Research

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Multiple pre- and postzygotic components of reproductive isolation between two co-occurring *Lysimachia* species

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Summary

- Genetic divergence between species depends on reproductive isolation (RI) due to traits that reduce interspecific mating (prezygotic isolation) or are due to reduced hybrid fitness (postzygotic isolation). Previous research found that prezygotic barriers tend to be stronger than postzygotic barriers, but most studies are based on the evaluation of F_1 hybrid fitness in early life cycle stages.
- We combined field and experimental data to determine the strength of 17 prezygotic and postzygotic reproductive barriers between two *Lysimachia* species that often co-occur and share pollinators. We assessed postzygotic barriers up to F_2 hybrids and backcrosses.
- The two species showed near complete RI due to the cumulative effect of multiple barriers, with an uneven and asymmetric contribution to isolation. In allopatry, prezygotic barriers contributed more to reduce gene flow than postzygotic barriers, but their contributions were more similar in sympatry.
- The strength of postzygotic RI was up to three times lower for F_1 progeny than for F_2 or backcrossed progenies, and RI was only complete when late F_1 stages and either F_2 or backcrosses were accounted for. Our results thus suggest that the relative strength of postzygotic RI may be underestimated when its effects on late stages of the life cycle are disregarded.

Introduction

Speciation is the result of reproductive barriers that progressively reduce gene flow between divergent lineages, until it is completely interrupted (Coyne & Orr, 2004; Schemske, 2010; Singh, 2022). A fundamental goal in speciation studies is thus to identify and assess the relative contribution of multiple forms of reproductive isolation (RI) operating between pairs of diverging lineages (Ramsey *et al.*, 2003; Coyne & Orr, 2004; Martin & Willis, 2007; Lowry *et al.*, 2008). The strength of a barrier must be assessed in terms of the degree to which it reduces gene flow in the context of other barriers acting earlier in the plant life cycle (Ramsey *et al.*, 2003; Coyne & Orr, 2004; Sobel *et al.*, 2010). Overlooking unidentified barriers may therefore lead to an inaccurate view of a particular barrier's actual contribution (Lowry *et al.*, 2008; Sobel & Chen, 2014; Karrenberg *et al.*, 2019), yet there are remarkably few studies that assess the contributions of numerous barriers together (e.g. Kostyun & Moyle, 2017; Cahenzli *et al.*, 2018; Cuevas *et al.*, 2018; Christie & Strauss, 2019; Karrenberg *et al.*, 2019). In a recent review, Christie *et al.* (2022) found that, over the last 15 yr, isolating barriers were studied only in 89 species pairs, and no study considered more than eight barriers.

Traditionally, RI barriers have been divided into two main groups according to whether they operate before (prezygotic) or after (postzygotic) syngamy. For most species pairs, RI is the consequence of multiple prezygotic and/or postzygotic barriers acting sequentially (Ramsey *et al.*, 2003; Rieseberg & Willis, 2007; Ritchie, 2007; Lowry *et al.*, 2008; Widmer *et al.*, 2009; Palma-Silva *et al.*, 2011; Christie *et al.*, 2022). In plants, ecogeographical and phenological differences, floral isolation by pollinators, and pollen precedence (Mayr, 1942; Grant, 1981; Howard, 1999) constitute important prezygotic barriers, while fruit or seed production, lower hybrid viability or fertility, and ecological inferiority of hybrids are important postzygotic barriers (Dobzhansky, 1937; Mayr, 1942; Rundle *et al.*, 2000; Schluter, 2000). Both prezygotic and postzygotic barriers may depend on genotype–environment interactions (ecological or extrinsic barriers), while others can be attributed to intrinsic genetic incompatibilities (Seehausen *et al.*, 2014; Karrenberg *et al.*, 2019). Whereas the strength of prezygotic barriers may fluctuate over time if environmental conditions change (Wellenreuther

et al., 2010; Ortego *et al.*, 2017), potentially reversing the speciation process (Grabenstein & Taylor, 2018) or giving rise to species assimilation through hybridization (Seehausen *et al.*, 2008), the evolution of postzygotic RI through genetic incompatibilities is generally irreversible (Sobel *et al.*, 2010) such that species identity is maintained in hybrid zones (Seehausen *et al.*, 2008).

A recurring question concerns the relative contributions made by extrinsic prezygotic vs intrinsic postzygotic barriers to RI and the maintenance of species integrity (Ramsey *et al.*, 2003; Carrió & Güemes, 2014; Cahenzli *et al.*, 2018; Christie & Strauss, 2018). To date, the most comprehensive reviews seem to support the view that prezygotic barriers contribute more to reducing gene flow than postzygotic barriers (Lowry *et al.*, 2008; Baack *et al.*, 2015; Christie *et al.*, 2022), but studies have assessed postzygotic RI only on the basis of F_1 hybrid fitness at early stages of the life cycle, and we know little about the fitness of F_2 hybrids and backcrosses (Carrió & Güemes, 2014; Karrenberg *et al.*, 2019; Christie *et al.*, 2022). In F_1 hybrids, the impact of isolating barriers is often highly variable, ranging from negative to positive values (Christie *et al.*, 2022). Hybrid progeny often shows hybrid vigour in early stages of the life cycle but reduced fertility due to hybrid breakdown in later stages (Rieseberg & Carney, 1998; Jiang *et al.*, 2000; Fishman & Willis, 2001; Carrió & Güemes, 2014; Fraïsse *et al.*, 2016). The relative absence of research of the fitness of F_2 hybrids and backcross hybrids is all the more notable, given that Dobzhansky–Muller incompatibilities are considered the main genetic source of low hybrid fitness (Coyne & Orr, 2004; Stacy *et al.*, 2017) and are expected to become manifest only as a result of recombination between divergent genomes (Fishman & Willis, 2001; Coyne & Orr, 2004; Gavrilets, 2004; Scopece *et al.*, 2010). Estimates of RI that rely solely on studies of early hybrid stages may lead to an overestimate of the strength of ecological prezygotic relative to postzygotic barriers (Schemske, 2010; Christie *et al.*, 2022), potentially overestimating the incidence in reversibility in the speciation process (Ortego *et al.*, 2017; Grabenstein & Taylor, 2018). It is therefore critical to include late intrinsic postzygotic barriers in studies of RI to obtain an accurate picture of the factors that maintain species integrity.

