Evidence for the long-term maintenance of a rare self-incompatibility system in Oleaceae

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Summary

- A rare homomorphic diallelic self-incompatibility (DSI) system discovered in *Phillyrea angustifolia* (family Oleaceae, subtribe Oleinae) can promote the transition from hermaphroditism to androdioecy. If widespread and stable in Oleaceae, DSI may explain the exceptionally high rate of androdioecious species reported in this plant family. Here, we set out to determine whether DSI occurs in another Oleaceae lineage.

- We tested for DSI in subtribe Fraxininae, a lineage that diverged from subtribe Oleinae *c.* 40 million yr ago. We explored the compatibility relationships in *Fraxinus ornus* using 81 hermaphrodites and 25 males from one natural stand and two naturalized populations using intra- and interspecific stigma tests performed on *F. ornus* and *P. angustifolia* testers.

- We uncovered a DSI system with hermaphrodites belonging to one of two self-incompatibility (SI) groups and males compatible with both groups, making for a truly androdioecious reproductive system. The two human-founded populations contained only one of the two SI groups.

- Our results provide evidence for the evolutionary persistence of DSI. We discuss how its stability over time may have affected transitions to other sexual systems, such as dioecy.

Introduction

Since Darwin (1877), evolutionary biologists have been attempting to reconstruct how flowering plants evolved from hermaphroditism, in which individuals produce female and male organs in the same flower (Yampolsky & Yampolsky, 1922), to dioecy, in which female and male organs are found in flowers of different individuals (Barrett, 2002; Charlesworth, 2006). Two main pathways for the evolution of dioecy have been proposed. The first involves the selection of major sterility mutations (reviewed in Westergaard, 1958). A population genetic model shows that dioecy can evolve from an ancestral hermaphrodite or from a monoecious (female and male organs found in separate flowers on the same individual) species via intermediate systems in which hermaphrodite individuals coexist with unisexual mutants that have lost either their male (gynodioecy) or their female (androdioecy) function (Charlesworth & Charlesworth, 1978). This pathway is considered to be the most common (Charlesworth & Charlesworth, 1978; Lloyd, 1980; Renner & Won, 2001; Torices *et al.*, 2011). The second pathway involves heterostyly, in which hermaphrodites possess a heteromorphic diallelic self-incompatibility (DSI) system with two types of genetically different individuals, one with short styles and long stamens, and one with long styles and short stamens (Lloyd, 1979). This system, heterostyly, has a temporal analog,

heterodichogamy, in which hermaphrodites are separated into two dichogamous, reciprocal morphs: protandrous and protogynous (Kubitzki & Kruz, 1984; Dommée *et al.*, 1990).

Gynodioecy characterizes > 20 000 (6%) angiosperm species (Renner & Ricklefs, 1995; Dufay *et al.*, 2014), whereas androdioecy has been described in only a dozen species (< 0.005%). For both reproductive systems, theory predicts that evolution from hermaphroditism should be difficult because – unless compensated by the doubling of their fitness in the remaining sexual function – unisexual individuals have half the reproductive capacity of hermaphrodites, which can produce seeds through both pollen and ovules (Lewis, 1942; Charlesworth & Charlesworth, 1978). The success of gynodioecy in flowering plants is explained by a nucleocytoplasmic conflict (Hurst *et al.*, 1996): male sterility is encoded by mitochondrial mutations that are transmitted maternally (Saumitou-Laprade *et al.*, 1994) and there is no reproductive disadvantage as a result of the loss of the male function. Therefore, females need only to produce slightly more seeds than hermaphrodites to increase in frequency in populations. Moreover, if hermaphrodites suffer from inbreeding depression associated with selfing, females – produced only from outcrosses – will enjoy a fitness advantage and gynodioecy can be maintained. By contrast, the rarity of androdioecy can be attributed to the purely Mendelian transmission of female sterility and to the enormous fertility advantage required of male individuals in partially

self-fertilizing populations caused by the reduced availability of ovules for outcrossing (Lloyd, 1975 #143; Charlesworth & Charlesworth, 1978; #95; Charlesworth, 1984 #97; Sakai *et al.,* 1997 #187; Van Etten *et al.,* 2008 #2495).

In the androdioecious Oleaceae species, *Phillyrea angustifolia* (Saumitou-Laprade *et al.,* 2010; Husse *et al.,* 2013; Billiard *et al.,* 2015), a homomorphic DSI system separates hermaphrodites into two morphologically identical self-incompatibility (SI) groups, such that individuals of a given SI group can only sire seeds on hermaphrodites from the other group, whereas males are compatible with both SI groups (Saumitou-Laprade *et al.,* 2010). Recent results from controlled crosses have revealed that progeny produced by a given male give rise to equal proportions of males and hermaphrodites on hermaphrodites of one SI group, but to males only on hermaphrodites of the other SI group (Billiard *et al.,* 2015). Mathematical modeling shows that the DSI system associated with male-biased segregation explains the success and spread of female-sterile mutants without requiring inbreeding depression or resource reallocation. Even males with lower male fitness than hermaphrodites can be selected for, and high frequencies (> 50%) of males can be observed for realistic values of the male fitness advantage compared with hermaphrodites (i.e. comprising between 1.3 and 4) (Billiard *et al.,* 2015). DSI provides the conditions under which female sterility can spread: males are compatible with both groups of hermaphrodites, giving them a twofold fitness advantage. Interestingly, in *P. angustifolia,* the female-sterility locus also causes full segregation distortion and strongly favors the transmission of the male-determining mutation. The way in which DSI paves the way to the evolution of androdioecy mirrors the way in which uniparental transmission of mitochondrial genes favors the expression of selfish cytoplasmic male-sterility mutations in gynodioecious species and provides a satisfactory explanation for the spread and maintenance of female sterility from hermaphroditism in *P. angustifolia* (Pannell & Voillemot, 2015).

