



Original Article

Reproductive Strategy of the Polyploid Species *Varronia curassavica* Jacq. in Restinga Environment

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Abstract

This study aimed to elucidate the breeding strategies of *Varronia curassavica*, an important medicinal species associated with Brazilian restinga. This was accomplished by combining phenological and genetic data. Every 2 weeks over a period of 2 years, we measured flowering and fruiting phenology to evaluate the activity and intensity of phenophases ($n = 60$). We evaluated the mating system, pollen ovule ratio and genotypes from progeny and mother plants using 8 nuclear microsatellite loci. We observed flowering and fruiting of *V. curassavica* at low intensity throughout the entire year, but with 2 distinct peaks, one of which was seasonal, corresponding to the period of gradual increase of temperature and photoperiod. Overlapping of flowering and fruiting strategies favors gene flow among different groups of individuals and between populations by attraction of fauna throughout the year. Analysis of the mating system indicates that *V. curassavica* is a typical outcrossed species ($\hat{t} = 0.98$; pollen/ovule ratio = 7087.50). Combining phenology with genetic studies improved our understanding of the reproductive strategies of this species. The typical outcrossing system of *V. curassavica* reflects the existence of functional self-incompatibility mechanisms still unaffected by changes in genetic balance by polyploidy.

Subject area: Reproductive strategies and kinship analysis

Key words: Atlantic forest, genetic conservation, microsatellite, medicinal plant, outcrossing rate, phenology

The mating system in plants is the main determinant of population genetic structure (Baker 1959; Bawa 1974; Hamrick and Godt 1989). The mating patterns, however, can be determined by both genetic and ecological factors. The degree of self-incompatibility is included among genetic factors (Karron et al. 1997; Goodwillie et al. 2005), and the behavior of foraging pollinators (Hirao et al. 2006)

and flowering phenology are among ecological factors (Oddou-Muratorio et al. 2006).

Varronia curassavica Jacq. (Boraginaceae) is a hermaphroditic species with known mechanisms of self-incompatibility (Opler et al. 1975; Ganders 1979; Taisma and Varela 2005), such as heterostyly and protogyny (Hoeltgebaum et al. 2017). Heterostyly in *Varronia*

has been known since Darwin (1877), and it is considered common among different genera of Boraginaceae (Opler et al. 1975). Species with this characteristic have intramorph incompatibility, and in most cases, only crossbreeding between flowers with different morphs can produce viable offspring (Barrett 2002; Barrett 2003). However, studies have shown that the restoration of self-compatibility may occur naturally in some species, or arise as a result of polyploidization (Schifino-Wittmann and Dall'Agnol 2002). Polyploidy may affect, or even prevent, self-incompatibility through changes in the genetic balance of a species (Entani et al. 1999; De Nettancourt 2000), and it has often been related to increased self-compatibility (Galloway et al. 2003).

Changes in compatibility associated with polyploidy can, in turn, result in changes in the mating system (Galloway et al. 2003). Effects of these changes, such as the appearance of inbreeding depression, are still little known in polyploid species and somewhat contradictory (vide Husband and Schemske 1997; Dudash and Fenster 2001; Galloway et al. 2003; Galloway and Etterson 2007; Barringer and Geber 2008).

Polyploidy is present in several species of Boraginaceae (Mable 2004; Meeus et al. 2012; Coppi et al. 2014). *Varronia curassavica* was recently characterized as an autotetraploid species (Hoeltgebaum and Reis 2017, unpublished data). Thus, the evaluation of mating systems in polyploid populations of *V. curassavica*, combined with phenological studies, is important to clarify the extent to which self-incompatibility mechanisms are retained in these populations.

The species presents a wide distribution, from Central America to the south of Brazil, where it occurs abundantly on sandbanks, which are locally known as restingas. Restinga areas have high structural complexity and highly adverse environmental conditions. Plant species, are, correspondingly, susceptible to these wide swings in environmental conditions (Falkenberg 1999). Currently, the restinga is an Atlantic Forest ecosystem threatened by human pressure, especially for its location along the Brazilian coast (Strohaecker 2008; Barcelos et al. 2012).

