rates

Seeds from mature capsules sown on vermiculite were scored for germination (appearance of cotyledons above the surface) with a mean of 43.4 % ± 18.7 [see Supplementary data Table S3]. There was no significant difference between this value and the 47.3 % ± 22.6 mean germination obtained for the three Castelar seed lots for the diallel parents (t = 0.276, df 9, p > 0.05).

Diallel analyses

Cumulative results for the four full sib diallels C179, C180, C190 and Cfusion are given in Fig. 4A-D and are summarised in Table 1. All four diallels showed full-sib pairs that were reciprocally cross-compatible (RCC), reciprocally cross-incompatible (RCI) and that differed in compatibility depending upon which plant functioned as pollen donor, i.e. they were non-reciprocally cross-compatible (NRC). There were also a number of pollinations for which reciprocal crosses were not possible due to limited

flowering of our material (NRP), some of which were compatible and others of which were incompatible.

In addition to results pertaining to SI, our diallels also showed evidence of other genetic mechanisms related to mating systems. Specifically, diallels C179 and C180 both contained male-sterile plants (6/26 and 4/41 plants, respectively). These two diallels were constructed from different fruit that represented crosses between the same two parental plants (pk2-8 x pk2-23), so they are in effect replicates of the same diallel. The occurrence of some male-steriles in these crosses suggest that the parent that they share in common but that they do not share in common with C190 (pk2-23) was segregating for a male-sterility gene. The low frequency of male-steriles further suggests that the male-sterility gene is recessive (Byers *et al.*, 2005) and that the parent all crosses share in common (pk2-8) was heterozygous for this gene.

The presence of genetic male-sterility factors raises the possibility that some results might be attributable to variation in maternal or paternal success rates (Elle and Meagher, 2000). However, comparison of these success rates (Supplementary data Fig. S1) does not show any strong asymmetries when male-sterile plants are excluded.

All of our incompatible crosses scored zero fruit set, but some of our crosses scored as compatible achieved only one fruit set following repeat pollinations, e.g. C179: 17 x 5 = 1/6 (one fruit from six pollinations), C180: 18 x 7 = 1/3; 31bis x 4 = 1/5, C190: 21 x 24 = 1/5. Mixed results (successes and failures among replicates of specific crosses) following repeat pollinations seem to occur in most diallel studies (although sometimes obscured by the use of '+' and '–') and are usually referred to as 'incongruities'. In our diallel results for *Handroanthus* the incongruity incidence was notably high: C179 = 31 % of a total of 142 repeat pollinations, C180 = 26.6 % of 192 repeat pollinations, C190 = 19.3 % of 83 repeat pollinations. All incongruities are listed for each diallel in Supplementary data Tables S4-7). We investigated the potential impact on our results of classifying cross pairs as compatible even if some attempts of that cross were unsuccessful by comparing cross success rates for ‘compatible’ RCC and NRC crosses (Supplementary data Table S8). For each cross

type, we assessed the crosses attempted and the proportion successful for each diallel. For three of the four diallels there was no significant difference in crosses attempted for the two types of cross outcome, and the fourth was only marginally significant. Thus, we conclude that number of crosses attempted was not driving our results. In terms of proportion of crosses successful, two of our diallels showed significant differences between RCC and NRC and two did not show significant differences. Our conclusion is that the cross-compatibility in NRC crosses might be slightly weaker in their cross-compatibility compared to RCC, which would also be consistent with the dominance model we propose in the Discussion. Thus, although we acknowledge that our criterion for designating crosses as 'successful' introduces a crucial assumption into our results, the number of pairs affected was small relative to the total, and modifying this criterion would both increase as well as decrease the number of non-reciprocal pairs. Given the absence of a consistent way to modify this criterion, we accept that it introduces a margin of error into our findings.