DSI may have evolved before androdioecy appeared in *P. angustifolia* and the occurrence of homomorphic DSI in other lineages is unknown. The maintenance of a DSI that is not associated with morphological differences (e.g. heterostyly in entomophilous species) is unexpected for two reasons. First, SI systems are susceptible to the rapid invasion of new incompatibility alleles, because of a strong frequency-dependent advantage of rare mating phenotypes (Wright, 1939). Second, in hermaphroditic species, self-compatible mutants are expected to invade a homomorphic DSI population regardless of the extent of inbreeding depression (Charlesworth & Charlesworth, 1979b). Hence, the persistence of homomorphic DSI over time is not expected, but it is required to explain recurrent events of transition from hermaphroditism to androdioecy within a plant family and the evolution from androdioecy towards dioecy. To investigate the evolutionary persistence of the DSI discovered in *P. angustifolia,* we screened another Oleaceae lineage, the genus *Fraxinus* (ash trees), for DSI. Based on flower morphology, male and hermaphroditic plants have been reported in *Fraxinus ornus* (flowering ash) populations (Dommée *et al.,* 1999; Verdu *et al.,* 2004, 2006) and, as in *P. angustifolia* (Lepart & Dommee, 1992;

Hao *et al.,* 2011; Husse *et al.,* 2013), *F. ornus* populations show high male frequencies (50% and higher; Dommée *et al.,* 1999). Furthermore, lineage differentiation times in the Oleaceae can be gleaned from recent phylogenetic analyses based on sequences of the nuclear ribosomal internal transcribed spacer-1 and four maternally inherited plastid regions (*trn*L-*trn*F, *trn*T-*trn*L, *trn*S-*trn*G spacers and the *mat*K region) of 61 Olea taxa and 17 Oleaceae species outside the genus *Olea,* including *Fraxinus americana, F. excelsior, F. quadrangulata* and *Phillyrea latifolia* (Besnard *et al.,* 2009). This study estimates the divergence time of the ash lineage from *Phillyrea* (subtribe Oleinae) at > 40 million yr ago (Ma) (Besnard *et al.,* 2009). Therefore, given this long inter-lineage divergence time (80 Myr) and its androdioecious populations coupled with high male frequencies, *F. ornus* is a good candidate for the study of the evolutionary persistence of the DSI system discovered in *P. angustifolia.*

To characterize the SI system in *F. ornus,* we investigated three populations showing high frequencies of males and analyzed incompatibility reactions at pre-zygotic stages by testing pollen germination on stigmas (the so-called stigma test) using hermaphroditic pollen recipients and hermaphroditic and male pollen donors. To assess the functional stability of SI, we extended the stigma test to the interspecific level using *P. angustifolia* pollen and/or stigmas. Our results provide compelling evidence for a DSI system in *F. ornus* with two mutually compatible SI groups of hermaphrodites – that is incompatible within a group but compatible between groups – with males being compatible with both groups. Our results and recent phylogenetic data shed light on the evolutionary persistence of the DSI system and its evolutionary consequences.

Materials and Methods

Plant material and populations studied

Fraxinus ornus is one of 16 species in section Ornus of the genus *Fraxinus* (Oleaceae). It is widespread in southern and central Europe (Supporting Information Fig. S1), with natural populations in southeastern France (Barbero *et al.,* 1971). In addition, populations of anthropogenic origin are currently expanding in the French Mediterranean region, particularly in river valleys, including the Vis Valley (Thébaud & Debussche, 1991) and Lez Valley near Montpellier, France (Fig. S1; Notes S1). There are two types of individual, distinguishable by their flowers: hermaphrodites with perfect bisexual flowers (Rouy & Foucaud, 1897) and males whose flowers have no stigmas or styles (Gillet & Magne, 1883). Both sexual morphs co-occur in populations (Dommée *et al.,* 1999).

Fraxinus ornus trees from three sites in southern France were studied (Fig. S1). At the first site, flowers were sampled in a natural population located within the natural species distribution of *F. ornus* in the Loup Valley (Alpes-Maritimes, France). This population is divided into two subpopulations: one is found along the Loup River near Saut-du-Loup (hereafter called SDL); the other is located on top of a southeast-facing cliff above the Loup Valley near Gourdon (hereafter called G). A seed collected in 1999 on an