Reduction and fragmentation of habitat, combined with the high incidence of extraction of this species what is used for medicinally (Passos et al. 2007; Hoeltgebaum et al. 2015), are factors that confer fragility to natural populations of *V. curassavica*. Thus, understanding the reproductive strategy is critical to support conservation efforts and sustainable use (Bawa and Ng 1990; Reis 1996; Nason and Hamrick 1997; Sebbenn et al. 2000; Mantovani et al. 2006; Nazareno and Reis 2012) of *V. curassavica* and it is particularly important for species widely used by both local populations and the pharmaceutical industry (Gandolfo and Hanazaki 2011; Goneli et al. 2014).

Therefore, to elucidate issues related to the reproductive strategy of *V. curassavica*, we connected genetic data with ecological data on flowering and fruiting and search answer the following questions: 1) What is the flowering strategy and fruiting species? 2) Is there a pattern of seasonality in reproductive phenology? 3) Considering the existence of mechanisms of self-incompatibility and polyploidy, which is the outcrossing rate?

Material and Methods

Study Area

We carried out this study in 2 areas of herbaceous shrub vegetation located in a restinga (sandy dune) on Joaquina Beach, Parque Municipal Dunas da Lagoa da Conceição (PMDLC) (27°37'46.19"S;

48°27'1.59"W), and on Campeche Beach (27°41'6.07"S 48°28'50.20"W), which is contiguous with PMDLC located east of Florianópolis, Santa Catarina, Brazil. These areas cover almost 563 hectares, and the combined area is considered the largest sand dunes in Florianópolis (Cecca 1997). Climate in both areas is humid temperate with warm summers and well-distributed rains throughout the year (Cfa, Köppen 1948). During the study period, the monthly average temperature was 21.6 °C, with maximum in January–February (25.8 °C) and minimum in July (17.43 °C). The average annual rainfall was approximately 1304 mm in 2014 and 2188 mm in 2015 (see Supplementary Figure 1). Day length in hours ranged from 13.75 h.day⁻¹ in December to 10.25 h.day⁻¹ in June.

Reproductive Phenology

Field Measurements

We performed phenological observations every 2 weeks from January 2014 to January 2016. We monitored 30 reproductive individuals taken randomly from each area, totaling 60 individuals marked with numbered aluminum tags. We evaluated the floral button phenophases, open flowers (anthesis), unripe and ripe fruit. We quantified the phenophases according to the methodology proposed by Fournier (1974), which individually measures the intensity of phenophases using an interval scale of 0 to 4, with an amplitude of 25% of each interval. 0 = absence; 1 = >0–25%; 2 = >25–50%; 3 = >50–75%; 4 = >75–100%.

Phenological Statistical Analyses

We calculated the percentages of individuals to give monthly phenophases, as well as phenological intensity. We calculated the intensity of each phenophase by the method proposed by Fournier (1974). We carried out verification of the normal distribution of the data using the Kolmogorov-Smirnov test (Zar 1999). We estimated Spearman rank coefficients (Zar 1999) to verify the relationship between phenophases and climate variables (temperature, precipitation and photoperiod) each month.

We used Circular statistics to determine seasonality, as proposed by Morellato et al. (2010). To calculate the statistical parameters, we converted months into angles such that 0° = January up to 360° = December in 30° intervals. We calculated the average angle as (\bar{a}) and the angular standard deviation and vector length as (r). Significance of the angle was determined by the Rayleigh test (Z) for circular distribution (Zar 1999). We used the Student *t*-test (t ; $P < 0.05$) to detect possible differences between areas and years studied. We analyzed data by using the Oriana program (Kovach 2004).

Genetic Analysis

Sampling, DNA Extraction, Amplification and Genotyping

We evaluated, to determine the mating system, genotypes from 283 progeny and 16 breeding mother plants randomly sampled from the Joaquina population. We kept the collected seeds immersed in water for 12 h and pulped before being germinated in trays in the greenhouse. We used the Nucleospin Plant II (250) kit (MACHEREY-NAGEL GmbH & Co. KG) for the extraction of the leaf genomic DNA, according to the manufacturer's instructions.

