

ARTICLE



Widespread coexistence of self-compatible and self-incompatible phenotypes in a diallelic self-incompatibility system in *Ligustrum vulgare* (Oleaceae)

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The breakdown of self-incompatibility (SI) in angiosperms is one of the most commonly observed evolutionary transitions. While multiple examples of SI breakdown have been documented in natural populations, there is strikingly little evidence of stable within-population polymorphism with both inbreeding (self-compatible) and outcrossing (self-incompatible) individuals. This absence of breeding system polymorphism corroborates theoretical expectations that predict that in/outbreeding polymorphism is possible only under very restricted conditions. However, theory also predicts that a diallelic sporophytic SI system should facilitate the maintenance of such polymorphism. We tested this prediction by studying the breeding system of *Ligustrum vulgare* L., an insect-pollinated hermaphroditic species of the Oleaceae family. Using stigma tests with controlled pollination and paternity assignment of open-pollinated progenies, we confirmed the existence of two self-incompatibility groups in this species. We also demonstrated the occurrence of self-compatible individuals in different populations of Western Europe arising from a mutation affecting the functioning of the pollen component of SI. Our results show that the observed low frequency of self-compatible individuals in natural populations is compatible with theoretical predictions only if inbreeding depression is very high.

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INTRODUCTION

Breeding system transitions are among the most frequent evolutionary transitions in a wide range of taxa including angiosperms, fungi, algae, and bryophytes (Billiard et al. 2011; Stebbins 1957). In particular, in flowering plants, losses of mechanisms preventing self-fertilization have occurred many times independently (Goldberg et al. 2010; Goodwillie 1999; Igic et al. 2008). Evolutionary transitions from self-incompatibility (SI) to self-compatibility (SC) generally seem to occur rapidly, as suggested by several documented cases of recent SI loss (Durand et al. 2020), especially between close species (e.g., Shimizu et al. 2008), or even within species (e.g., among northern American populations of *Arabidopsis lyrata*, Foxe et al. 2010, Mable et al. 2017). This particular breeding system transition is well understood and supported by theoretical models which show that SI should disappear whenever mate availability limits reproductive success and inbreeding depression is low enough (Charlesworth and Charlesworth 1979; Fisher 1941; Gervais et al. 2014; Porcher and Lande 2005).

However, the big picture might not be that simple since theoretical studies also suggest that intermediate evolutionary states can be found, with both outcrossing [SI] phenotypes and selfing [SC] phenotypes stably coexisting within populations. In theory, such SI/SC polymorphism can only be stable under a subtle balance between

inbreeding depression and mate availability (Charlesworth and Charlesworth 1979; Fisher 1941; Porcher and Lande 2005; Van de Paer et al. 2015). If [SC] mutants achieve high selfing rates, high values of inbreeding depression would result in their exclusion from the population, while low values would lead to a rapid fixation. In addition, the number of functional S-alleles should be limited, otherwise the reproductive advantage of SC genotypes relative to SI ones in terms of mate availability and transmission advantage would be offset, again resulting in the exclusion of [SC] mutants. This particular condition should seldom be met in natural populations, where the number of functional S-alleles is typically very large owing to high levels of negative frequency-dependent selection (Castric and Vekemans 2004). Overall, theoretical models thus predict that populations with stable SI/SC polymorphism should be rather rare in natura.

Empirical evidence of such polymorphisms has been documented in some species (e.g., Mable et al. 2005; Ortiz et al. 2006). In many cases, however, the observed polymorphisms might only be transient since populations generally show recent demographic events in their history which likely resulted in a decrease in the number of S-alleles or in inbreeding depression, hence creating novel situations where a SC mutant invades (Busch and Schoen 2008). For instance, [SC] genotypes have been documented in *Aster furcatus* populations with reduced diversity at the S-locus

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(Reinartz and Les 1994). Similarly, natural populations of *Laevenworthia alabamica* have been shown to display SI/SC polymorphisms (Busch et al. 2010), with peripheral populations tending to loose [SI] completely (Busch 2005). Overall, it remains unclear whether or not stable SI/SC polymorphisms are present in natural populations as predicted by theoretical models.

Any species with an intrinsically low number of S-alleles should be more prone to display such stable polymorphism. In particular, in species with homomorphic diallelic self-incompatibility systems (DSI), where the number of functional S-alleles is reduced to its bare minimum, the conditions for SI/SC polymorphism should be broader than in the more common multi-allelic systems where very large numbers of S-alleles are typically found (Castric and Vekemans 2004). Van de Paer et al. (2015) investigated the fate of a [SC] mutant within a hermaphroditic DSI population using a phenotypic model where the mutation could affect either both the pollen and stigma genes or only one gene (two SC mutants are thus possible: a pollen-part or a pistil-part mutant). Results suggest that a [SC] mutant should always invade a SI population when it has only two S-alleles, with three possible stable outcomes. When inbreeding depression is below 0.5, SI is lost as the [SC] phenotype invades and goes to fixation. For higher values, the [SC] phenotype necessarily stably coexists with either a single [SI] phenotype, or with both [SI] phenotypes (Van de Paer et al. 2015). Hence, populations of a hermaphroditic DSI species should be prone to display stable SI/SC polymorphisms.