open-pollinated tree in Gourdon was planted in the CEFE-CNRS experimental garden in Montpellier (France); this tree is hereafter called G_{1999}-48. At the second site, hereafter referred to as Vis Valley, seeds were collected in 1991 on two open-pollinated trees located in Vis Valley and germinated in glasshouse conditions. Seedlings were planted in 1994 as 2-yr-old plants in the CEFE-CNRS experimental garden (Dommée *et al.*, 1999). At the third site, hereafter called Lez Valley, flowers were collected on trees located along the Lez River in Castelnau-le-Lez, France. The second and third sampling sites, Vis Valley and Lez Valley, are located outside the natural species distribution of *F. ornus*. This secondary species distribution originates from two plantings grown from seeds harvested on a single tree located in the Montpellier Botanical Garden (Notes S1). The first planting, initially composed of 100 trees planted in 1920, was established in the 'Arboretum du Grenouillet' near the Vis River (Gorniès, France). The second planting, composed of a few trees planted *c.* 1920, was established in a private garden near the Lez River facing the town of Castelnau-le-Lez. The progeny of these planted trees have naturalized and spread across the Vis, Lez and Hérault Valleys (i.e. 42 km) over the last 100 yr (Thébaud & Debussche, 1991), giving rise to hundreds of thousands of trees. All three study sites harbor populations growing under natural conditions with frequencies of hermaphrodite and male individuals of *c.* 50%.

In total, 81 hermaphrodites and 25 males were collected and included in cross-compatibility tests.

Compatibility/incompatibility assessments

Three sets of stigma tests were performed in 2012, 2014 and 2015 to assign sampled *F. ornus* trees to SI groups.

Incompatibility tests To ensure receptive stigmas free of contaminant pollen, we bagged branches (*c.* 50 cm long, with several terminal buds) on tester recipients 3 wk before stigmas became receptive (using two PBS3d/50 bags, size $16 \times 50 \times 16$ cm^3 with a 10×25 cm^2 PVC window on the front; PBS International, Scarborough, UK). To synchronize pollen availability of some tested genotypes with the stigma receptivity of tester recipients, we stored freshly collected pollen at $-80°C$ until application on flowering recipients (Methods S1).

To observe compatibility/incompatibility reactions in optimal conditions, we developed a standardized protocol in which recipient flowers were held, matured and fertilized *in vitro* (Methods S1). Under these conditions, pollen grains and pollen tubes were easily distinguished from maternal tissues (Fig. 1). The presence of several pollen tubes converging through the stigma tissue towards the style and successive attrition until a single tube reaches the style (Fig. 1b,c) was used as the determining criterion to score compatibility between pollen recipient and donor. The absence of pollen tubes or the presence of only short pollen tubes growing no further than the first cell layers of the stigma was used as the
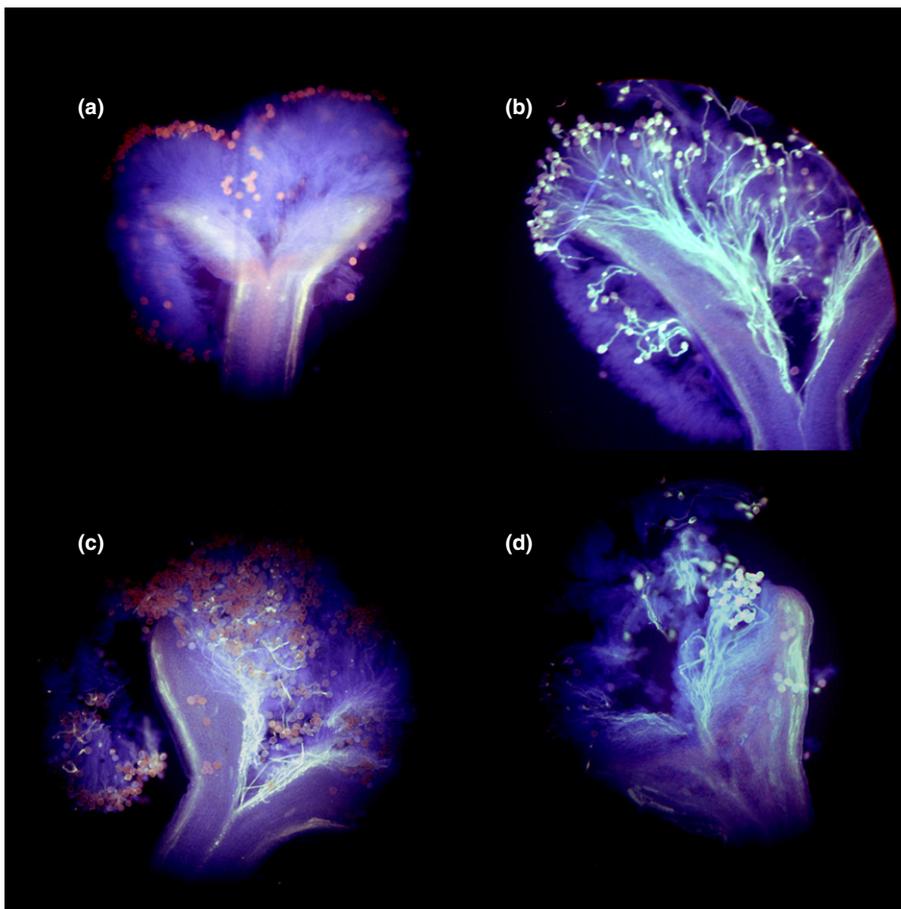
Fig. 1 Stigma tests performed to assess self-incompatibility, incompatibility and compatibility in *Fraxinus ornus*: example of the A17 and G_{1999}-48 hermaphrodites. Hermaphrodite A17 pollen does not germinate on its own stigmas (a), indicating self-sterility. A17 pollen germinates on the G_{1999}-48 hermaphrodite (b), attesting to its viability. Germination of pollen from the D27 male (c) and the SDL17 hermaphrodite (d) on A17 stigmas, attesting to the receptivity of A17.

criterion to score incompatibility (Fig. 1a). As a result of the risk of contamination, the test was always performed on three flowers. The same protocol has been used to investigate cross-compatibility in *P. angustifolia* (Saumitou-Laprade *et al.*, 2010).