Extracted DNA was amplified by Polymerase Chain Reaction (PCR), and we used 8 polymorphic microsatellite loci developed for the species by Figueira et al. (2010) (Table 1). To amplification primers, we used DNA diluted in water at 9:1 μ L and KAPA PCR kit (KAPA Biosystems) with a volume of 10 μ L per reaction. Each primer for each locus was labeled with the fluorochromes

Table 1. Microsatellite loci used in genotyping *Varronia curassavica* Jacq. (Boraginaceae) with repeated motifs and sequence of primers developed by Figueira et al. (2010), labeled with the NED fluorescent (yellow), VIC (green), PET (red) and FAM (blue) in 2 multiplex combinations; Fluor = inflorescence

Combination	Locus	Motif	Sequence (5′–3′)	Fluor	T _a (°C)
Multiplex 1	MCvCIRCPQ14	(TG) ₁₃	F:CTTGTAGCTGCCACTTCCT R:GAATAATGCACAACGAGTCA	NED	61
	MCvCIRCPQ3	(AC) ₁₀ (AAAATT) ₃	F:ATTAGGCGTTTGGTGCTAC R:GCAGCGTATTTTAGCAGAGA	VIC	61
	MCvCIRCPQ8	(AC) ₉	F:CCAAGTGTGCGTAATACACTT R:TCTTCTGACGTTTTCTCAT	PET	61
	MCvCIRCPQ6	(CA) ₈	F:TACTAGCACCGTTGAATAGG R:TAGGGACCGTAAAAGACAT	FAM	61
Multiplex 2	MCvCIRCPQ11	(TC) ₈ (AC) ₁₆	F:ATTGCCCTTAGCCGGTAG R:CGAATGTGAATTGCGAGTT	VIC	61
	MCvCIRCPQ7	(TG) ₉	F:GGCAACAATAAGGTTAGAGC R:TCACTCTCATGTTTACGC	PET	61
	MCvCIRCPQ16	(CA) ₈	F:CTCCTTCACTGTTTCAAAGG R:GGAAGAAGCTGATCTCTGTG	NED	61
	MCvCIRCPQ15	(TG) ₈	F:TTAGGAACATCTCCTTAGTGG R:TGGCTCCCCATTATATTTTC	FAM	61

Table 2. Results of analyses of circular statistical tests for seasonal and phenological behavior of *Varronia curassavica* Jacq. (Boraginaceae) in the restingas of Campeche (CAM) and Joaquina (JOA), Florianópolis/SC—Brazil between 2014 and 2015

A	Phenophase	Obs. (N)		Mean angle (\bar{a})		Mean date		Length of mean vector (r)		Rayleigh (z) test of uniformity (P)	
		2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
CAM	Flower bud	360	405	296°	304°	24/10	01/11	0.13	0.20	0.002*	1.1E-07*.**
	Anthesis	301	308	329°	321°	13/10	18/11	0.14	0.31	0.003*	<1E-12*.**
	Unripe fruit	241	298	282°	331°	10/10	28/11	0.16	0.23	0.002*	8.4E-08*.**
	Ripe fruit	64	86	310°	355°	07/11	22/12	0.33	0.34	9.5E-04*.**	6.5E-05*.**
JOA	Flower bud	372	376	274°	297°	02/10	25/10	0.19	0.29	1.2E-06*.**	<1E-12*.**
	Anthesis	287	325	289°	297°	17/10	25/10	0.29	0.30	5.1E-11*.**	<1E-12*.**
	Unripe fruit	226	220	289°	301°	17/10	29/10	0.33	0.48	1.9E-11*.**	<1E-12*.**
	Ripe fruit	88	78	296°	307°	24/10	04/11	0.67	0.71	<1E-12*.**	<1E-12*.**

A = area; Obs. (N) = Number of observations.

* $P < 0.05$; ** $P < 0.001$.