DISCUSSION

Ovule penetrations

Estimates of the number of penetrated ovules in immature fruits from compatible crosses were twice those for self- and incompatible crosses. This incidence of non-penetrated ovules echoes somewhat the incidence of "non-fusion" ovules that characterize *Theobroma cacao*, and it raises the possibility that self or incompatible cross pistil abscission is triggered by the level of non-penetrated ovules in the pistil. Given the mass of pollen tubes that reach the ovary in self and incompatible cross pollinations (Fig. 2E) we do not believe that lower ovule penetrations are due to lack of pollen tubes. A lower incidence of ovule penetrations in selfed pistils was also reported in LSI species *Narcissus triandrus* and *Ipomopsis aggregata* (Sage *et al.*, 1999; Sage *et al.*, 2006). However, a caveat is needed for such a pre-penetration (or lack of penetration) type mechanism since our results for *H. heptaphyllus* differ from observations reported for a number of other species of Bignoniaceae with LSI: *Dolichandra cynanchoides* and *Tabebuia nodosa* (Gibbs and Bianchi, 1999), *Spathodea*

campanulata (Bittencourt *et al.*, 2003), *Zeyheria montana* (Bittencourt and Semir, 2004), *Handroanthus impetiginosus* (Bittencourt and Semir, 2005) and *Jacaranda racemosa* (Bittencourt and Semir, 2006). In these species, although selfed pistils show lower values for ovule penetrations than in crossed pistils at 24h and 48h intervals, by 96h, and well before pistil abscission, the numbers of penetrated ovules in selfed and crossed pistils show parity. Effectively, in these taxa there is an initial lag in selfed vs. crossed ovule penetrations but this is made up prior to abscission. We think that it is quite likely that both our lower, and in other bignons slower, ovule penetration may be due to hostile 'cross-talk' between self- pollen tubes in the style – ovules, with an interaction that triggers the initiation of pistil abscission *before* any ovules are penetrated by self tubes and fertilization occurs. Or failure to achieve a minimum level of ovule penetration within a brief time interval may induce abscission as a consequence of resource allocation (Gibbs and Bianchi, 1999; Gibbs, 2014).

A major gene control model

A working hypothesis is to consider major gene control of LSI in *H. heptaphyllus*, as found in conventional SI mechanisms, and as was postulated for the diallel cross results with LSI species *Theobroma cacao* (Knight and Rogers, 1955) and *Asclepias exaltata* (Lipow and Wyatt, 2000). Conventional homomorphic SI in most elucidated cases involves a single multiallelic locus, as in the GSI or SSI models, or an oligolocic control with two or, rarely, more loci. The marked occurrence of full-sib pairs showing non-reciprocal incompatibility in our four diallels rules out a one-locus GSI type mechanism, but unilateral compatibility can occur in both SSI, due to dominance effects between *s* alleles, and also in two-locus GSI as found in the grasses. In the latter, the complementary action of alleles that is a feature of this mechanism (i.e. both *s* alleles in the pollen must be present in the pistil for incompatibility to occur) can show non-reciprocal compatibility in crosses depending upon which plant acts as the pollen parent.

Since the plants in our diallels derive from a cross between two related individuals, it is likely that there are very few, and at most four, SI alleles in each diallel. Thus, the genetic composition of

our diallels arising from full sib progeny between, say, S1/S2 and S3/S4, then there are four progeny genotypes that would have been included in the diallel, S1/S3, S1/S4, S2/S3, S2/S4. An SSI-type scenario, in which the two parental plants for the diallels comprise four *s* alleles of which one is dominant to the other three, could produce results similar to those in our diallels. Since all three diallels share the same maternal parent, assuming a maternal hierarchy with the dominant *s* allele, i.e. S1>S2=S3=S4 and paternal hierarchy S1=S2=S3=S4, then a diallel of these four progeny would yield the phenotypes shown in Table 2.

Thus, under this hypothesized genetic model, the frequency of reciprocal incompatibility, reciprocal compatibility, and non-reciprocal compatibility are all a third. This is very close to the observed pattern in our data (Table 1). If we construct model diallels increasing the number of full sib plants, these proportions change a little due to the increase of off-diagonal replications of cross types, but the overall pattern remains similar (Supplementary data Table S9). Thus, a simple genetic system of SSI-type with a dominance hierarchy among alleles represents a robust explanation for our diallel results. We note that different co-dominance relations between the same alleles present in the pollen or pistil are a feature of conventional SSI. For example, Kowyama *et al.* (1994, Fig. 2) for SSI in *Ipomoea trifida* propose, amongst many, that allele s_1 is dominant to s_6 and s_{19} in the pistil, but co-dominant with these alleles in the pollen.