Here, we assess the strength of 17 potentially pre- and postzygotic reproductive isolating barriers between two species of the plant genus *Lysimachia*. Until recently, the two species were considered to be different flower colour morphs of the single species *Lysimachia arvensis*, one with blue and the other with orange flowers, but patterns of sequence divergence at the internal transcribed spacer (ITS) locus, suggest that they are separate species, and they have been described as *L. arvensis* (the former orange morph) and *Lysimachia loeflingii* (the former blue morph; Jiménez-López *et al.*, 2022). The two species differ in their geographic distribution, although with areas of sympatry in the Mediterranean Basin (Arista *et al.*, 2013). They also show subtle differences in flowering phenology, with substantial overlap (Jiménez-López *et al.*, 2020b). In sympatric populations, the same solitary bee species visit the flowers of both species, potentially facilitative gene flow (Ortiz *et al.*, 2015; Jiménez-López *et al.*, 2020a). Hand crosses between both species produce viable F_1 progeny with salmon-coloured flowers, but hybrids are rarely observed in the wild (Jiménez-López *et al.*, 2020a). To understand the basis of RI between *L. arvensis* and *L. loeflingii*, we asked three main questions: (1) What is the relative strength of their prezygotic and postzygotic RI barriers? (2) What barriers act to maintain divergence in sympatry? (3) What is the relative contribution of postzygotic isolating barriers in the late stages of the life cycle after hybridization with respect to those most frequently studied in the early stages? Not only does our study cover a large number of isolating barriers, but it also goes further into the life cycle than most previous studies, making it important to better understand the processes that lead to divergence between species and that maintain species boundaries in the wild.

Materials and Methods

Study species

Lysimachia arvensis (L.) U. Manns and Anderb. and *L. loeflingii* F.J. Jiménez-López & M. Talavera (LA and LL hereafter) are annual forbs native to the Mediterranean Basin and Europe and naturalized throughout much of the rest of the world. They co-occur in the Mediterranean Basin, though LL predominates in sympatric populations. Phylogenetic analysis suggests that these species diverged over 2.5 million yr ago (Jiménez-López *et al.*, 2022). Both species are hermaphrodite and tetraploid ($2x = 40$). Their flowers exhibit lateral and vertical herkogamy, but due to differences in herkogamous traits, all LA plants allow delayed selfing when pollinators are scarce, while some LL plants are susceptible to competing selfing throughout flower anthesis and others are incapable of autonomous selfing (Jiménez-López *et al.*, 2019, 2020c). In sympatry, there is a marked preference of pollinators for LL flowers (Ortiz *et al.*, 2015; Jiménez-López *et al.*, 2020a). Mating system analysis based on microsatellite markers indicates that LA has a higher selfing rate, probably due to low pollinator visitation and delayed selfing (Jiménez-López *et al.*, 2020b).

Assessment of reproductive isolating barriers

We studied five prezygotic RI barriers and 12 postzygotic RI barriers, nine in F_1 and three in F_2 and backcrosses. To measure the overall degree of RI, we used the unified RI indices as described by Sobel & Chen (2014) and Sobel & Streisfeld (2015), which are directly related to gene flow and take values that range from −1 (all matings are interspecific) to 1 (no interspecific mating), with zero indicating random mating. Because RI indices for different isolating barriers are calculated on an equivalent basis, we could also combine the values for the component barriers to produce an index of cumulative RI. As RI barriers are often asymmetric between species (Tiffin *et al.*, 2001; Martin & Willis, 2007) and parents (Sobel & Chen, 2014; Sobel & Streisfeld, 2015), the RI index for each barrier was calculated separately for each species and parent (i.e. specifying which species was the sire and dam) and, depending on the nature of each barrier, a different equation was applied (see Supporting Information Table S1;

Sobel & Chen, 2014). We estimate 95% confidence intervals of RI indices for all barriers to assess their significance and excluded those with confidence intervals that overlapped zero from our calculation of cumulative RI (see Karrenberg *et al.*, 2019).

Geographic isolation and differentiation of climatic niches

To assess the importance of ecogeographic isolation, environmental niche models (ENM) were calculated for each species to assess its potential distributions under current bioclimatic conditions. The presence of each species was recorded for 547 and 558 LA and LL locations, respectively. Locality data were collected from our own records (e.g. Arista *et al.*, 2013; Jiménez-López *et al.*, 2020c), as well as GBIF occurrences (http://www.gbif.org/) when it was possible to verify flower colour. A set of the 19 layers of bioclimatic variables (Hijmans *et al.*, 2005) was obtained from WorldClim (www.worldclim.org), with a resolution of 30 s. Using DIVA-GIS 7.5.0 (Hijmans *et al.*, 2001), a section of each layer was used, including the Mediterranean region and adjacent areas where the presence of the species had been described.