FAM (blue), PET (red), NED (yellow) and VIC (green) for the 8 microsatellite loci (Table 2), forming 2 multiplex systems. The set of cycles and temperatures used were 95 °C for 3 min of denaturation, followed by 30 cycles, each cycle consisting of 3 phases: the first, 95 °C for 30 s; the second, 61 °C for 30 s and the third, 72 °C for 30 s for elongation, followed by a final elongation step at 72 °C for 30 min. We performed Capillary electrophoresis using 1 µL of diluted PCR product in ultrapure water (15: 2 µL) and adding 0.25 µL of GS600 LIZ® and 8.75 µL of formamide HIDITM. We carried out reading of the alleles in an ABI 3500XL Sequencer (Applied Biosystems) with 24 capillaries. We used Gene Mapper v.3.2 (Applied Biosystems) to interpret the electropherogram peaks.

Determination of the Mating System

Outcrossing Rate

We estimated the outcrossing rate indirectly from the maximum likelihood method by means of the estimated pairwise kinship and individual coancestry coefficient for polyploid species, as proposed by Ritland (1996). The coefficient of coancestry considers the probability that 2 alleles from random individuals will be identical

by descent (r), and it was calculated using Poly Relatedness V1.6 (Huang et al. 2015). The estimator used supports ambiguous genotypes, that is, when the heterozygous alleles dosage is not known. We used the coancestry coefficient obtained to calculate the outcrossing rate as proposed by Wright (1921): $\hat{t} = (1 - \hat{f}) / (1 + \hat{f})$, assuming that the cause of inbreeding in the population has resulted from self-pollination.

Pollen Ovule Ratio

Additionally to the outcrossing rate, we also evaluated the reproductive system by the pollen/ovule ratio (P:O), considered a skillful alternative and precise technique (Cruden 2000), directly related to the offering of floral resources and pollination mode (Cruden 1977).

We carried out quantification of pollen grains in 10 flowers obtained from 10 individuals. We stained 4 anthers from each flower with Carmine Acetic 2% and stored in 2 ml Eppendorf® tubes containing 500 µL of lactic acid. For pollen count, subsamples of 10 µL for each flower were used and analyzed in Neubauer Chambers under an optical microscope (12×). We determined the number of eggs from 5 flowers. We cut carpels longitudinally and analyzed under stereomicroscope (16×) to count the number of ovules in the

ovary of the flower and calculate the mean and standard deviation. We calculated the P:O ratio following [Cruden \(1977\)](#).

Data Archiving

In fulfillment of data archiving guidelines ([Baker 2013](#)), we have deposited the primary data underlying these analyses in the Dryad Digital Repository: DOI:10.5061/dryad.44ns0.

Results

Reproductive Phenology

Varronia curassavica are with flower almost throughout the year, but with 2 distinct peaks (see Supplementary Figure 2), one between February and March and another, more intense, between August and October. In the Campeche population, maximum flowering intensity was recorded in September, reaching 78% and 76% for flower buds and open flowers, respectively. In the Joaquina population, these values were 94% and 93% for those phenophases in the same period. During the second year, the pattern was similar, with peaks between January and February and August and November. Variations between populations and between years, however, were not significant ($t = -0.64$; $P > 0.05$). One of flowering peaks was seasonal, having an average date in October and November (Table 2). This period is characterized by high rainfall and temperature. However, no significant correlation was noted between flowering and climate variables.

Like flowering, fruit production showed a similar pattern (see Supplementary Figure 2). For the unripe fruit, intensity peaks first occurred between March and April, with a second, more intense peak between August and November. The latter was seasonal for both populations, with mean date in October (Table 2). The ripeness of the fruit, however, was significantly different in both evaluated years, as shown by production intensity of the 2 areas ($t = 3.11$ for Campeche and $t = 2.72$ for Joaquina; $P < 0.05$). In 2015, the largest number ripe fruit was made in Joaquina, but the intensity peaks were more pronounced in Campeche (52%), showing a higher concentration of maturation events. In the Joaquina population, a peak of ripeness was only observed to occur between September and November (33% in October). This was less intense than that observed for the Campeche population (52% in September) which bore fruit with more intensity from March to April and from August to October. In the second year, the intensity of maturation was significantly lower, with 2 peaks, one in February and another from September to October, but not exceeding 13% on the Fournier scale for all individuals assessed, although reaching 60% intensity in the first year. Peaks of fruit maturation were also seasonal and significant with average mean date between October and November (Joaquina) and November and December (Campeche) (Table 2). No significant correlation was noted between fruiting and climate variables.