The common privet (*Ligustrum vulgare*, also called European privet), in the Oleaceae family, is a hermaphroditic species that is ideal for testing these theoretical predictions. While nothing is known about its breeding system, all other Oleaceae species studied so far have a sporophytic DSI, as first evidenced in *Phillyrea angustifolia* (Saumitou-Laprade et al. 2010) and confirmed in *Fraxinus ornus* (Vernet et al. 2016), *F. excelsior* (Saumitou-Laprade et al. 2018), *Olea europaea* subsp. *europaea* (Mariotti et al. 2020; Saumitou-Laprade et al. 2017a), and *O. europaea* subsp. *laperrinei* (Besnard et al. 2020). The first goal of this work was to test whether the common privet expresses DSI, which was explored at the prezygotic stage using stigma tests, as well as at the postzygotic stage using genotyping and paternity analysis. If *L. vulgare* populations indeed have only two S-alleles, the SC phenotype is expected to coexist stably with one or two [SI] phenotypes (Van de Paer et al. 2015). The second goal of this work was to determine whether [SC] phenotypes can indeed be found in the study species in natura. To do so, we screened a set of wild populations for SI/SC behavior using stigma tests. We also transferred a set of these individuals to an experimental garden and performed controlled stigma tests in a diallelic crossing design in order to assess compatibility relationships among [SI] and [SC] individuals used as pollen recipients and pollen donors. Third, we applied the model developed by Van de Paer et al. (2015) to our experimental results to formulate quantitative predictions on the level of inbreeding depression required to maintain the breeding system observed in *L. vulgare* in natural populations.

MATERIALS AND METHODS

Plant material and study populations

Ligustrum vulgare in the Oleaceae family. *L. vulgare* is a deciduous or semievergreen Oleaceae shrub, native to Europe, North Africa, and western Asia (Fig. 1). It is an insect-pollinated species with petaliferous, fragrant, and nectariferous flowers. It has a generalist pollination system, with *Bombus* sp. as main pollinators. At the flowering peak, adult shrubs are covered in pyramidal panicles, each comprising several tens of flowers (see Fig. 1). One individual plant can produce more than 10,000 fruits, each carrying 1–4 seeds (Obeso and Grubb 1993). Seed dispersal can be facilitated by birds or animals during winter. Individuals can also reproduce vegetatively.

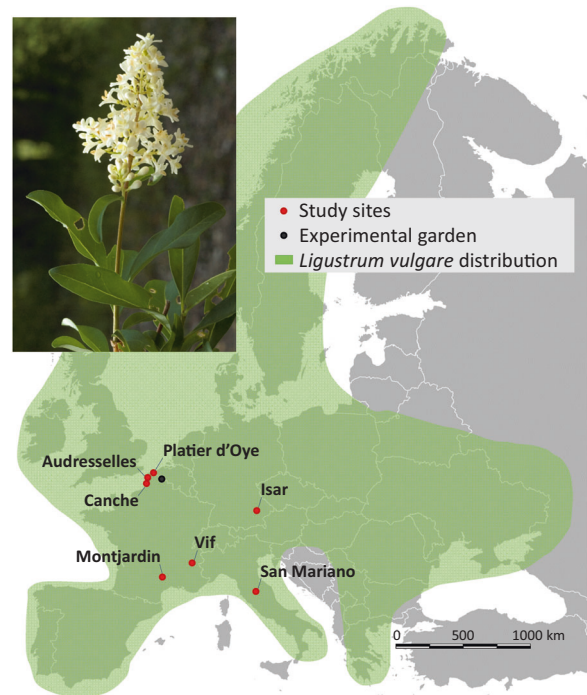


Fig. 1 *Ligustrum vulgare* geographic species range across Europe and position of the seven studied natural populations. Photo credit: Marijke Verhagen, Saxifraga Foundation.

Identification of tester genotypes. To test for DSI in *L. vulgare*, we first identified two cross-compatible genotypes whose pollen could be used to test a set of wild populations. If the species indeed possesses only two DSI groups, any individual used as a recipient will be compatible with only one of these tester genotypes. Compatibility was assessed on six individuals in a study site at Marais de Péronne (N 50°33′30.298″, E 3°9′59.778″) using stigma tests and following a protocol that was successfully used in other Oleaceae species (Saumitou-Laprade et al. 2010; Saumitou-Laprade et al. 2017a; Vernet et al. 2016). Briefly, these in vitro tests involve depositing a small amount of pollen on stigma from a dissected emasculated flower placed on agar medium and observing pollen germinations under the microscope (see Supplementary File S1). When the recipient is compatible with the donor's pollen, pollen tubes grow through the stigmatic tissue to the style and then towards the ovules. Absence of pollen tubes (or presence of only short pollen tubes that do not reach the style) was scored as incompatibility. Compatibility scoring was thus binary (compatible or incompatible). Once a cross-compatible tester pair (MP-01 and MP-04, referred to hereafter as tester A and tester B) had been identified, we collected fresh inflorescences with a few open flowers from them, and transferred them to the laboratory. Open flowers were eliminated and inflorescences were then placed in controlled conditions until flower buds opened. The four-lobed corolla with attached anthers were collected with forceps and stored in a Petri dish. Dehiscence was then induced by placing corollas with anthers in a dry atmosphere for 4 h and pollen was immediately stored at -80°C for subsequent stigma tests.

Screening wild populations for DSI and [SI]/[SC] phenotypes. Pollen from the two tester genotypes was used to assess compatibility on 184 individuals sampled in seven European natural populations from five geographically distinct regions in France (North Sea coast, Cevennes, Alps), Germany (Bavaria), and Italy (Umbria) (see Fig. 1 and Table 1). Because of the clonal nature of the study species, care was taken to only include ramets separated by at least 4 m within each population, thereby limiting the chances of including clones in the data set. As described above, compatibility scoring for the 184 individuals was binary (compatible or incompatible, Supplementary File S1). Individuals incompatible with tester A and compatible with tester B were classified as belonging to an incompatibility group called “G_A”, and individuals compatible with tester A and not tester B were classified as “G_B”. If more than two compatibility groups occur in this species, some individuals would be compatible with

Table 1. Screening of natural population of *Ligustrum vulgare* for incompatibility groups using stigma tests.