We selected only uncorrelated bioclimatic variables contributing significantly to the model, based on the Jack-knife index (Table S2). The maximum entropy algorithm implemented in MAXENT v.3.4.1 (Phillips *et al.*, 2016) evaluated the potential distribution of each species using ENM. To do this, the presence data were randomly divided into two sets: training data were used to build the model (75%), and testing data were used to test accuracy (25%). For each species, we included 200 replicates per run, with 500 iterations, 10^4 background points, and using default options.

Isolation by asynchrony in seasonal flowering phenology

We evaluated isolation due to asynchrony in the seasonal flowering phenology of each species in 11 sympatric natural populations in the southern Iberian Peninsula. These populations were selected to encompass most of the variability in elevation and in environments typically occupied by both species (Table S3). In each population, we conducted a weekly census throughout the flowering period. Because the populations were typically small, we were able to count the total number of open flowers of each species in each population. Flowers of both species have a similar life span (3 d), and we thus assumed that all flowers had the same probability of crossing.

Isolation by asynchrony in daily patterns of flower anthesis

To estimate the strength of the isolation barrier due to asynchrony in flower opening, we studied the daily pattern of flower anthesis of each species at three populations close together where the two species co-occurred (ESP_H_Hin, ESP_SE_St, ESP_SE_DH; Table S3). At each site, floral anthesis was recorded for 10 d during peak flowering. At dawn of each day, 100 floral buds of each species were randomly labelled, and the number of open flowers was recorded every hour between 08:00 and 20:00 h. In each population, data recorded over 10 d were averaged to calculate the mean pattern of flower anthesis for each species.

Pollinator-mediated isolation

Pollinator-mediated pollen flow within and between species was evaluated in the same three natural sympatric populations for which daily patterns of flower anthesis were studied. We established experimental plots with different proportions of species by thinning the plants of each population to have the required mix of proportions for each species. Three types of plots were constructed in each population: 'balanced', with the same proportion of flowers of both species; 'LL-biased', with 80% LL and 20% LA flowers; and 'LA-biased', with 20% LL and 80% LA. Each experimental plot consisted of 12–20 plants, with a total of 200 flowers of both species intermingled. For each population and plot type, pollinator activity was recorded for 15-min censuses from 09:00 h to 14:00 h on 1–5 sunny days, accumulating a total of between 5 and 10 observation hours. During each census, we recorded the sequence of flowers visited by each pollinator; in all, we recorded 4003 flower-to-flower transitions (LL–LL, LA–LA, LL–LA and LA-LL).

Isolation by pollen precedence

Seeds from the three sympatric populations mentioned above were collected and combined in approximately equal proportions. Plants grown in the glasshouse of this seed pool were used to study both pollen precedence and postzygotic barriers (see below).

To assess the role of pollen precedence as a prezygotic barrier, we conducted controlled pollinations on flowers of 27 LL and 32 LA plants randomly separated into three experimental groups. The flowers were emasculated before anthesis, avoiding the deposition of any self-pollen on the stigmas. As both species produce the same amount of pollen (Arista *et al.*, 2013), equal numbers of anthers from each species were placed in microtubes and sonicated to extract equal amounts of pollen. Then, on their first day of anthesis, pollen from this 1 : 1 pollen mix was deposited on the stigmas until saturation. The hand-pollinated flowers were left to set fruits, their seeds were sown, and the resulting plants (823 in total) were grown in the glasshouse until flowering to check for flower colour. We interpreted progeny with a flower colour similar to that of their maternal parents as the result of intraspecies fertilization, and progeny with salmon-coloured flowers as the result of interspecies fertilization (Jiménez-López *et al.*, 2020a). In the absence of pollen precedence, flowers hand-pollinated with a 1 : 1 pollen mix are expected to produce a similar number of both types of offspring.

Postzygotic isolating barriers

To assess postzygotic barriers acting in the F_1 hybrids, we compared fitness components throughout the life cycle of progeny from intraspecific and interspecific crosses produced by hand-pollination of glasshouse-grown plants from the above-mentioned mixed seed pool. The following fitness components were considered: fruit-set of maternal parents; seeds per fruit of maternal parents; germination of seeds; survival until flowering

of seedlings; pollen grains and ovules per flower of daughter plants; fertility of daughter plants (proportion of fertile plants); fruit-set of fertile plants; and seeds per fruit of fertile plants. We carried out hand-pollinations on flowers, previously emasculated, from 39 LL to 65 LA plants grown in the glasshouse and randomly separated into four experimental groups, each consisting of nine to 17 plants per species. In total, we performed 71 interspecies crosses (32 LL×LA; 39 LA×LL) and 113 intraspecies crosses (58 LL×LL; 55 LA×LA), producing four offspring classes (F_1 hybrids from LA maternal parent, F_1 hybrids from LL maternal parent, pure LL and pure LA; Fig. S1). Fruit-set was 100% in all these crosses (fruit-set by maternal parents); ripe fruits were collected and the number of seeds per fruit was counted (seeds set by maternal parents).