Stigma test In this study, a stigma test corresponds to one pollen sample tested on a pair of reference recipients previously verified for their cross-compatibility (i.e. belonging to two different SI groups). In each test, we scored the incompatibility of the tested pollen with one of the two reference recipients (indicating that pollinator and recipient belong to the same incompatibility group) and its compatibility with the other recipient (thereby assessing viability of the tested pollen). We also scored the cases in which tested pollen would be compatible with both reference recipients, indicating that the tested pollen donor would belong to a third SI group different from the two groups represented by tester recipients (Tables 1, S1). Pairs of reference testers were also used as pollen donors for some tested recipients when bagging and prevention of contamination were possible.

Selection of reference testers for intraspecific stigma tests In 2012, some flowering trees of the *F. ornus* collection growing in the CEFE-CNRS experimental garden were tested for compatibility/incompatibility relationships in order to select the pairs of reference recipients for further testing. The early-flowering hermaphrodite A17 and the late-flowering hermaphrodite B02 trees from the Vis Valley collection, and the hermaphrodite tree G_{1999}-48 from the Loup Valley collection, were designated as reference recipients to assess compatibility phenotypes of the males and hermaphrodites included in this study. Some hermaphrodites were phenotyped using the stigma test with pollen from SDL_{2012}-17 from the Loup Valley collection as a reference pollen donor.

Interspecific stigma tests with *P. angustifolia* testers To (1) assess the functional stability of DSI, (2) substantiate our conclusions on its occurrence in *F. ornus*, and (3) determine whether SI groups are conserved among Oleaceae species, we performed interspecific stigma tests with *P. angustifolia* hermaphrodites belonging to the G1 and G2 SI groups identified previously (Saumitou-Laprade *et al.*, 2010). Using either frozen pollen from *P. angustifolia* on fresh receptive stigmas from *F. ornus* (in early May), or frozen *F. ornus* pollen on fresh *P. angustifolia* stigmas (at the flowering period of *P. angustifolia*, late March), non-ambiguous, repeatable compatibility and incompatibility reactions were tested in this study (Fig. 2).

Results

A total of 207 compatibility tests was performed on a total of 81 hermaphrodites and 25 males assessed for their compatibility/incompatibility phenotype (Tables 1, S1). We succeeded in performing full stigma tests for 53 hermaphrodites and 12 males, 16 and two of which, respectively, were assessed in intra- and interspecific stigma tests (Table 1).

Self-incompatibility in *F. ornus*

Self-incompatibility was tested on seven different hermaphrodites (Table 1) and self-pollen tubes were not detected in any of the observed stigmas (e.g. for A17, Fig. 1a). Pollen viability and stigma receptivity were verified for A5, A17, SDL_{2015}-16 and G_{1999}-48 (Table 1). Pollen from A5, A17 and SDL_{2015}-16 germinated on G_{1999}-48 stigmas, and the pollen from G_{1999}-48 germinated on G_{2014}-3, G_{2014}-6, G_{2014}-9, G_{2014}-20, G_{2014}-23, G_{2014}-27, G_{2014}-28 and G_{2014}-29 stigmas. Pollen tubes from several males and/or compatible hermaphrodites germinated on the stigmas of A5, A17, SDL_{2015}-16 and G_{1999}-48 (Table 1). Given that self-pollination was unsuccessful, although pollen was clearly viable and stigmas were receptive, we concluded that there is SI in *F. ornus*.

Two incompatibility groups observed in four *F. ornus* hermaphrodite testers

To select testers for intraspecific stigma tests, compatibility tests were first performed in 2012 among four hermaphrodite recipients (A17, B02, SDL_{2012}-17 and G_{1999}-48). Pollen from B02 on A17 stigmas yielded no pollen tubes, resembling self-pollinated stigmas (Fig. 1a). A similar result was obtained with pollen from SDL_{2012}-17 on G_{1999}-48. However, pollen from A17 and B02 tested on the G_{1999}-48 stigmas produced pollen tubes growing through the stigma tissue towards the style. Compatibility was also observed with the pollen of SDL_{2012}-17 and G_{1999}-48 on B02 stigmas. We classified these four hermaphrodites into two different incompatibility groups and used them in pairs as testers in the intraspecific stigma tests.

Interspecific stigma tests reveal correspondence between the two incompatibility groups in *P. angustifolia* and *F. ornus*

Stigma tests were performed using A17 and G_{1999}-48 as recipients of *P. angustifolia* pollen harvested from hermaphrodites belonging to the G1 and G2 SI groups, respectively (Billiard *et al.*, 2015). We observed incompatibility between A17 stigmas and *P. angustifolia* G1 pollen and between G_{1999}-48 stigmas and *P. angustifolia* G2 pollen (Fig. 2a,d). However, we observed compatibility between A17 stigmas and *P. angustifolia* G2 pollen and between G_{1999}-48 stigmas and *P. angustifolia* G1 pollen (Fig. 2b,c). In addition, crosses were compatible between a single *P. angustifolia* male and both *F. ornus* recipients (data not shown). Therefore, we assigned the hermaphrodite A17 and all of its incompatible mates to the G1 SI group, and the hermaphrodite G_{1999}-48 and all of its incompatible mates to the G2 SI group.