However, two features suggest some caution is necessary apropos this hypothesis for genetic control of incompatibility in LSI *H. heptaphyllus*. One is the relatively high number of 'incongruities' that occur in the crosses, with fruit set or lack of fruit set as a result for the same cross. In a study of GSI in *Anagallis monelli*, Talavera *et al.* (2001) reported such incongruous results from repeat pollinations in three of their five diallels, with an incidence 1.6 %, 3.7 % and 6.2 % of total pollinations. In our diallel results for *Handroanthus* the incongruity occurrence was notably high: C179 = 31 % of a total of 142 repeat pollinations, C180 = 26.6 % of 192 repeat pollinations, and C190 = 19.3 % of 83 repeat pollinations.

We have considered various factors that might cause variable results from repeat pollinations. Firstly, temperature variation in the glasshouse, since pollinations were made throughout the flowering season (April to November) over five years, and temperature has been reported to influence pollen germination and pollen tube growth in various species (Austin *et al*., 1998; Pham *et al.*, 2015). However, in our studies of pistils that were fixed, from pollinations carried out at different phases of the flowering period, and over different years, all were observed to have prolific pollen tube growth to the ovary, and we conclude that any glasshouse temperature fluctuations were not relevant. Secondly, a number of studies have shown that plants produce many more flowers than initiate fruits, and with the bignon species *Catalpa speciosa* Stephenson (1979) showed that only three flowers in each 15 flower inflorescence produced fruits; after successful pollination of any three flowers, remaining flowers ceased to be effectively female-fertile. However, these cases of maternal resource limitation mostly involve successful initial fruit set to be followed by failure. With *H. heptaphyllus* it seems very unlikely that pollinations with successive flowers on the same plant influenced fruiting, and so contributed to the incongruities, since the sequence of whether successful fruit initiation was followed by flowers with pistil abscission, or the reverse, varied almost equally in all diallels (Supplementary data Tables S4-7).

Another cause for caution is the fact that despite our model indicating the occurrence of four mating groups in the full sib progeny, we are unable to discern clearly such groups in any of the diallels. We attribute this to biological limitations on the completeness of our diallels (treelets with sparse flowering). Consequently, our results to not lend themselves to testing hypotheses that involve more complex genetic control of SI, such as more involved dominance hierarchies or multiple SI loci. Moreover, we have no evidence that these anomalies are caused by other gene loci additional the main S locus, as proposed for *Theobroma cacao* (Cope, 1962; Da Silva *et al.*, 2016; Lanaud *et al.*, 2017) and *Asclepias exaltata* (Lipow and Wyatt, 2000).

We recognize that our proposed 'sporophytic' model for the control of LSI in *H. heptaphyllus* presents an enigma since in conventional SSI species, pollen recognition occurs at the stigma whereas in our species self and cross-incompatible pollen tubes reach the ovary. However, we would note that

studies in three families with SSI - Asteraceae, Brassicaceae and Convolvulaceae - indicate that each has a distinct molecular mechanism (Hiscock and Tabah, 2003), and moreover, there are several studies which implicate a gametophytic SI element in species with SSI (Lundquist, 1990). We would also note that two previous studies that have employed full sib diallels with LSI species - *Theobroma cacao* (Knight and Rogers, 1955) and *Asclepias exaltata* (Lipow and Wyatt, 2000) - both concluded that a sporophytic mechanism best explained their results.

LSI or EID?

Seavey and Bawa (1986) proposed the term late-acting self-incompatibility in a review of published reports of fruit-set failure following self pollination despite self pollen tubes reaching the ovary/ovules. These authors were well aware that in a situation where self pollen tubes penetrate ovules prior to pistil abscission, the concept that this may be due to an enigmatic SI mechanism sits uncomfortably with the alternative, perhaps more straightforward explanation, that lack of fruit set is due to inbreeding effects. Unsurprisingly, the authors of most studies on species that present the LSI syndrome also comment that, as an alternative explanation, their results could be a consequence of early acting inbreeding depression (EID). Klekowski (1988) and Nic Lughadha (1998) have presented a case for EID as a general explanation for LSI type observations, whilst other authors invoke both possible LSI and EID effects to explain their results (Dorken and Husband, 1999; Hao *et al.*, 2012).