To assess germination and survival under natural conditions, seeds from these crosses (376 F_1 hybrids from LL maternal parents, 387 F_1 hybrids from LA maternal parents, 767 pure LL and 768 pure LA) were sown in pots, and those from each experimental group were arranged separately in the field close to one of the origin populations (ESP_SE_DH, Table S3). The pots were surveyed twice a week to control germination and seedling survival until flowering; pollen grains and ovules per flower were quantified. Some of these plants from each experimental group (51 pure LL, 33 F_1 from LL maternal parents, 33 F_1 from LA maternal parents and 62 pure LA) were moved to the glasshouse to carry out controlled crosses preventing pollinator visits. As all these plants were fertile, we then performed hand crosses between plants within each of the four offspring classes from each experimental group to assess the fruit-set and seeds per fruit (Fig. S1). As both the fruit-set by maternal parents and the fertility of daughter plants were 100% for all cross types, we concluded that RI due to any of the associated fitness components was zero and did not consider these further.

To assess the strength of barriers in the F_2 and backcrosses, controlled crosses were carried out in the glasshouse on 179 daughter plants: 51 pure LL, 33 F_1 from LL maternal parent, 33 F_1 from LA maternal parent and 62 pure LA; again, these plants were randomly separated into four experimental groups. Seven classes of crosses between these plants were considered: both parents being pure LL; both parents being pure LA; both parents being F_1; backcrosses with LL (F_1×LL and LL×F_1); and backcrosses with LA (F_1×LA and LA×F_1; Fig. S1). Between 164 and 893 plants from each of these cross classes were grown in the glasshouse, and controlled crosses were performed within each class to assess the proportion of fertile plants as well as fruit-set and seeds per fruit of the fertile plants. The barrier strength due to each fitness component was then calculated by comparing the fitness components of F_2 or backcrossed plants with those of the pure LL or LA plants.

Cumulative RI and prezygotic vs postzygotic RI

We calculate cumulative RI by all barriers acting sequentially using the Excel sheet provided by Sobel & Chen (2014) in their supplementary material (evo12362-sup-0003). This sheet was used for each species/parent combination separately, alternatively considering backcrosses or F_2. RI barriers due to the ovule production of daughter plants were not considered, because these would be redundant with RI barriers due to seeds set by those plants (as the second component directly depends on the first). We estimate total RI and the relative contribution of each barrier by combining all RI barriers studied (Table S4) and without considering the geographical barrier (as gene flow would occur mainly in sympatry; Table S5) (Runquist *et al.*, 2014; Sobel & Chen, 2014; Sobel & Streisfeld, 2015). Furthermore, we calculated total prezygotic RI, sympatric prezygotic RI (excluding the geographical barrier) and total postzygotic RI (without the prezygotic barriers), as well as total postzygotic RI on the basis of F_1, F_2 and backcross progeny.

Statistical analysis

Data were analysed using generalized linear models (GLMs), with link functions and error distributions appropriate for the type of variable response modelled (see Table S6). All analyses were performed using the SPSS GLM module (IBM SPSS Statistic 25, 2017, USA) with a Type III test. When the GLM showed significant differences, the means of each treatment were compared using *t*-tests based on the standard errors calculated from the specific models. In addition, chi-square tests were applied to pollinator transitions to assess the significance of deviations of observed frequencies from expectation.

Results

Prezygotic barriers

Based on the jack-knife index of the ENM for both combined species, eight environmental variables per species were selected to estimate the distributional area of each species (Table S2). Four variables were the same for both species and were related to temperature and precipitation. All projections showed excellent predictive success, with values > 0.9 under the curve (AUC). The ENM for the current environmental conditions was highly consistent with the current distribution of each species. The potential contact area was predicted in 37.14% of the distributional area, with the probability of co-occurrence of both species being higher in the Iberian Peninsula and the Mediterranean Basin than elsewhere in Europe. LA had a wider projected area than LL, mainly in central Europe (Fig. 1), and the area projected for LL was highly associated with the Mediterranean Basin. LL occurs in 62.9% of the combined distribution of both species, sharing 74.54% of its area with LA, while LA shared only 42.5% of its area with LL (Fig. 1). This unequal overlap resulted in an asymmetric component of isolation by geographic distribution, with a value of 0.255 for LL and 0.575 for LA (Fig. 2; Table S4).

Regarding the other prezygotic barriers measured, significant differences were observed for the beginning and range of flowering times, the daily patterns of flower anthesis, deviations between observed and expected transitions, and the effect of pollen precedence for several effects (Table 1). Specifically, nine of the 11 populations differed in the beginning of flowering

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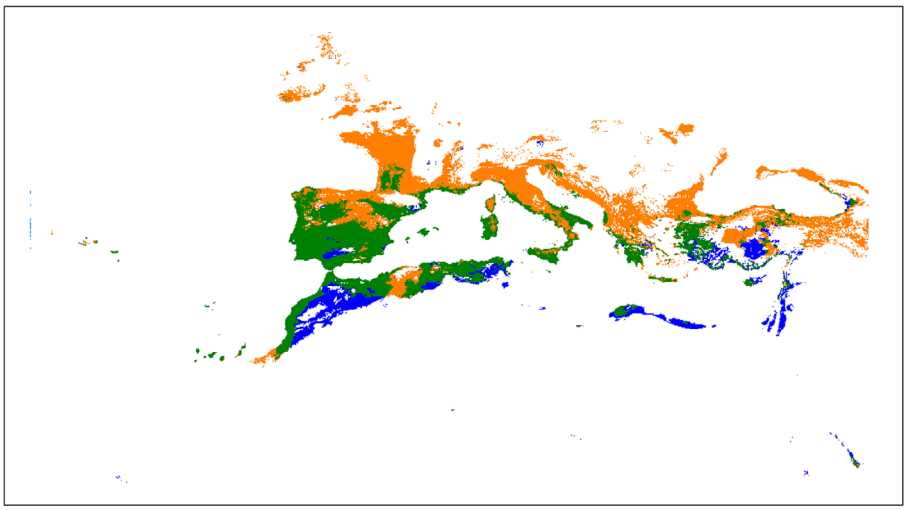

Fig. 1 Allopatric range of distribution of *Lysimachia loeflingii* (blue) and *L. arvensis* (orange) and range of overlap between both species (green) according to environmental niche models (ENM) by MaxEnt. Bioclimatic variables used in this model are shown in Supporting Information Table S2.