Only two incompatibility groups are found among *F. ornus* hermaphrodites

Using *F. ornus* hermaphrodites from the two incompatibility groups as testers, we performed intraspecific stigma tests with 53 different hermaphrodites and 12 males (Table 1). The results showed that 47 hermaphrodites were incompatible with a G1

Table 1 *Fraxinus ornus* samples phenotyped for self-incompatibility (SI) group using intra- and interspecific stigma tests

Population	Site and/or year of sampling	ID of tested pollen donor	Year of typing	Sex	Compatible with		Pair of *F. ornus* testers	SI type scored on *P. ang.*	SI group	SI/SC
					G1	G2				
Gourdon	G_{1999}	2	2012	H	0	1	B02/SDL_{2012}-17	—	G1	—
		46	2012	H	0	1	B02/SDL_{2012}-17	—	G1	—
		47	2012	H	0	1	B02/SDL_{2012}-17	—	G1	SI
		48	2012	H	1	0	B02/SDL_{2012}-17	G2	G2	—
	G_{2014}	3	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		6	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		9	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		20	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		23	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		27	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		28	2014	H	0	1	A17/G_{1999}-48	—	G1	—
		29	2014	H	0	1	A17/G_{1999}-48	—	G1	—
	SDL_{2012}	2	2012	H	1	0	A17/G_{1999}-48	—	G2	—
		17	2012	H	1	0	A17/G_{1999}-48	—	G2	—
	SDL_{2015}	1	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		2	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		3	2015	H	1	0	B16/G_{1999}-48	G2	G2	—
		5	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		7	2015	H	0	1	*	G1	G1	—
		8	2015	H	1	0	*	G2	G2	—
		9	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		10	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		11	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		12	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		13	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		14	2015	H	0	1	*	G1	G1	—
		15	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		16	2015	H	0	1	B16/G_{1999}-48	G1	G1	SI
		17	2015	H	0	1	B16/G_{1999}-48	—	G1	—
		18	2015	H	0	1	B16/G_{1999}-48	G1	G1	—
		19	2015	H	0	1	B16/G_{1999}-48	—	G1	—
		20	2015	H	1	0	*	G2	G2	—
		21	2015	H	0	1	*	G1	G1	—
		5	2015	M	1	1	B16/G_{1999}-48	Compatible with G1 and G2	G2	—
		6	2015	M	1	1	B16/G_{1999}-48	Compatible with G1 and G2	G1	—

Table 1 (Continued)

Population	Site and/or year of sampling	ID of tested pollen donor	Year of typing	Sex	Compatible with		Pair of _F. ornus_ testers	SI type scored on _P. ang._	SI group	SI/SC
					G1	G2				
Vis Valley	1999	A01	2012–2014	H	0	1	B02/SDL$_{2012}$-17	–	G1	–
		A05	2012–2014	H	0	1	A17/G$_{1999}$-48	–	G1	SI
		A07	2012–2014	H	0	1	A17/G$_{1999}$-48	–	G1	SI
		A08	2012–2014	H	0	1	A17/G$_{1999}$-48	–	G1	SI
		A13	2012–2014	H	0	1	*	G1	G1	–
		A15	2012–2014	H	0	1	A17/G$_{1999}$-48	–	G1	SI
		A16	2012–2014	H	0	1	B02/G$_{1999}$-48	–	G1	–
		A17	2012–2014	H	0	1	B02/G$_{1999}$-48	–	G1	SI
		B01	2012–2014	H	0	1	B02/SDL$_{2012}$-17	–	G1	–
		B02	2012	H	0	1	A17/G$_{1999}$-48	–	G1	–
		B06	2012–2014	H	0	1	*	G1	G1	SI
		B14	2012–2014	H	0	1	B01/G$_{1999}$-48	–	G1	–
		B15	2012–2014	H	0	1	A17/G$_{1999}$-48	–	G1	–
		B16	2012–2014	H	0	1	B02/SDL$_{2015}$-103	G1	G1	–
		B18	2012–2014	H	0	1	B16/G$_{1999}$-48	G1	G1	–
		B19	2012–2014	H	0	1	*	G1	G1	–
		C08	2012–2014	H	0	1	B16/G$_{1999}$-48	–	G1	–
		C10	2012–2014	H	0	1	B02/SDL$_{2012}$-17	–	G1	–
		C13	2012–2014	H	0	1	B16/G$_{1999}$-48	G1	G1	–
		C20	2012–2014	H	0	1	*	G1	G1	–
		A02	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		A11	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		A14	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		A18	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		B03	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		B11	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		B12	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		B13	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		C07	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1
		C19	2012–2014	M	1	1	A17/G$_{1999}$-48	–	Compatible with G1 & G2	Compatible with G1