Klekowski (1988) proposed that a genetic load model could cause embryo abortions within a short developmental period, and that the concept of lethal equivalents based on the homozygous occurrence of many recessive genes with small detrimental effects might allow clear ratios in progeny. Particularly in woody species, where longevity could lead to an accumulation of deleterious recessive genes, EID might mimic the effects of self-incompatibility. In a comprehensive study of seed abortion in species of the genus *Stylidium*, Burbidge and James (1991) reported ISI values (the ratio of seed set after selfing to that after crossing) of 0.2 or less (the value employed as a cut off point to indicate self-incompatibility) in 37 of 53 species. No evidence of self-incompatibility was found in

these species, but a sound case was made for the role of recessive lethal equivalents that act to abort seed formation in selfed ovules. This study showed that EID can mimic the effects of self-incompatibility.

However, a notable feature of the hand cross- and self-pollination studies made by Burbidge and James (1991) is that in most *Stylidium* species many selfed flowers produced some fruits that were shown to contain reduced seed set accompanied by ovules that manifested arrested growth and abortion. As they commented: "The stage of maturation at which abortion occurred varied within capsules, and, along with the proportion of seeds that aborted, it varied between capsules, plants, populations and species. Nevertheless, some evidence of post-self-pollination ovule development was always encountered in these species, but early abortion was more common than late."

In contrast, in our *Handroanthus* plants, self-pollinations and cross-incompatible pollinations all resulted in pistil abscission after a few days, and the distribution of such fully compatible or fully incompatible crosses is not consistent with EID. Zero fruit set following self-pollinations is a feature that has been reported for LSI taxa of diverse families, e.g. Apocynaceae (Broyles and Wyatt, 1993), Bignoniaceae (Gibbs and Bianchi, 1999; Bittencourt and Semir, 2004, 2005; Gandolphi and Bittencourt, 2010; Bittencourt, 2017), Bombacaceae (Oliveira *et al.*, 1992; Gribel and Gibbs, 2002), Byttneriaceae (Knight and Rogers, 1955), Lardizabalaceae (Kawagoe and Suzuki, 2005), Leguminosae (Borges *et al.*, 2008), Sterculiacae (Taroda and Gibbs, 1982). Moreover, in species of Bignoniaceae, Bombacaceae and Byttneriaceae, fertilization - self or cross - is followed by a resting zygote, such that the selfed pistils are abscised well before any cell divisions to initiate the embryo occur: in *Theobroma cacao*, following successful crosses, embryo initiation occurs some 44 days post-pollination (Cheesman, 1927) whilst self pistils are aborted within a few days. In this situation it is difficult to see how deleterious recessives can act post-zygotically to trigger early pistil rejection.

In addition, seed set in mature capsules from cross-compatible full-sibs did not show marked presence of aborted seeds or lack of viability. Given that crosses between full-sibs involve a marked

level of inbreeding it would be surprising if there was no impact of inbreeding depression in our diallel results, but we find little evidence for notable ovule abortion due to recessive lethals.

In conclusion, our diallel crosses support the mechanism of late-acting self incompatibility (LSI) over early-acting inbreeding depression (EID). The former could be driven by a single locus whereas the latter would presumably be driven by a whole genome effect. If crossing success rates were a consequence of EID, one would expect to see a broad distribution of success rates in crosses. However, although there was a relatively high incidence of 'incongruity' with mixed success rates in our results, overall there was still a much higher proportion of clearly compatible or incompatible crosses than one would expect under EID. Thus, the diallel results corroborate the ovule penetration and pistil abscission results in supporting genetically controlled LSI in *Handroanthus*.

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FIG. 1. Precociously flowering plants of *Handroanthus heptaphyllus*. (A) Plants of diallel C190 at two years after sowing. (B) Diallel C180: immature fruit set at 8 days following cross-pollinations between individuals 18bis x 33 and 18bis x 34. Scale bar (A) = 26 mm; (B) = 10 mm.