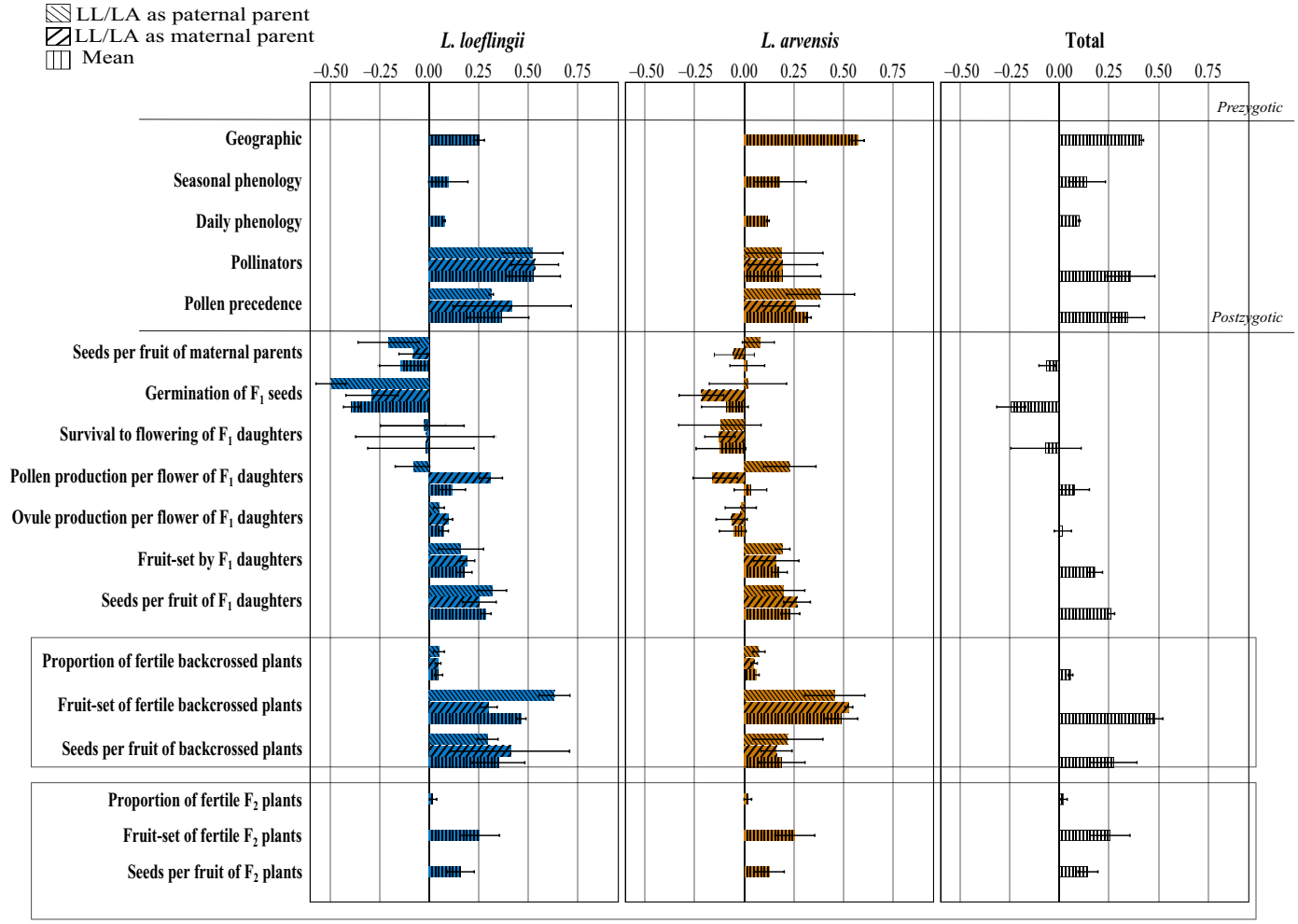

Fig. 2 Individual strength and 95% confidence intervals of pre- and postzygotic reproductive isolation (RI) barriers between *Lysimachia loeflingii* and *L. arvensis* calculated after Sobel & Chen (2014). Isolation of *L. loeflingii* (from *L. arvensis*) is shown in blue, while isolation of *L. arvensis* (from *L. loeflingii*) is shown in orange. Mean isolation between the two species is shown in grey. RI barriers for each species when acting as paternal parent (pollen donor) or maternal parent (pollen receptor) are shown separately, as well as the mean between parents. Postzygotic barriers at the F_2 were calculated in two parallel ways that are shown separately, considering either F_2 crosses or backcrosses.

between species, with LL being earlier. The flowering period ranged from March to July (Fig. S2), with LL showing a significantly longer period (mean ± SD, 75.6 ± 6.02 d for LL; 57.3 ± 17.0 d for LA). The strength of the RI barrier due to asynchrony in seasonal flowering phenology had a mean value of 0.139, the value for LA being almost twice that for LL (Fig. 2; Table S4). On a daily basis, flowers of LL always opened earlier and closed later than those of LA (Fig. S3). Consequently, the period of LA anthesis was always included within that of LL; flowers of LL were open for a mean of 10.3 h d^{-1} (±0.65 SE), while those of LA were open 8.17 h d^{-1} (±0.79 SE). The mean strength of the RI barrier due to asynchrony in daily flower anthesis was relatively low (0.0994), although it was higher for LA than for LL (Fig. 2; Table S4).