The compatibility with the pair of testers belonging to SI group G1 or G2 was assessed using _F. ornus_ in intraspecific stigma tests and/or _Phillyrea angustifolia_ in interspecific stigma tests.
Sex, sexual phenotype of the sampled tree; H, hermaphrodite; M, male; 0, incompatibility observed; 1, compatibility observed; (–), not tested; SI/SC, self-incompatibility/self-compatibility observed.
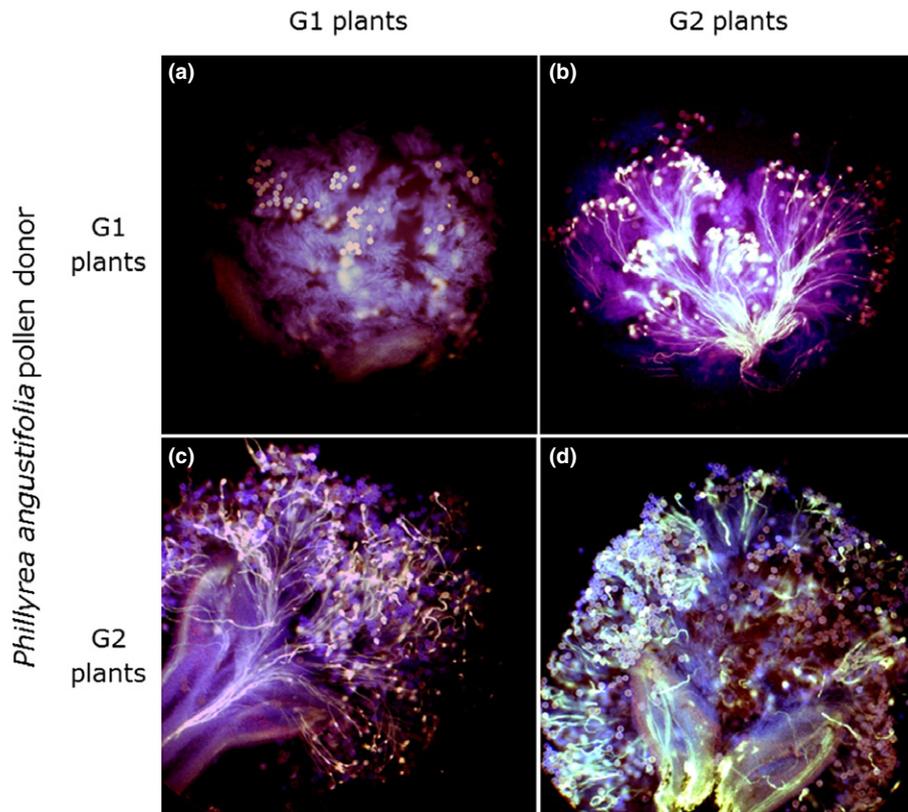
Fig. 2 Transgeneric conservation of the self-incompatibility reaction between *Phillyrea angustifolia* and *Fraxinus ornus*. Pollen from *P. angustifolia* can germinate on the stigmas of *F. ornus* according to their respective incompatibility groups. Pollen from hermaphrodites classified as G1 in *P. angustifolia* (Saumitou-Laprade *et al.*, 2010) fails to germinate on stigmas from the *F. ornus* A17 hermaphrodite (a) (and all of its incompatible mates) scored as G1 in the present study, but germinates on the G_{1999}-48 hermaphrodite (b) (and all of its incompatible mates) scored as G2. Symmetrical results are observed with pollen from *P. angustifolia* hermaphrodites classified as G2 that germinate on stigmas from A17 (c), but not on stigmas from hermaphrodites classified as G2 (d).

tester, but compatible with a G2 tester. We scored these hermaphrodites as belonging to the G1 incompatibility group. The six remaining hermaphrodites were incompatible with G2 testers, but compatible with G1, and therefore scored as belonging to the G2 incompatibility group. No hermaphrodites tested in this study appeared to be compatible with both groups of testers. We concluded that, in the tested *F. ornus* sample, two and only two incompatibility groups are present.

A perfect match of SI assignment in intra- and interspecific stigma tests

Among the 53 *F. ornus* hermaphrodites phenotyped in this study, 18 were tested simultaneously with pairs of testers belonging to *F. ornus* and *P. angustifolia*. All intra- and interspecific stigma tests assigned *F. ornus* hermaphrodites to one and only one of the two SI groups (Table 1), demonstrating full conservation of the compatibility/incompatibility specificities across these two divergent species.

Males are compatible with hermaphrodites of both SI groups

We performed intraspecific stigma tests with 12 different males (Table 1). Among them, two were also phenotyped using interspecific stigma tests. For every tested male, pollen tubes were observed in the stigmas of both groups of testers, indicating compatibility with both SI groups of hermaphrodites. Three

additional male genotypes were tested for compatibility with a single group of testers (Table S1); in all cases, compatibility was observed.