Population name	Population identifier	Region	Country	GPS coordinates	Altitude (m)	Year of sampling	G _A individuals			G _B individuals			G _N individuals	SI (G _A + G _B)	SC (G _A + G _B)	Estimated frequencies (95% confidence interval)	
							Total	SI	SC	Total	SI	SC				G _A	G _B
Canche ^a	CA	North sea	France	N 50° 31' 25.813 E 01° 37' 32.311	30	2012–2017	21	17	1	3	28	23	0	5	0	0.41 (0.27–0.56)	0.56 (0.41–0.71)
Audresselles ^a	AU	North sea	France	N 50° 49' 10.662 E 01° 35' 44.796	10	2013	9	7	1	1	11	10	0	1	0	0.39 (0.22–0.65)	0.56 (0.39–0.82)
Platier d'Oye ^a	PO	North sea	France	N 50° 59' 50.118 E 02° 31.984	0	2013	9	9	0	0	8	7	0	1	0	0.56 (0.37–0.83)	0.44 (0.25–0.71)
Montjardin ^a	MJ	Cévennes	France	N 44° 07' 48.089 E 03° 24' 10.226	1000	2015–2017	24	14	3	7	20	13	0	7	0	0.47 (0.3–0.66)	0.43 (0.27–0.62)
Vif ^a	VIF	Alpes	France	N 45° 00' 23.965 E 05° 40' 46.549	515	2015	7	2	1	4	3	0	0	3	0	0.67 (0.33–1.0)	0.0 (0.0–0.35)
Isar ^b	G	Bavaria	Germany	N 48° 25' 27.181 E 11° 53' 26.653	450	2015	9	7	1	1	13	10	0	3	0	0.39 (0.22–0.65)	0.56 (0.39–0.82)
San Mariano	SM	Umbria	Italy	N 43° 04' 54.625 E 12° 18' 28.759	300	2014	15	9	0	6	7	7	0	0	0	0.56 (0.37–0.83)	0.44 (0.25–0.71)
Total							94	65	7	22	90	70	0	20	0	0.46 (0.38–0.55)	0.49 (0.41–0.58)

Sampled individuals were tested as pollen recipients using the pollen from two inter-compatible tester plants and, when enough flowers were available, using self-pollen (nt: individuals for which self-pollen was not tested). G_A: individuals incompatible with tester A and compatible with tester B. G_B: individuals compatible with tester B. G_N: individuals compatible with both tester A and tester B. SI: individuals for which self-pollen did not germinate on their own stigmas. SC: individuals for which self-pollen germinated on their own stigmas. The frequencies given in the three last columns were calculated on plants assessed both for SI group (using pollen from tester A and B) and self-pollen.

^aPopulations from which cuttings of individual genotypes were transferred to the experimental garden.

^bPopulation from which collected seeds were sown and grown in the experimental garden.

both testers, forming a potential third group ("G_N"). A subsample of 142 shrubs was also tested for self-compatibility by self-pollinating their stigmas to identify potential [SC] phenotypes (Table 1). We estimated the frequencies of G_A, G_B and [SC] individuals along with their confidence intervals in each population using the method described in Sison and Glaz (1995), which allows constructing two-sided confidence intervals for multinomial proportions (MultinomCI function in the DescTools package in R, Signorelli 2019). For further tests, 236 individuals (plant cuttings or seeds) from six of the same natural populations were transferred to the experimental garden at the University of Lille and genotyped (see below). 88% of individuals in the experimental population were also assessed for SI and assigned to a SI group, and we detected two [SC] individuals (one from the Montjardin population in France, and one from the Isar population in Germany).

Pre- and postzygotic analysis of cross-compatibility behavior among [SI] and [SC] *L. vulgare*

- *Prezygotic analysis*: Stigma tests performed in a diallelic design

To precisely describe the cross-compatibility behavior of [SC] individuals detected in the population screening, we performed controlled pollinations in a full diallelic crossing scheme using plants from the experimental garden. Crosses involved ten distinct genotypes: four G_A (CA-44, MP-01, VIF-09, and VIF-16), four G_B (CA-07, CA-31, G13-08, and MP-04), and two SC genotypes (G16-10 and MJ-05). In this reciprocal diallelic design, each genotype was tested as a pollen donor and pollen recipient for cross-compatibility behavior with all other genotypes using stigma test (Saumitou-Laprade et al. 2017a; Saumitou-Laprade et al. 2017b). Each of these 100 crosses was replicated on three flowers. As described above, compatibility scoring was binary (compatible or incompatible, Supplementary File S1).

- *Postzygotic analysis*: paternity success and selfing assessed in an experimental population

The results of these diallelic stigma tests were then confirmed at the postzygotic stage using paternity analysis in the experimental garden. The artificial population included 236 adult shrubs originating from the wild populations described above and collected as seeds or cuttings. Individuals were arranged in four densely planted plots separated by less than 50 meters. Each of these plots contained a mixture of G_A and G_B genotypes. In autumn 2017, we collected seeds on all plants that were included in the diallelic crosses (except VIF-16, which did not produce enough seeds) and on one additional G_B plant (MJ-06-1). The ten mother plants were distributed in three of the four plots. Each of the ten maternal progenies was divided into two groups. In the first group, offspring were genotyped at the embryo stage (498 offspring, with 50 embryos per mother plant, except for MP-04 for which only 48 seeds were available). In the second group, seeds produced by the six mother plants that had large enough progenies (two [G_A]: CA-44 and VIF-09; two [G_B]: CA-31 and G13-08; and the two [SC]: G16-10 and MJ-05) were germinated in the greenhouse and grown until each seedling had several leaves before genotyping (711 offspring collected on six mother plants, with an average of 118 seeds per mother plant).