FIG. 2. *Handroanthus heptaphyllus* abscised pistils: pollen tube growth observed with fluorescence microscopy. (A-D) Selfed pistils with (A) pollen grain germination at the stigma, (B) pollen tubes (pt) in the style, and (C-D) penetrated ovules showing pollen tube 'tail' at the micropyle (one non-penetrated); (E) Pollen tubes in the ovary (ov) in a non-reciprocal incompatible cross C190 6x11. Scale bar (A-B) = 3 mm; (C-D) = 500 µm; (E) = 1 mm.

FIG. 3. *Handroanthus heptaphyllus* ovule penetration in abscised pistils (self and incompatible cross pollinations) and harvested immature capsules (compatible crosses). Mean for abscised selfs = 39.86 % (n = 1613 ovules from 12 pistils), and abscised crosses 37.03 % (n = 1461 ovules from 13 pistils). Difference not significant (X^2_c = 2.61, df 1, α= 0.005). Mean for compatible crosses 79.5 % (6675 young seeds from 42 pistils, expressed for a mean of 200 ovules per pistil). Error bars = 95% confidence interval. Difference with abscised pistils significant (t = 23.33, df = 65, p < 0.05). Different letters above columns denote significant differences among treatments.

FIG. 4. Diallel results for *Handroanthus heptaphyllus* full sib progeny: (A) C179, (B) C180 (C) C190, and (D) Cfusion. In each cell, the upper number is the number of fruit set, and the lower number is the number of crosses done. Cells are color coded as to cross type: = reciprocally cross-incompatible (RCI), = reciprocally cross-compatible (RCC), = not reciprocally compatible (NRC - incompatible), = not reciprocally compatible (NRC - compatible), = self-pollinated, = not reciprocally pollinated.

TABLE 1. *Counts of reciprocally cross incompatible (RCI), reciprocally compatible (RCC) and non-reciprocal incompatible/compatible (NRC) from Figure 4A-D for diallels C179, C180, C190 and Cfusion [179-180] (excluding male-sterile plants in C179 and C180). Also non-reciprocal pollinations (NRP),* ***C****: compatible;* ***I****: incompatible*

Full-sib Diallels	**C179**		**C180**		**C190**		**Cfusion [179-180]**	
Full-sib pairs interaction		(%)		(%)		(%)		(%)
Reciprocally cross-incompatible **[RCI]**	22	(34)	39	(33.3)	21	(38)	6	(18)
Reciprocally cross-compatible **[RCC]**	23	(36)	39	(33.3)	16	(29)	15	(45)
Non-reciprocal (unilateral) compatibility **[NRC]**	19	(30)	39	(33.3)	18	(33)	12	(36)
Total pairs	64		117		55		33	
Non-reciprocal pollinations **[NRP]**	17 **C** 19 **I**		14 **C** 14 **I**		15 **C** 11 **I**		3 **C** 2 **I**	
Total **[NRP]**	36		28		26		5	

TABLE 2. *Model for sporophytic control of SI in* Handroanthus heptaphyllus *with expected compatibility interactions and phenotypes generated in a full sib diallel between two plants with S1S2 x S3S4. Model postulates S1 is dominant to the other three alleles in the maternal genotype, i.e. S1>S2=S3=S4, with all alleles co-dominant in the paternal genotype, i.e. S1=S2=S3=S4. (Allele* ***S1*** *in bold is dominant; + compatible crosses, - incompatible crosses, -- incompatible self pollinations. Grey shaded cells show non-reciprocal compatibility)*