Reproductive System

Considering all families as a single population, the apparent outcrossing rate (\hat{t}) (Table 3) was 0.98 (± 0.06), indicating that *V. curassavica* is a typical outcrossing species. The percentage of inbreeding found was not significant. Considering the outcrossing rate by family, the values were all close to one and showed little variation.

The average number of pollen grains per flower was 28 350, and ovules per flower were equal to 4; thus, P:O was equal to 7087.50.

Table 3. Estimates of the mating systems for each *Varronia curassavica* Jacq. (Boraginaceae) seed tree and for species populations in the restinga of Joaquina—Florianópolis, Santa Catarina State, Southern Brazil

Family (<i>n</i>)	<i>r</i>	\hat{t}	95% CI
01 (14)	-0.055	1.12	1.057–1.183
02 (13)	-0.065	1.14	1.093–1.187
03 (20)	-0.037	1.08	1.006–1.154
04 (20)	-0.068	1.15	1.087–1.213
05 (20)	-0.043	1.09	1.027–1.153
06 (20)	-0.029	1.06	1.003–1.117
07 (20)	-0.049	1.10	1.035–1.165
08 (20)	-0.064	1.14	1.079–1.201
09 (20)	-0.027	1.06	0.994–1.126
10 (20)	-0.063	1.13	1.071–1.189
11 (20)	-0.013	1.03	0.938–1.122
12 (19)	-0.035	1.07	1.036–1.104
13 (20)	-0.049	1.10	1.018–1.182
14 (20)	-0.046	1.10	1.085–1.115
15 (09)	-0.038	1.08	0.992–1.168
16 (08)	-0.068	1.15	1.032–1.268
População	0.012	0.98	0.925–1.035

n = number of progenies; *r* = coancestry coefficient of [Ritland \(1996\)](#); \hat{t} = apparent outcrossing rate; CI = confidence interval calculated by *t* distribution.

Discussion

Reproductive Phenology

The evaluated phenophases were not significantly correlated with climatic variables, although seasonality was observed. The study areas are characterized by a climate with moderate seasonality, suggesting that factors apart from climate might influence the frequency of phenophases (vide [Koptur et al. 1988](#); [Morellato and Leitão-Filho 1990](#); [Talora and Morellato 2000](#)). For tropical species, endogenous factors, as well as selective and biotic pressures, may play a role in determining phenological change ([Borchert 1980](#); [Talora and Morellato 2000](#); [Medeiros et al. 2007](#)).

Flowering individuals can be observed at low intensity throughout the entire year, but with 2 peaks correlated with seasonality, corresponding to the period of gradual increase of temperature and photoperiod. Studies carried out by [Morellato \(1991\)](#), [Talora and Morellato \(2000\)](#) and [Staggemeier et al. \(2007\)](#) all suggest that flowering is triggered by increasing photoperiod, temperature and humidity in the transition from dry to wet season. Flowering that time of the year would benefit the species by increased availability of light, amount of nutrients available to the plants and the activity of pollinators ([Morellato 1991](#); [Morellato and Leitão-Filho 1992](#)).

Overlapping flowering strategies whereby species maintain a continuous pattern of flowering over a peak annual flowering time allows for the regulation of pollen flow and maintenance of pollination throughout the year, favoring random mating between different groups of individuals and a broad guild of floral visitors. Regulating pollen flow includes avoidance of self-pollination by reducing the number of flowers per day and promoting the movement of pollinators between plants ([Sakai 2001](#)). At the same time, high intensity and synchronization peaks mark different periods of the year when pollinators are attracted and reducing uncertain pollination ([Augspurger 1980](#)). Thus, the overlapping phenologies cycles, as represented by these patterns, tends to maximize the possibility of cross-fertilizing and promoting gene flow of the species.