DNA extraction and genotyping. Total genomic DNA from the 236 adults in the experimental plot and the 1209 offspring (498 embryos and 711 seedlings) was extracted and purified using the NuclioSpin 96 Plant II Kit (Macherey Nagel, Duren, Germany) following the manufacturer's protocol. For adults and seedlings, DNA was extracted from 10 to 20 mg of lyophilized leaves. For embryos, the full embryo was separated from the endosperm before lyophilization and DNA extraction. Five polymorphic microsatellite loci were developed for the study and amplified in a single multiplex reaction (see Supplementary File S2). Forward primers from each microsatellite locus were labeled with fluorescent dyes (Applied Biosystems, Foster City, California, USA and Eurofin MWG, Paris, France): HEXTM (for Lv-01), FAMTM (for Lv-03 and Lv-16), ATTO565TM (for Lv-09), and ATTO550TM (for Lv-19). We performed PCR in a 10 µL volume containing 1× multiplex PCR master mix (Qiagen Hilden, Germany), 5–20 ng of genomic DNA, and 0.2 µM of labeled forward and unlabeled reverse primers. The PCR cycling program included an initial denaturation step (95 °C for 15 min) followed by 32 cycles of denaturation (94 °C for 30 s), annealing

(55 °C for 1 min 30 s) and extension (72 °C for 1 min) and a final extension (60 °C for 30 min). We mixed 1.5 µL of the PCR reaction with 0.25 µL of the GeneScan 500 LIZ size standard (Applied Biosystems) and 9.75 µL of deionized formamide. Alleles were size separated by electrophoresis on an ABI PRISM[®] 3130 sequencer and scored with GeneMapper version 5 (Applied Biosystems). Among the 236 individuals, we identified 123 unique genotypes. Each genotype was represented by 2.03 copies on average. These clones derive from plant cuttings that were collected on neighboring individuals in natura. Each unique genotype was considered as a potential father in the paternity analysis

Paternity analysis. For all offspring, paternity was assigned using the maximum-likelihood method described in Kalinowski et al. (2007) and implemented in the program CERVUS version 3.0.7 (Marshall et al. 1998). All unique genotypes in the experimental garden were considered as potential pollen sources. For each offspring-putative father combination, a paternity likelihood was estimated using a ratio of probabilities (LOD score, i.e., the likelihood that the examined plant is the true father divided by the likelihood that the examined plant is not the true father). To decide whether paternity could be assigned to the individual with the highest LOD score, the difference in LOD scores between the two most-likely fathers was calculated and compared to a critical difference in LOD scores below which paternity could not be attributed at either a relaxed (80%) or a strict (95%) level of confidence. This critical value was obtained using simulations, that were performed using the following parameters: 10,000 offspring, 123 candidate fathers, all candidate fathers sampled, and 0.01 as the proportion of mistyped loci. Seedlings that were genotyped on less than three markers were excluded from the analysis. For each offspring, paternity analysis produced three alternative outcomes: (i) no assignment because two or more putative fathers were compatible with the offspring, and the difference in LOD scores was too low to attribute paternity to the most-likely father at the chosen confidence level, (ii) paternity was attributed to the mother, allowing us to estimate a selfing rate for each mother, or (iii) paternity was attributed to another shrub. No wild populations of *Ligustrum vulgare* were growing in close proximity to the common garden, so all seeds were necessarily sired by plants in the experimental population.

Conditions for the maintenance of SC within a DSI system

Predictions of inbreeding depression and prior selfing consistent with estimated [SC] phenotype frequencies. Van de Paer et al. (2015) developed a phenotypic model to study the coevolution between SI and unisexuality. In particular, they studied the conditions for the maintenance of polymorphism with a pollen-part [SC] phenotype and two [SI] phenotypes, assuming: (i) a prior selfing rate of γ (proportion of ovules that are self-fertilized, with self-fertilization occurring before cross-fertilization, i.e., no competition between self and cross-pollen for the access to ovules), and (ii) that selfed seeds suffer from inbreeding depression (denoted by δ and defined as the decline in fitness of individuals produced by selfing relative to the fitness of outcrossed individuals, see Equation 15 in Van de Paer et al. 2015). The expected frequency of the pollen-part [SC] phenotype when maintained with both [SI] phenotypes is given by

$$f_{[SC]} = \frac{1 + \gamma}{2\gamma\delta} - 1 \quad (1)$$

Given the estimated frequency of the [SC] phenotype $\hat{f}_{[SC]}$ in our set of wild populations and its 95% confidence interval, Eq. (1) was used to determine the sets of values $\{\delta, \gamma\}$ consistent with our observations.

RESULTS

Phenotypic characterization of diallelic SI in *L. vulgare*

Based on patterns of cross-compatibility assessed using stigma tests in seven wild populations, only two SI groups were detected: plants either belonged to the G_A SI group (94 plants; incompatible with tester A, compatible with tester B), or to the G_B SI group (90 plants; incompatible with tester B, compatible with tester A). Among the 142 individuals tested with their own pollen, 135 displayed a typical incompatibility reaction, but pollen tubes germinated and grew through the stigma and style in seven individuals (5%, see Table 1; Fig. 2 panel C3). These [SC] individuals were detected in five populations, in geographically and ecologically different regions, from the North Sea coast in France

to Bavaria in Germany and from 10 to 1000 m in elevation (Table 1). All of them belonged to the G_A group.

Compatibility/incompatibility relationships among [G_A], [G_B], and [SC] individuals assessed using stigma tests in a diallelic design

The diallelic stigma tests supported the conclusions from the wild populations: this species has DSI (pollen from putative G_B plants always germinated on stigmas of G_A plants and vice versa, Table 2, Fig. 2 panels A1, B2, D4, and E5). For the two [SC] genotypes, however, the results depended on whether they were used as a recipient or a donor of pollen. Used as recipients, both [SC] individuals showed incompatibility with G_A [SI] tester individuals (Table 2, Fig. 2 panels C1, C2) and compatibility with G_B [SI] individuals (Table 2, Fig. 2 panels C4, C5), confirming that they belong to the G_A group. As pollen donors, they showed compatibility with G_A individuals, but also with G_B individuals, and were self-compatible (Table 2, Fig. 2 column 3).