♀\♂	S1/S3	S1/S4	S2/S3	S2/S4
S1/S3	--	-	+	+
S1/S4	-	--	+	+
S2/S3	-	+	--	-
S2/S4	+	-	-	--

Figure 1

Figure 2

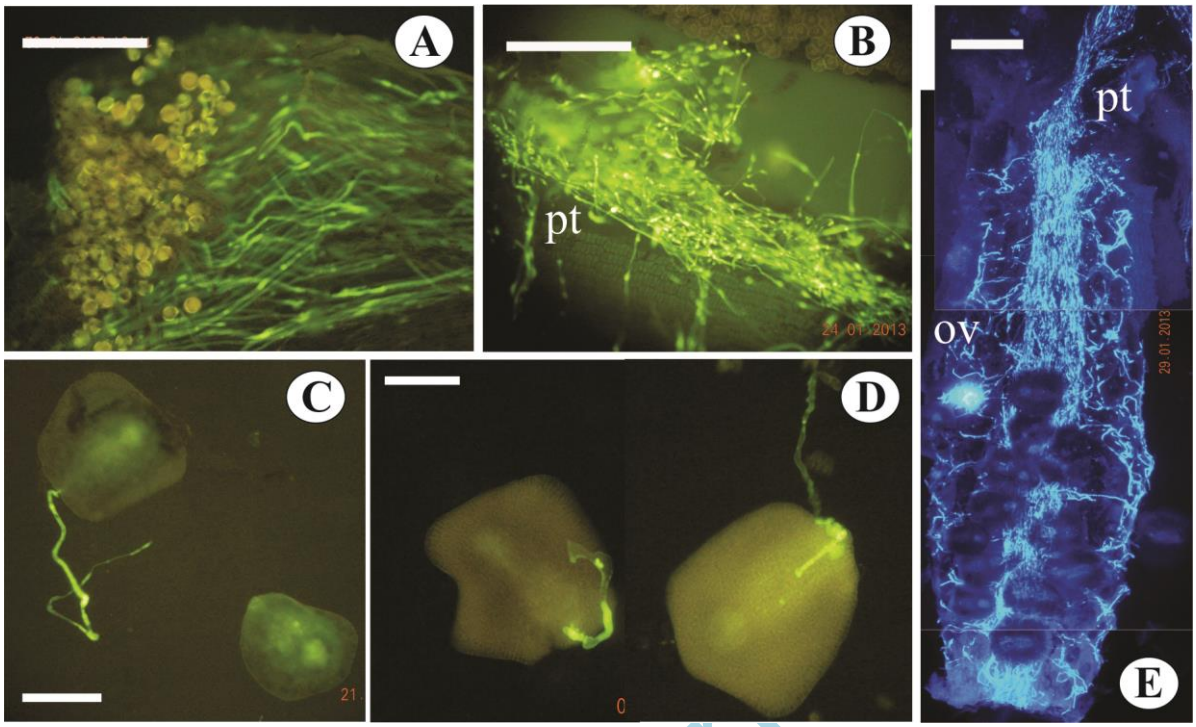

Figure 3

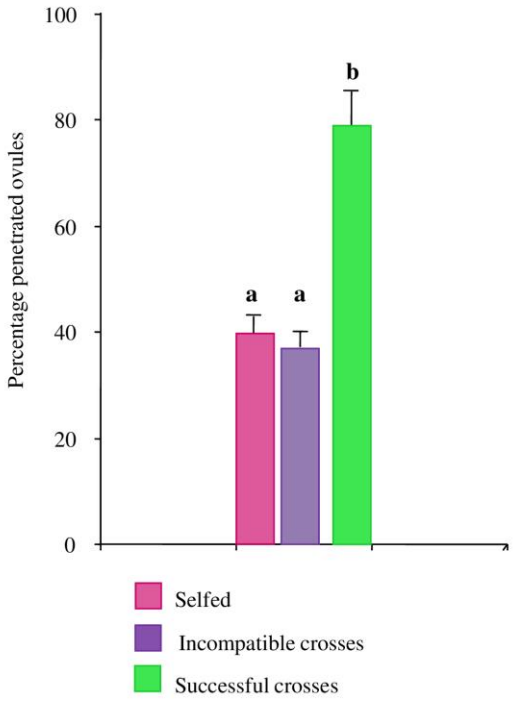

FIG. 4. (A) *Handroanthus heptaphyllus* diallel C179 [pk 2-8 x pk 2-23]