LA and LL shared the same pollinators, with bee species of the genera *Lasioglossum* and *Halictus* being the main visitors. From a total of 4003 pollinator transitions recorded, 3037 were intraspecific (2013 LL to LL and 1024 LA to LA; Fig. 3) and 966 were interspecific (490 LL to LA and 476 LA to LL). Transitions from LL to LL were significantly more frequent than expected in all three experimental-array types, while those from LA to LA were significantly more frequent than expected only in LL-biased stands; the opposite pattern occurred in balanced stands and was not different from the expected in LA-biased stands (Fig. 3). Interspecific transitions were always significantly less frequent than expected. The strength of isolation due to pollinator behaviour showed a very high mean value (0.360), with marked asymmetry between species (0.528 for LL vs 0.193 for LA), but was similar through male and female functions in each species (Fig. 2; Table S4).

In the pollen precedence experiment, the proportion of hybrid progeny sired by pollination with a 50 : 50 mixture of pollen from both species was 29 ± 2.1% (mean ± SE) for LL maternal parents and 38 ± 2.5% for LA maternal parents (Fig. S4).

Table 1 Summary of generalized linear model (GLM) results for different effects: species (*Lysimachia arvensis*/*L. loeflingii*), population, plot type, transition type, cross type or seed origin (see the Materials and Methods section for details).

	Effects	Wald-chi^2	df	*P*
Prezygotic barriers				
Beginning of flowering	Population	3483.818	10	**0.000**
	Species	1910.227	1	**0.000**
	Interaction	1031.273	10	**0.000**
Flowering period	Population	244 455	10	**0.000**
	Species	1854 727	1	**0.000**
	Interaction	1018 273	10	**0.000**
Daily pattern of flower anthesis	Population	37 355	2	**0.000**
	Species	2092.060	1	**0.000**
	Interaction	1.769	2	0.413
Deviation between observed and expected pollinator transitions	Population	4.343	2	0.114
	Transition type	38.826	3	**0.000**
	Plot type	4.008	2	0.135
	Interaction*	54.886	6	**0.000**
Pollen precedence	Species	6.680	1	**0.010**
Postzygotic barriers in F_1				
Seeds produced by maternal parents	Cross type	24.936	3	**0.000**
Germination	Origin	201.448	3	**0.000**
Survival	Origin	18.900	3	**0.000**
Pollen production	Origin	25.700	3	**0.000**
Ovule production	Origin	50.157	3	**0.000**
Fruit-set	Origin	176.000	3	**0.000**
Seeds per fruit	Origin	132.000	3	**0.000**
Postzygotic barriers in F_2				
Fertility	Origin	61.600	6	**0.000**
Fruit-set	Origin	317.517	6	**0.000**
Seeds per fruit	Origin	129.004	6	**0.000**

*Interaction for transition type and plot type (see the Materials and Methods section for details) in this barrier. Significant *P*-values are in bold.