Compatibility/incompatibility relationships among [G_A], [G_B], and [SC] individuals assessed by paternity analysis in the experimental population

Paternity tests were conducted both at the embryo stage (ten progenies) and, when enough seeds were available, at the seedling stage (six progenies). Germination rates were high (88% on average) and consistent across mother plants. All offspring that were successfully genotyped on 3 or more markers (1190 on 1209, 98%) were compatible with at least one father in the experimental plot. However, we were unable to assign a father to 152 offspring (13%) at the relaxed confidence level and to 287 offspring (24%) at the strict confidence level, because there were two or more possible fathers with LOD-score differences too low to attribute paternity to the most-likely father. To reliably identify compatible genotypes, we needed to limit the occurrence of type I errors (false assignments). Therefore, only the results obtained with the strict 95% threshold are presented below (but see Supplementary File S3 for results obtained using the relaxed 80% threshold). The overall mean selfing rate was 0.22 in the experimental population. Among the 201 offspring produced by selfing, 200 were from the two previously identified SC genotypes. The selfing rates differed between these two genotypes ($\chi^2_{(1)} = 16.77, P < 0.001$), with values of 0.77 for G16-10 and 0.52 for MJ-05, but were consistent in the two stages at which offspring were genotyped (embryos vs. seedlings, $\chi^2_{(1)} = 7.31 \cdot 10^{-31}, P = 1$ for G16-10 and $\chi^2_{(1)} = 0.965, P = 0.326$ for MJ-05). Except for one seedling, outcrossed offspring from the [SC] G16-10 genotype were all sired by a shrub belonging to the G_B group (43 pollination events by five different fathers, see Fig. 3 and Supplementary File S3). Outcrossed offspring from the other SC genotype, MJ-05, were sired either by a G_B father (29 pollination events by five different fathers) or by G16-10 (14 pollination events, see Fig. 3 and Supplementary File S3). Both SC genotypes sired seeds on both G_A and G_B mothers (see Fig. 3 and Supplementary File S3).

In the eight SI plants, 98% of the offspring from the three G_A mothers were sired by G_B fathers (236 pollination events by 15 different fathers) and 99% of offspring of the five G_B mothers were sired by G_A fathers (188 pollination events by 10 different fathers, see Fig. 3 and Supplementary File S3), confirming - at the postzygotic stage - the occurrence of a functional DSI system.

Aside from a few unexpected siring events, which could either be ascribed to a limited degree of leakiness in the system or to paternity miss-assignments (one selfing event of the CA-44 mother plant, which was otherwise behaving as a SI genotype, and five within-group fertilizations by SI individuals, see Fig. 3 and Supplementary File S3), paternity analyses thus confirmed the results of the stigma tests described above. (1) *Ligustrum vulgare* has a DSI system, (2) in some individuals, this system was dysfunctional, resulting in SC behavior, and (3) when this was


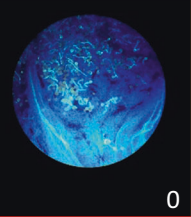
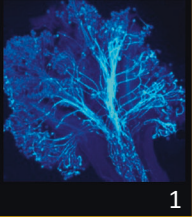
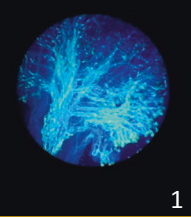

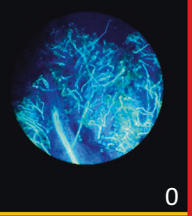
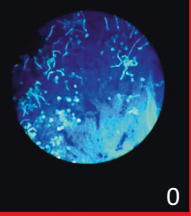
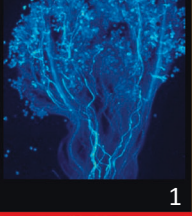

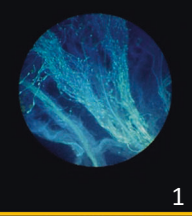
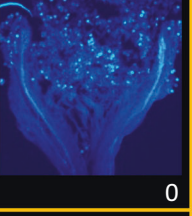
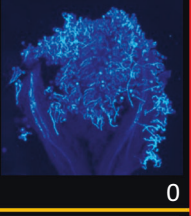
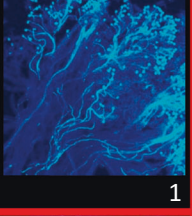
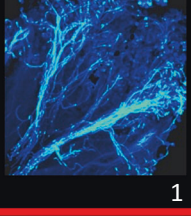
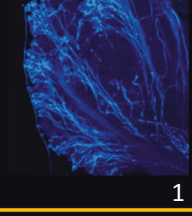
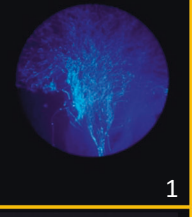
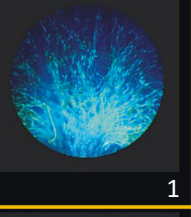
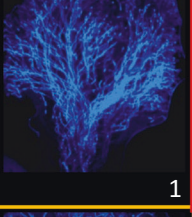
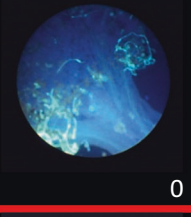
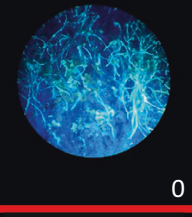

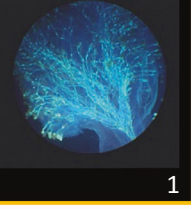
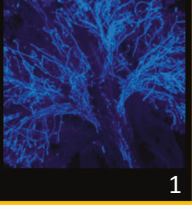
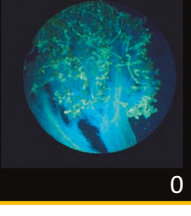
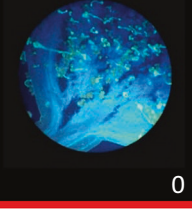
		1	2	3	4	5
		MP-01 (tester A) G_A [SI]	CA-44 G_A [SI]	G16.10 G_A [SC]	CA-07 G_B [SI]	MP-04 (tester B) G_B [SI]
Donnor	Recipient					
A	MP-01 (tester A) G_A SI	 0	 0	 1	 1	 1
B	CA-44 G_A SI	 0	 0	 1	 1	 1
C	G16.10 G_A [SC]	 0	 0	 1	 1	 1
D	CA-07 G_B [SI]	 1	 1	 1	 0	 0
E	MP-04 (tester B) G_B [SI]	 1	 1	 1	 0	 0