♀ \ ♂	2	3	4	5	7	8	9	10	12	13	14	16	17	18	19	20	21	22	23	24	25	26
2	0 2	0 2	2 2	0 2				0 2		1 1			1 1		0 1							
3	0 2	0 2	3 3	2 4		3 3	1 1						2 2									0 3
4	0 1	2 2	0 3	0 3	0 1	3 5	2 3	4 5	1 1	0 2	0 2		3 6	1 1	0 3				2 3			
5	0 1	4 4	0 4	0 2	0 2								0 3									
7				1 2	0 1	1 1																
8		1 3	1 3	1 2		0 2	3 3	0 2	0 1	3 6			3 3	0 2	3 3				0 1	0 2		0 3
9		0 1	0 4	0 1	0 1	3 3	0 1				2 2			3 6	0 3				0 3	0 4		
10	0 2		5 5			2 4	0 2	0 2	0 2	2 4			0 1	3 4	8 9			0 2	10 13	0 4		
12	0 1		5 5		0 2	3 5	0 3	0 5	0 1	0 2	1 4		2 8	0 1		0 5	0 2					
13								1 3		0 1		0 1							5 5			0 2
14			3 3	1 1		0 3	1 1			2 2	0 1	0 1	1 1									
16			4 4					0 9		0 2	1 2	0 1	2 4	2 4	0 5				3 4			
17		1 1	0 8	1 6		4 5		0 3	1 5		1 1		0 2			0 3		1 1			0 3	
18			0 4			0 1	1 6	0 3					1 1	0 1	4 4	0 3			0 4			
19	0 1		2 3			2 2	0 3	2 7						3 4	0 1		0 1		7 7	2 2		
20			2 2						0 3				0 4	1 3		0 1	0 1					
21									0 1		2 3		1 1		0 2	0 4	0 1			0 3		
22													0 1					0 1	0 1			
23		1 1	3 3			0 3	3 3	8 11		7 7				0 2	8 8			0 1	0 1	3 3		
24		0 1				1 2	3 5	0 3							1 1		0 2		2 3	0 1		
25		0 1											0 2	0 2							0 1	
26		0 3				3 4															0 1	0 1

FIG. 4. (B) *Handroanthus heptaphyllus* diallel C180 [pk 2-8 x pk 2-23]