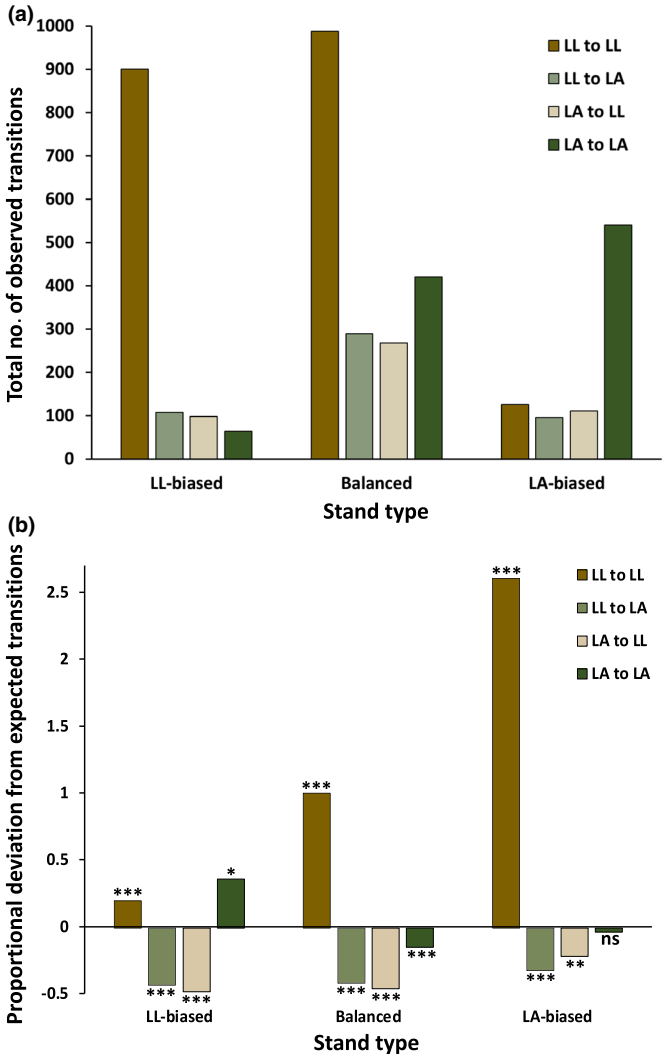

Fig. 3 (a) Pollinator transitions observed among *Lysimachia loeflingii* (LL) and *L. arvensis* (LA) in experimental arrays differing in proportions of flowers of each species (LL-biased 80 LL : 20 LA, balanced 50 LL : 50 LA and LA-biased 80 LA : 20 LL), placed in three natural mixed populations. (b) Proportional deviations of observed transitions from expected values according to colour proportions in these experimental arrays. Positive or negative values of bars indicate observed values higher or lower than expected, respectively; significance of deviations was determined by a chi-square test: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Consequently, the strength of the RI barrier by pollen precedence was slightly higher for LL, with a mean of 0.345 (Fig. 2). The strength of that barrier showed overall contrasting asymmetries and was higher for LL as maternal parents and for LA as paternal parents (Fig. 2; Table S4).

Postzygotic barriers in F_1

Postzygotic barriers manifest in F_1 progeny were heterogeneous for both species, ranging from negative to positive. For seeds produced by maternal parents, germination, survival, pollen and ovule production, fruit-set and seeds per fruit of daughter plants differed significantly according to their origin (Table 1). In general, LA maternal parents produced more seeds per fruit than LL, and in both cases seed number per fruit was slightly, but not significantly, higher after interspecific crosses (Fig. S5). The strength of RI due to this fitness component varied from positive to negative, depending on species and parent, with the confidence interval including zero in all cases except when LL was the paternal parents (Fig. 2; Table S4).

Higher germination rates were observed for F_1 seeds than for pure seeds (Fig. S5). RI due to differences in germination was negative in all cases (mean = −0.30), with important asymmetries between species and parents (Fig. 2; Table S4). Pure seedlings showed slightly lower survival than F_1 seedlings, although differences were significant only for pure LA seedlings (Fig. S5). RI due to the fitness component of the seedlings was negative in all cases (mean = −0.0698), but their confidence intervals typically included zero, except when LA was the maternal parent (Fig. 2; Table S4). Despite significant differences in pollen and ovule production, there was no apparent trend. RI due to either pollen or ovule production showed important asymmetries between species and parents, ranging from positive to negative (Fig. 2; Table S4). However, the mean values were positive for both fitness components (0.0752 and 0.0162 for pollen and ovule productions, respectively). Finally, pure plants produced significantly more fruits and seeds than F_1 plants (Fig. S5). RI due to fruit-set or seeds per fruit was always positive and significant, irrespective of species and cross direction, with mean values of 0.1774 and 0.2598, respectively (Fig. 2; Table S4).

Postzygotic barriers expressed in F_2 and backcrossed individuals

Unlike F_1, the postzygotic barriers in F_2 and backcrosses were always positive. Fertility, fruit-set and the number of seeds per fruit varied significantly among pure, F_2 and backcrossed plants (Table 1). While all pure plants were fertile, almost 4% of F_2 plants were totally sterile (they produced no fruit either as maternal or paternal parent), and between 7% and 14% of backcrossed plants showed total sterility (Fig. S6). Among fertile plants, both fruit-set and seeds per fruit showed the highest values for pure plants and the lowest values for backcrossed plants (Fig. S6).

RI attributed to the fitness components expressed by F_2 and backcrossed plants was always positive and significant, regardless of species and parent (although with some asymmetries); the strength of these barriers was always higher when considering backcrossed plants (Fig. 2; Table S4). The strongest RI barrier was observed in terms of fruit-set (mean value in backcrosses: 0.480) and the weakest in terms of plant fertility (mean value in backcrosses: 0.051; Fig. 2; Table S4).

Cumulative RI and prezygotic vs postzygotic RI

When accounting for the sequential action of multiple barriers, both species were almost completely isolated, with an RI ranging from 0.9496 when considering F_2 plants to 0.9783 when considering backcrossed plants (Fig. 4; Table S4). In sympatry, cumulative RI was also almost complete, ranging from 0.9097 (F_2) to 0.9606 (backcrosses) (Fig. 4; Table S5). Cumulative RI for LL was higher when individuals were the maternal parents and higher for LA when they were the paternal parents (Table 2). The barriers contributing most to total RI were prezygotic: geography, pollinator behaviour and pollen precedence (Fig. 4; Table S4). Among postzygotic barriers, reduced fruit and seed set in the F_1 and reduced fruit-set in the F_2 and backcrosses were the most effective in reducing gene flow (Fig. 4).

Most barriers were asymmetric between species (Table S4). Pollinators, geography and F_1 germination were the most asymmetrical barriers, while reduced fruit-set of F_1 plants was the least asymmetrical. Overall, the barriers were slightly stronger for LA, indicating a higher potential for gene flow from LA to LL.

When considering the total prezygotic and postzygotic barriers separately, the prezygotic barriers were stronger (0.8309) than the postzygotic barriers, which reached values of 0.5625 for F_2 progeny and 0.7875 for backcrossed progeny. These differences were more marked in LL (Fig. 5). In sympatry, however, the strength of the prezygotic barriers was still higher than that of the postzygotic barriers for F_2 progeny but lower than that for backcrossed progeny, although this difference was due to LA (Fig. 5). The barrier strength due to reduced F_1 progeny fitness was half that due to F_2 progeny and less than a third of that of backcrosses. These differences were much more marked for LL (Fig. 5).