Fig. 2 Illustration of the different compatibility relationships observed using stigma tests in *Ligustrum vulgare*. Each column (1–5) corresponds to a genotype used as the pollen donor on five recipient genotypes (rows A–E). Panels outlined in red correspond to self-pollinations. G_A [SI] and G_B [SI]: self-incompatible genotype belonging to the self-incompatibility groups A and B respectively; G_A [SC] self-compatible genotype which stigmas are incompatible with pollen from G_A genotypes. The scoring was binary, with “0” indicating incompatibility (absence of pollen tubes, or very short pollen tubes that do not reach the style) and “1” indicating compatibility (pollen tubes grow through the stigmatic tissue to the style and then toward the ovules).

the case, an asymmetry was observed: [SC] individuals could sire both G_A and G_B individuals, but as recipients they behaved like members of the G_A group.

Conditions for the stability of a polymorphic [SC]/[SI] population in a DSI

Overall, [SC] phenotypes are clearly present, though rare, in five out of the seven *L. vulgare* wild populations studied (see Table 1). We used Van de Paer et al. (2015) model to determine the range of inbreeding depression values consistent with maintenance of a stable polymorphism for both [SI] and [SC] phenotypes at the

observed frequencies (Table 1). We found that, under prior selfing hypothesis, the observed frequencies are consistent with inbreeding depression higher than 0.95, depending on the value of the selfing rate, which was required to exceed 0.9 (Fig. 4).

DISCUSSION

DSI is widely present in the Oleaceae tribe

Phylogenetic analyses of the Oleaceae (Wallander and Albert 2000) suggest that the subtribe Ligustrinae forms the basal clade of Oleaceae tribe and represents the first lineage that diverged after

Table 2. Compatibility relationships between 10 *Ligustrum vulgare* genotypes, characterized at the prezygotic stage using stigma tests in a full diallelic crossing scheme.

SI group		Genotype	Pollen donor									
			G _A				[SC]		G _B		G _B	
			CA-44	MP-01	VIF-09	VIF-16	G16-10	MJ-05	CA-07	CA-31	G13-08	MP-04
			(tester A)						(tester B)			
Pollen recipient	G _A	CA-44	SI	0	0	0	1	na	1	1	1	1
	G _A	MP-01 (tester A)	0	SI	0	0	1	1	1	1	1	1
	G _A	VIF-09	0	0	SI	0	1	1	1	1	1	1
	G _A	VIF-16	0	0	0	SI	1	na	1	1	1	1
	[SC]	G16-10	0	0	0	0	SC	1	1	1	1	1
	[SC]	MJ-05	0	0	0	0	1	SC	1	1	1	1
	G _B	CA-07	1	1	1	1	1	1	SI	0	0	0
	G _B	CA-31	1	1	1	1	1	1	0	SI	0	0
	G _B	G13-08	1	1	1	1	1	1	0	0	SI	0
	G _B	MP-04 (tester B)	1	1	1	1	1	1	0	0	0	SI

Germination of pollen tubes on stigmas were scored in 100 crosses (three flowers pollinated in each cross) performed between four G_A, four G_B, and two [SC] genotypes (G16-10 and MJ-05). As pollen donors, [SC] individuals were compatible with both G_A and G_B genotypes, but as pollen recipients, they were incompatible with G_A genotypes and compatible with G_B genotypes.

SI Self-incompatibility reaction observed (pollen tube growth stopped within stigma), SC self-compatibility reaction observed (pollen tubes converged in the stigmatic tissue and grew toward the style), 0 incompatibility reaction, 1 compatibility reaction, na missing data.

an allotetraploidization event. Recent work by Olofsson et al. (2019) defines the Schreberineae (including the genera *Schrebera* and *Comoranthus*) as the basal clade and the Ligustrinae as the next derived clade before the two other subtribes split off: Fraxininae (including the genus *Fraxinus*) and Oleinae (with 12 genera including *Olea* and *Phillyrea*). The existence of two SI groups in *L. vulgare*, as well as all other Oleaceae species belonging to the allotetraploid Oleeeae tribe so far tested (Saumitou-Laprade et al. 2018; Saumitou-Laprade et al. 2010; Saumitou-Laprade et al. 2017a; Vernet et al. 2016) suggests that a homomorphic DSI has been present in the Oleaceae species since the allotetraploidization event. Heterostyly or distyly is reported in all ancestral diploid subtribes, except in Fontanesieae (Darwin 1877; Dommée et al. 1992; Ganders 1979; Kiew 1984; Kim 1999; Ryu 1976; Woodson et al. 1976). It is not known whether the SI in the species with homomorphic DSI is inherited from a system already present in heterostylous species and has persisted since the loss of floral heterostyly (with retention of homologous incompatibility genes) or whether it has evolved following the allotetraploidization event. Detailed genetic studies and phylogenetic analyses are needed to assess a possible homology of DSI between heterostylous and non-heterostylous taxa in the Oleaceae (Barrett 2019).