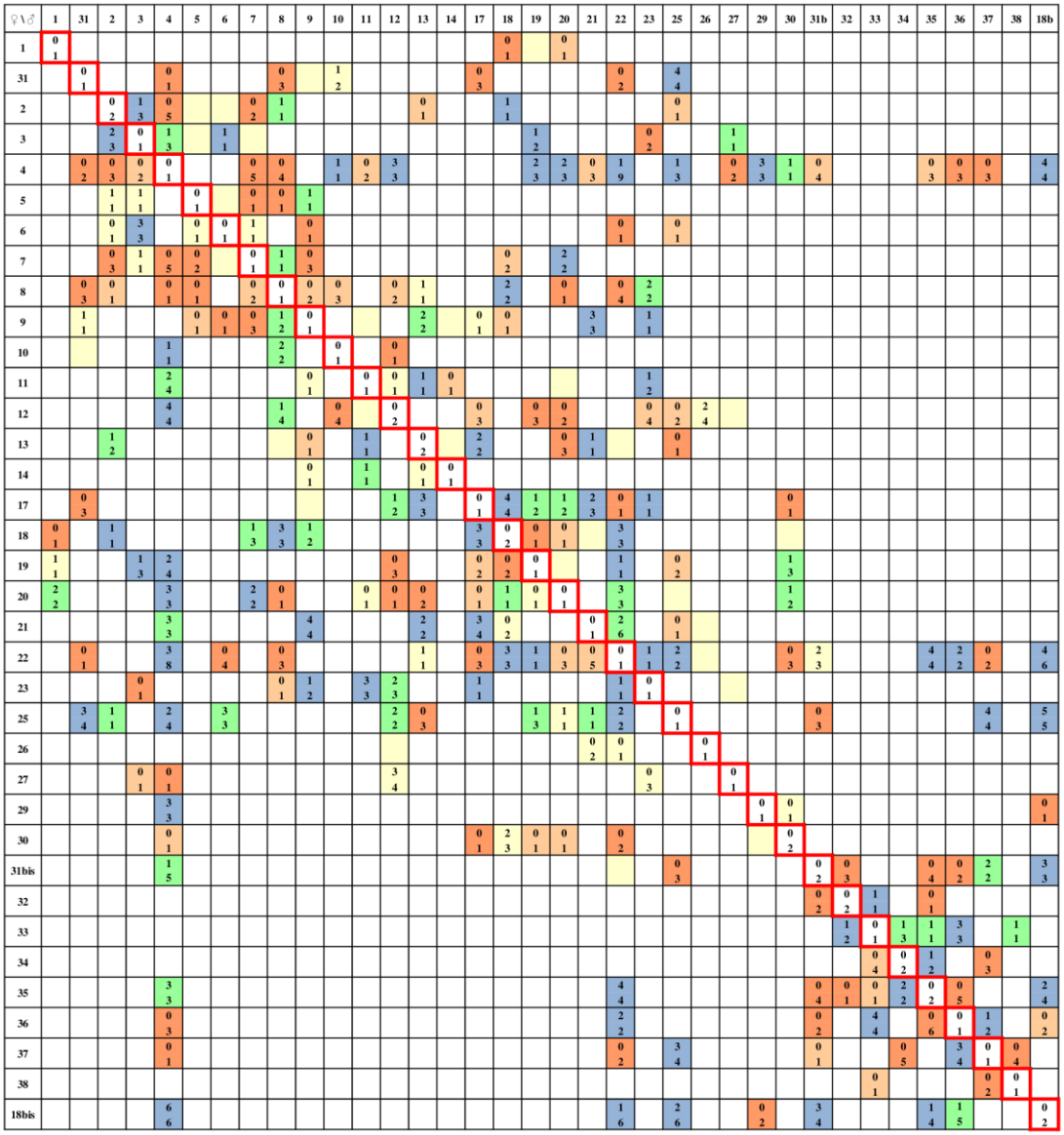

FIG. 4. (C) *Handroanthus heptaphyllus* diallel C190 [pk 2-8 x pk 3]

♀ \ ♂	1	2	3	4	6	7	8	9	10	11	13	14	15	16	17	18	19	20	21	22	23	24
1	0 4	0 3		0 2	0 5	0 2		0 4	0 2			5 5							3 3	0 2		
2	0 2	0 2		0 8	0 1	0 1	0 1	0 1		2 2										0 4		
3			0 2	1 2																		
4		8 9	0 1	0 2	3 3		0 2				1 1							1 1	2 3		1 1	
6	0 2	0 2		1 2	0 2			0 1		2 3		3 3		1 1				0 1	2 4		0 1	1 1
7	2 2			0 1		0 1		0 1		1 1				0 1				1 1			1 1	
8		0 1		0 2										1 1	0 1				1 1			
9	0 4	0 1			0 2	0 2		0 2	0 2			2 2		1 2	1 1				2 3	0 4		
10								0 1	0 1													
11		0 1			1 2	2 3				0 1		2 3				1 2	0 4		1 1			
13				0 1							0 2	0 1										
14	0 2			0 1	2 4	1 1		0 2		0 2	2 2	0 2			0 1			0 1	0 3		1 1	
15													0 1	0 1								
16				0 1	2 2	2 3	0 1					0 1	0 2	0 2	0 2	1 1						
17		1 1		1 1	1 1		0 1						1 1	0 2	0 2	1 1						
18										0 1						0 1						
19										0 8					0 2		0 1					
20	1 1			0 1	2 2	0 1						0 1						0 2	0 2			1 1
21	3 3	0 1		0 3	2 2		1 1	3 3		0 2		0 2		0 1				0 1	0 1	3 3	1 1	1 5
22	0 3	0 2						0 3										1 1	2 2	0 3		
23				1 1	0 1	1 2						1 1		1 1	1 1			1 1	1 1		0 1	4 4
24					0 1														3 3		2 2	0 2

FIG. 4. (D) *Handroanthus heptaphyllus* diallel Cfusion [C179-C180, pk 2-8 x pk 2-23]

♀ \ ♂	1	2	3	4	5	6	7	8	9	10	13	14	15	16
C179-13 **1**	02		02	04	55		44			12	03		06	01
C180-35 **2**		02	44	05	12		01				01		24	
C180-22 **3**	13	44	03	22				04		44		01		
C180-36 **4**	44		22	05			44	03					46	
C179-23 **5**	77	22			01			33	33	02	88			
C180-25 **6**			22			01							55	
C180-33 **7**	44	11		33			05		44				22	
C179-24 **8**	14		04	34	23			03	35		11			
C179-9 **9**					03		44	04	02	36	03			
C179-18 **10**	02		04		04				16	01	45	11		
C179-19 **13**	02	44			77			22	03	78	04			
C153 **14**			34							11		03		
C180-18bis **15**	03	14	17	02		26	01						02	
C179-16 **16**	02										05			01