Contrast between the natural *F. ornus* population and the two naturalized populations

The three populations investigated in this study included both males and hermaphrodites. We did not estimate their respective frequencies, which would require sex phenotyping over several years, because many individuals do not flower in any given year (Dommée *et al.*, 1999; Verdu *et al.*, 2004, 2006). Our study revealed a difference between the natural and naturalized populations (Table 1). In the natural Gourdon population, we scored 27 hermaphrodites as G1 and six as G2. By contrast, the 20 hermaphrodites from the naturalized Vis Valley population phenotyped using a full stigma test belonged to the G1 incompatibility group (Table 1). Because they belong to the same incompatibility group, these hermaphrodites cannot fertilize each other. The stigma test nevertheless showed that these hermaphrodites have viable pollen (see Table 1). Therefore, in the naturalized Vis Valley population, the hermaphrodites have functional pollen that is useless as a result of incompatibility; they behave functionally as females that require the pollen from males to produce seeds. This apparently androdioecious population is therefore a cryptic dioecious population. An additional 22 hermaphrodites from Vis Valley and 10 hermaphrodites from Lez Valley were phenotyped using their pollen on a G1 tester

only (in a so-called 'partial' stigma test). Pollen from all 32 hermaphrodites failed to germinate on the stigma of the tester; however, because the viability of their pollen was not assessed on a compatible tester, we concluded that the phenotype of these hermaphrodites was G1 or male-sterile (Table S1). Regardless of the viability of the pollen they produce, the Vis and Lez Valley hermaphrodites tested cannot sire seeds on the other hermaphrodites found at their respective sites, and their ovules therefore can be fertilized only by males.

Discussion

Intra- and interspecific stigma tests convincingly showed that *F. ornus* possesses the same unusual incompatibility system recently discovered in *P. angustifolia*. In both species, hermaphrodites belong exclusively to one of two SI groups and males can successfully pollinate all hermaphrodites. Thus, the SI system, together with its two specific SI mating types and the full compatibility of males, has been conserved in both species. This conservation of DSI in both species indicates that symplesiomorphy (conserved, ancestral trait) is more likely than homoplasy (trait shared by virtue of recurrent evolution) in the history of DSI in Oleaceae.

The conservation of DSI has three major consequences for mating system evolution in the Oleaceae. First, it indicates that DSI has been maintained over a long period of time. The DSI system discovered in *Phillyrea* was also detected in *F. ornus*, an Oleaceae lineage that diverged > 40 Mya from the Oleinae branch that contains *Phillyrea* (Besnard *et al.*, 2009). The shared DSI attests to the stability of the homomorphic DSI system, which is probably found in other species descending from the common ancestor of the Oleinae and Fraxininae subtribes (Wallander & Albert, 2000). Nevertheless, the maintenance of the homomorphic DSI system for over 40 Myr is unexpected, given the susceptibility of SI systems to the invasion of new SI alleles (Charlesworth & Charlesworth, 1979a; Uyenoyama *et al.*, 2001; Gervais *et al.*, 2011) and to the invasion of self-compatible mutants (Charlesworth & Charlesworth, 1979a,b). One possible explanation is the occurrence of molecular constraints that impede the formation of new incompatibility alleles. However, recent theoretical investigations have suggested that androdioecy and DSI help to maintain each other: DSI facilitates the maintenance of males (Pannell & Korbecka, 2010; Husse *et al.*, 2013; Billiard *et al.*, 2015) and the full compatibility of males hinders the invasion of self-compatible mutants. Under DSI, males and hermaphrodites have the same number of potential mating partners, and thus identical male fitness, but males – unlike selfing hermaphrodites – do not suffer from inbreeding depression (Van de Paer *et al.*, 2015). In addition, the full compatibility of males restricts the number of SI alleles to two, assuming that the emergence of new SI alleles requires at least two successive steps (Uyenoyama *et al.*, 2001; Gervais *et al.*, 2011): (1) the invasion of a self-compatible mutation affecting the genes involved in the male component of the SI recognition mechanism; (2) a second compensatory mutation affecting the female component, restoring SI recognition. Because the fully compatible males prevent

the invasion of self-compatible mutants (see earlier), the first step cannot be achieved, thus hindering the emergence of new SI alleles (Van de Paer *et al.*, 2015). The co-occurrence of DSI and androdioecy may explain their evolutionary persistence. Testing for the effect of androdioecy on the maintenance of DSI is possible in Oleaceae. Possible future investigations include the assessment of DSI in hermaphroditic lineages, such as the subgenus *Olea*, which contains only hermaphrodite species and has diverged > 30 Mya from the lineage containing androdioecious taxa, such as *Osmanthus* and *Phillyrea* (Besnard *et al.*, 2009).

Second, the conservation of DSI in the Oleaceae also explains stable androdioecy with high male frequencies in *F. ornus*. In *P. angustifolia* and *F. ornus*, the reproductive disadvantage that males face as a result of the loss of female function is offset by their full compatibility with all hermaphrodites. Phenotypic (Pannell & Korbecka, 2010) and genotypic (Husse *et al.*, 2013) theoretical models have shown that DSI allows the maintenance of high male frequencies in natural populations despite a weak or even non-existent male fitness advantage (Billiard *et al.*, 2015). The presence of DSI revealed in the current study provides a satisfactory explanation for the high male frequencies and hermaphrodites with viable pollen in natural *F. ornus* populations, as demonstrated using paternity analyses (Verdu *et al.*, 2006). *Fraxinus ornus* is a true functionally androdioecious species in which high stable male frequencies are expected, as demonstrated previously in *P. angustifolia* (Pannell & Korbecka, 2010; Husse *et al.*, 2013; Billiard *et al.*, 2015).