SC behavior in *L. vulgare* involves a mutation affecting the functioning of the pollen component of SI

Our wild populations screening allowed us to validate the theoretical prediction that [SC] genotypes should be found in populations of hermaphroditic species with DSI. We found evidence that these variants occurred at low frequencies in populations that were several hundreds of kilometers apart. The [SC] behavior was further confirmed with diallelic stigma tests and paternity analysis in our experimental population, which further suggested an asymmetrical compatibility for two [SC] genotypes. Indeed, (i) pollen from [SC] genotypes was compatible with individuals from both compatibility groups, but (ii) when used as recipients (in the diallelic stigma tests) or as mother plants in the experimental garden (in the paternity analysis), [SC] genotypes

were only compatible with/sired by individuals from the G_B group. Therefore, our results indicate that the breakdown of SI in *L. vulgare* involves a mutation affecting the functioning of the pollen component of SI in plants of the G_A group. While our wild population screening only examined individuals as recipients (using the two tester plants' pollen and self-pollen), all detected [SC] individuals also behaved as members of the G_A group. Two scenarios could explain the fact that only one incompatibility group was affected: (i) either the pollen-part locus in the G_A group is somehow more susceptible to breakdown or (ii) [SC] individuals all trace back to the same mutation and it is just a matter of chance that this incompatibility group was affected and not the other. SI breakdown has been observed in various other species and, interestingly, it often involves a breakdown in pollen-part incompatibility (e.g., Koseva et al. 2017; Sonneveld et al. 2005; Tsukamoto et al. 2003; Wu et al. 2011). An open question is whether this modification is due to mutations in a specific S-allele or to the epistatic effect of a modifying factor unlinked to the S-locus but affecting the expression of the pollen gene from a specific S-allele (Wu et al. 2011). Furthermore, in order to improve theoretical models describing the dynamics of SC variants in natural populations, future studies should estimate the ability of SC genotypes to produce offspring, both as father and mother and using both self- and cross-fertilizations. This could be done using manual-pollinations to compare seed production with self- versus allo-pollen or with monitored mixtures of both.

Evolution and maintenance of the [SI]/[SC] polymorphism in *L. vulgare*

Whether [SC] genotypes appeared several times or only once, the observed frequencies of SC individuals in wild populations (around 5%) cannot be explained by recurrent mutational breakdown, because this would require implausibly high mutation rates. Our results rather support a role of negative frequency-dependent selection (Van de Paer et al. 2015), along with a combination of high selfing rates (>0.90) and high levels of inbreeding depression (>0.95, see Fig. 4).

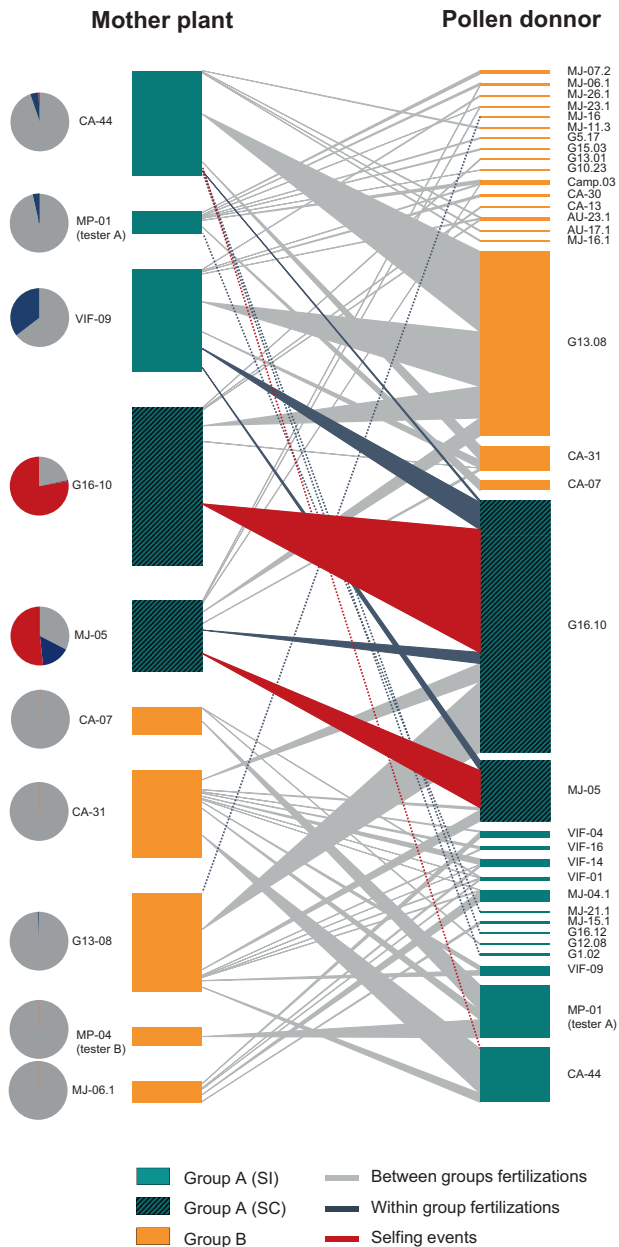


Fig. 3 Diagram representing all pollination events detected by paternity analysis in the experimental garden. On the left, each of the ten mother plants (pollen recipients) is represented by a rectangle whose length is proportional to the number of assigned offspring (from 23 for mother plant MP-04 to 198 for G16-10). On the right, confirmed father plants (pollen donors) are also represented by rectangles whose length is proportional to the number of offspring they sired according to paternity analysis (1–315). The width of the links between pollen donors and mother plants are again proportional to the number of detected events and are colored according to the type of event that occurred (i.e., between-group fertilization, within-group fertilization or selfing event). Dashed lines correspond to the siring events that did not conform with the hypothesis of a functional DSI system in *L. vulgare* (selfing events in SI individuals in red and within-group fertilizations by SI individuals in blue). Pie charts on the left represent the proportion of the different types of cross (between self-incompatibility (SI) groups, within SI group and selfing) detected by paternity assignment for each mother plant.