Like flowering, fruiting showed a similar pattern with peaks in February and March and between August and November for unripe fruit. Fruit ripening lasted until April, at the first peak. Also similar to flowering, low seasonality offers little restrictive conditions for both development and ripening of fruit throughout the year (Martin-Gajardo and Morellato 2003).

Compared to the amount of flowering records and unripe fruits, maturation was observed in a few individuals and with less intensity. Maturation was also significantly lower in the second year of assessment when rainfall was about 3 times higher than that observed in the first year in the period of fenofase. The production of flowers and fruits may change as a result of intense rainfall, reduced activity of pollinators or damage to the reproductive structures (Newstrom et al. 1994), thus interfering with flowering. Since differences were found in the maturation phase, this low-intensity ripe fruit compared to immature fruit could be a result of rapid removal by frugivores. Moreover, monitoring of inflorescence development of *V. curassavica* (Hoeltgebaum et al. 2017) demonstrated that the species has a high number of abortions, both flowers and fruits. Fruit abortion has been explained by paternity selection of the zygote (Bawa and Webb 1984). For *V. curassavica*, self-incompatibility intramorphs, which is typical of heterostylous species (Opler et al. 1975), could be one of the factors contributing significantly to this result.

Mating System

Estimates of outcrossing rate obtained for *V. curassavica* showed the prevalence of cross-fertilization in the reproductive mode of the species, characterizing it as a typical outcrossing species. The values of both population and families are similar, and with minor variation, suggest the timing at flowering. Reinforcing the results obtained from the use of molecular markers, the value obtained by the P:O ratio classifies the species as obligatory outcrossing according to the model proposed by Cruden (1977). This result demonstrates that the existing polyploidy in the population probably did not interfere yet in self-incompatibility mechanisms thereof.

Apart from enabling the maintenance of genetic variability, cross-fertilization also favors the hybrid vigor of species by the occurrence of new gene combinations coding for traits of interest, such as the production of secondary metabolites (Facanali et al. 2009). In addition, typical outcrossing species have high diversity within populations with high adaptive flexibility (Loveless and Hamrick 1984; Kageyama et al. 2003; Gonçalves et al. 2010). This flexibility can be advantageous to pioneer species with populations subjected to extreme conditions and variables, such as those affecting *V. curassavica* in restinga environments.

The predominance of outcrossing in *V. curassavica* is consistent with the phenological strategy and the existence of self-incompatibility mechanisms registered for the species, such as heterostyly and protogyny (Hoeltgebaum et al. 2017). However, evidence of these possible changes in mating systems caused by polyploidy indicates the need to monitor and maintain the population over the long term.

Changes in self-incompatibility systems have been registered for *V. curassavica*. Taisma and Varela (2005) studied the self-incompatibility system through controlled intersections and direct observation of pollen tubes. In the population studied, they observed partial compatibility between brevistylous morphs, suggesting morpho-specific differences in incompatibility reactions, as identified in other distylous species (Favre 2002). Brandão et al. (2015) evaluated the reproductive system through the P:O ratio for plants from a germplasm bank, and the results showed that the species could be classified as “optional outcrossing,” that is, able to reproduce by autogamy or

outcrossing. These variations, however, were not observed in our study population, suggesting efficacy of the self-incompatibility system and pollination.

In conclusion, this study has shown that the combination of phenology and genetics resulted in a greater understanding of the reproductive strategies of *V. curassavica*. The population of *V. curassavica* studied was typical of outcrossing which supports the existence of functional self-incompatibility mechanisms that remain unaffected by changes in the genetic balance, as a result of polyploidy, conferring, in consequence, adaptive advantages for support of populations in environments where environmental adversity is a constant. However, the viability of populations depends on the conservation of associated biota, in this case, pollinating fauna.

Supplementary Material

Supplementary data is available at *Journal of Heredity* online.

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Data Availability

Data deposited at Dryad: <http://dx.doi.org/10.5061/dryad.44ns0>

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