Third, stable DSI may have driven several transitions from hermaphroditism to dioecy in *Fraxinus*. The occurrence of DSI in Oleaceae, combined with the way in which it favors the selection of the female-sterility mutation, may be the key to understanding the pervasiveness of morphological androdioecy in Oleaceae (10 species in the Fraxininae subtribe and 27 in the Oleinae subtribe) (Wallander, 2001a). *Fraxinus* includes 48 species, both wind and insect pollinated (Wallander, 2013), and their breeding systems range from hermaphroditism to dioecy, including numerous intermediates, such as androdioecy and trioecy (co-occurrence of males, hermaphrodites and females). The mapping of sexual morphological traits on phylogenies (Wallander, 2001b, 2008; Hinsinger *et al.*, 2013) suggests that dioecy may have evolved three times independently within the genus: twice via trioecy (or polygamy as mentioned by Wallander (2008)) in sections *Fraxinus* and *Melioides*, and once via androdioecy in the section *Ornus* (Wallander, 2008). This latter section includes 16 species (three hermaphrodite, 11 androdioecious and two dioecious), among which dioecy appears to have evolved from hermaphroditism via androdioecy (Wallander, 2013). To date, a transition from androdioecy to dioecy is very unlikely because of the high constraints on androdioecy. However, when associated with the DSI system, androdioecy under nuclear sex determination can evolve as easily as gynodioecy under nucleo-cytoplasmic genetic control (Husse *et al.*, 2013; Pannell & Voillemot, 2015); both cases require only a slight resource reallocation to the remaining sexual function for the evolution of unisexual individuals. Therefore, if shared among Oleaceae species, DSI can explain the extraordinarily high frequencies of

androdioecious species reported in the family (Wallander, 2001b), and the section *Ornus* represents a good model to study if the transition from hermaphroditism to dioecy via androdioecy has occurred.

Both incompatibility groups were detected among the hermaphrodites in the natural population in Loup Valley. These hermaphrodites thus probably all have the potential to reproduce through their male function, making this population functionally androdioecious. Nevertheless, the observed frequencies of G1 and G2 (82% and 18%, respectively) were significantly different from the frequencies predicted by models: 50% G1 in the absence of distortion and any male advantage (Husse *et al.*, 2013), and between 20% and 40% with distortion (Billiard *et al.*, 2015) and a moderate male advantage. This discrepancy suggests that the studied population may not have reached the equilibrium predicted by theory. The Vis and Lez Valley sites appear to lack one of the two SI groups. Under the hypothesis of an expected minimum frequency of 20% for the G2 hermaphrodites (as observed in Loup Valley), the probability of observing no G2 hermaphrodites in our sample is 1.2% ($n = 20$) for Vis Valley and 10.7% ($n = 10$) for Lez Valley. Therefore, the most likely conclusion is that there are no G2 hermaphrodites at these two sites and they therefore lack one of the two SI groups. In this case, the Vis and Lez Valley hermaphrodites act solely as females. This system corresponds to one of the unstable equilibria predicted by theory (Husse *et al.*, 2013; Billiard *et al.*, 2015) when one SI allele is missing (e.g. caused by planting trees of a single SI group). Our results show that a functionally dioecious *F. ornus* population can persist and even expand (Thébaud & Debussche, 1991) when founded with only one S allele and isolated from (long-distance) dispersal and the introduction of the missing S allele. However, the century-old naturalized populations expanding from plantings outside the natural species distribution do not provide any clues as to how frequently this occurs in nature.

What can be said today about the origin of homomorphic DSI?

The homomorphic DSI of Oleaceae is old and stable, and may have driven the evolution of dioecy within the family either from distyly or from hermaphroditism. In general, distyly involves both a DSI system and reciprocal heterostyly. Phylogenetic analysis suggests that the ancestral state of Oleaceae is diploid and distylous (Taylor, 1943). Derived allopolyploid taxa, including the Oleinae and Fraxininae subtribes, to which *P. angustifolia* and *F. ornus*, respectively, belong, have undergone hybridization and genome doubling and are mainly homostylous (except for the genus *Shrebera* reported as heterostylous (Wallander & Albert, 2000)). DSI may have been conserved during the breakdown of heterostyly, following, for example, allopolyploidization or a change in the pollination system (Beach & Bawa, 1980). Alternatively, DSI may have arisen *de novo* in association with allopolyploidization. In any case, whether DSI in the heteromorphic diploid lineages is the same system as found in homomorphic allopolyploid taxa remains unknown. The few interspecific stigma tests performed using *Jasminum fruticans* (diploid and distylous) and *P. angustifolia* were unsuccessful (data not shown) and thus inconclusive. Molecular characterization of the incompatibility locus in both systems is a promising avenue of research to resolve the issue on the origin of homomorphic DSI.

Author contributions

All authors contributed extensively to the work presented in this article. P.V. and P.S-L. jointly designed and carried out the sampling design and phenotyping strategies with J.L. They performed phenotyping and analysis together with P.L. and S.B. B.D. and A.B. oversaw the germination and growth of the biological material. P.V., S.B. and P.S-L. wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Geographic distribution of *Fraxinus ornus* L. and localization of the three studied populations.

Table S1 *Fraxinus ornus* samples phenotyped for self-incompatibility (SI) group using only one of the two SI groups as tester

Methods S1 Two methods facilitating controlled stigma tests: pollen freezing at −80°C and compatibility tests on agar medium.

Notes S1 History of *Fraxinus ornus* introductions along the Vis and Lez Valleys.

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