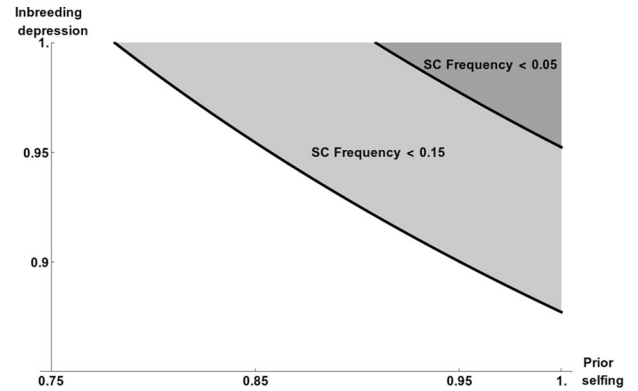


Fig. 4 Predicted inbreeding depression and prior selfing rate consistent with the estimated observed frequencies of the self-compatible (SC) phenotype in natural populations of *Ligustrum vulgare*. The curves show the values predicted for the mean (≈ 0.05 , top) vs. the lower 95% bound (≈ 0.15 , bottom) estimates of the SC phenotype frequency. The gray zone above the curves show the range of values that are consistent with a SC phenotype frequency lower than 0.05 and 0.15. Predictions were obtained using the model built by Van de Paer et al. (2015).

In our experimental population, selfing rates were assessed for the two [SC] individuals and were found to be high (0.77 and 0.52) but below that threshold. One possible explanation for this is that selfing rates likely depend on the environmental conditions. Because the experimental population was located in the species distribution area, plants were exposed to a pollinator community similar to what could be experienced by wild populations in the region. However, in natural populations, individual plants can reach important sizes due to their age and the clonal behavior of the species, meaning that geitonogamy might be higher in the wild than in our experimental setting, where plants were young. The artificial population was densely planted, with branches of neighboring individuals often being intertwined; density and abundance of flowering shrubs may have attracted pollinators and/or facilitated the transfer of cross-pollen among plants (Arista et al. 2017). In natural populations, low pollinator abundances and/or low plant densities might create conditions where pollination mainly involves self-pollen landing by gravity on stigma. More work is warranted to quantify selfing rates in the wild and verify whether typical populations (with lower densities and potentially comprising large clones, favoring geitonogamy) actually display levels of selfing compatible with the model's predictions.

Regarding the predicted high levels of inbreeding depression needed to maintain 5% of [SC] genotypes in natural populations, our data set only allows us to evaluate what happens in the first stages of the life cycle of this long-lived shrub. The fact that (i) germination rates in seeds derived from [SI] and [SC] plants were similar and that (ii) paternity analyses did not reveal differences in selfing rates between the embryo and the seedling stage in [SC] offspring suggest a limited inbreeding depression effect at the germination stage. Several explanations for the maintenance of the polymorphic breeding system observed in *L. vulgare* can be formulated. (i) There is inbreeding depression at the germination stage, but it was not detected in greenhouse conditions. (ii) Strong inbreeding depression is expressed either at earlier stages of the life cycle (before germination, with seed abortions for instance, which would not have been detected here), or later on, with reduced survival and/or reproduction after the seedling stage. This remains to be estimated since understanding the evolution of breeding systems requires correctly estimating lifetime inbreeding depression, and not only what happens during the early stages (Husband and Schemske 1996; Lesaffre and Billiard 2019). This is

particularly challenging in perennials, and long-term studies are now needed to ascertain whether or not the level of inbreeding depression in *Ligustrum vulgare* is sufficient to explain the low frequencies of [SC] individuals that were observed in natura.

Could [SC] genotypes constitute a pathway toward androdioecy?

Finally, theoretical models also suggest that SI breakdown in pollen (or in pistils) may represent an evolutionary pathway towards a third SI group through SC intermediates if a compensatory pistil- (or pollen-) part mutation restores SI (Gervais et al. 2011; Uyenoyama et al. 2001; Van de Paer et al. 2015) or toward androdioecy (Van de Paer et al. 2015). The transition from hermaphroditism to stable androdioecy requires at least two mutations: one producing female sterility and one rendering males compatible with both SI groups. Theory helps predicting in which order these mutations should appear so as to be selected for. Under nuclear control of sex, models predict that male individuals are eliminated from hermaphroditic populations due to the strong compensation required *via* a fitness advantage for the loss of one sexual function (Charlesworth and Charlesworth 1978; Lewis 1941; Lloyd 1975). It is thus unlikely that female sterility appears first. Additionally, if DSI allows the maintenance of stable androdioecy because males are compatible with both groups of hermaphrodites (Pannell and Korbecka 2010), androdioecy itself facilitates the maintenance of DSI (Van de Paer et al. 2015). The occurrence of compatible males that cannot self and thereby avoid the effects of inbreeding depression prevents the invasion of a SC mutation in the population. Thus, when males are present, [SC] hermaphrodites not only suffer from inbreeding depression, but also from the direct competition with males to sire the SI hermaphrodites (Van de Paer et al. 2015). Therefore, it is more parsimonious to hypothesize that the [SC] mutant would appear first, followed by a female sterility mutation closely linked to the SC mutation. In that sense, the SI breakdown in *L. vulgare* may represent the first step in the evolution toward androdioecy in Oleaceae. Whether the same evolutionary scenario gave rise to the androdioecy documented in other Oleaceae species such as *P. angustifolia* (Saumitou-Laprade et al. 2010) and *F. ornus* (Vernet et al. 2016) remains an open question, but the abundance of androdioecious species in Oleaceae (12% of species) compared to other angiosperms (0.01%) supports a facilitating role of DSI in this process.

In the future, full-sib progenies from our experimental population will be surveyed. Crosses segregating for both SI/SC behavior and SI group will be completed by controlled crosses involving the same parents in order to establish large mapping populations. This will allow us to compare the position of the locus controlling SI/SC behavior and the S-locus in *L. vulgare* with the loci controlling sexual phenotype and SI in *Phillyrea angustifolia*, an androdioecious Oleaceae for which a high-density genetic map is available (Carre et al. 2021). This should help shedding light on the hypothesized role of DSI and SC mutants in the emergence of males.

DATA AVAILABILITY

Data for this study (results of the stigma tests performed on the plants in the experimental garden, multilocus microsatellite genotypes of all adults from the experimental population, along with multilocus microsatellite genotypes of 1209 offspring collected on ten mother plants) are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.pvmcvdmb>.

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COMPETING INTERESTS

The authors declare no competing interests.

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