Discussion

Although niche distribution modelling indicates that *Lysimachia arvensis* (LA) and *L. loeflingii* (LL) have somewhat divergent ecological niches, with LL preferring drier environments than LA (see also Arista *et al.*, 2013), we infer that the two species co-occur over 37% of their natural ranges. Given their overlapping flowering phenology, shared pollinators and fertile F_1 progeny, there would thus appear to be substantial opportunity for gene flow between the two species, yet hybrids are rare in the field. We thus asked what maintains the integrity of the two closely related species, despite ample apparent opportunity for gene flow between them. Our study has revealed numerous components of RI, helping to explain this puzzle. Below, we discuss the role played by prezygotic and postzygotic RI, in turn, and conclude by drawing attention to the value of assessing fitness components

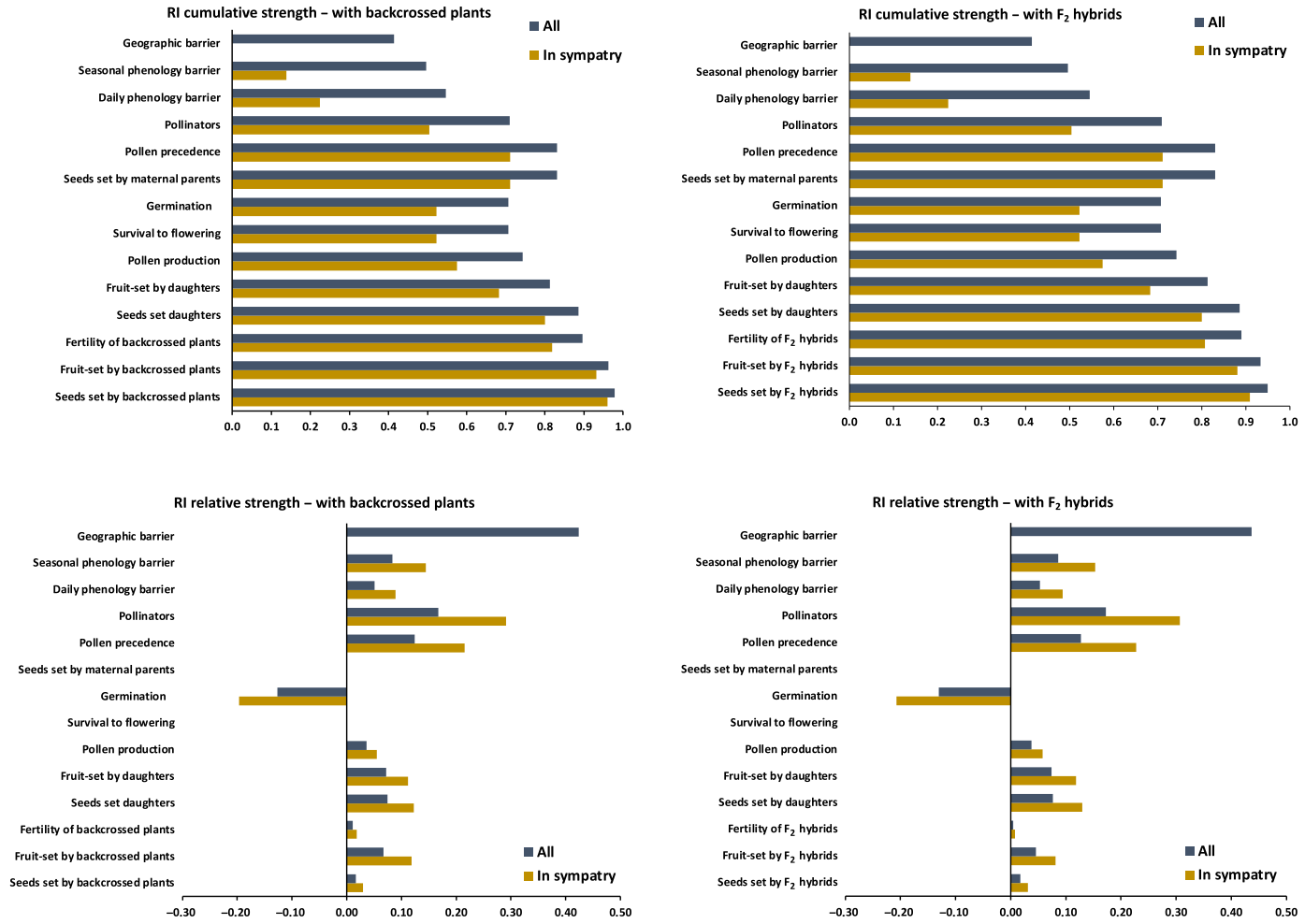

Fig. 4 Cumulative strength and relative contribution of reproductive isolation (RI) barriers between *Lysimachia loeflingii* and *L. arvensis*, considering either the fitness of progeny from backcrosses or F_2 crosses. Shown are RI considering all the barriers and RI excluding the geographical barrier (in sympatry).

in progeny beyond the F_1 and for both early and late stages of the plant life cycle.

Prezygotic RI barriers in sympatric populations

Our study has revealed evidence for multiple reproductive isolating barriers between sympatric populations of LL and LA, thus helping to explain these patterns. By assessing the strength of numerous barriers to gene flow in sympatric populations, we have been able to estimate the relative importance of prezygotic vs postzygotic RI between these two species. The low frequency of hybrid individuals in natural sympatric populations of LA and LL (Jiménez-López *et al.*, 2020a) contrasts with the high number of hybrids obtained by hand-pollinations, pointing to an important role for prezygotic RI barriers between the two species. These prezygotic barriers involved flowering phenology, pollen precedence and pollinator interactions, which together decreased the expected frequency of hybrid seed production by more than 71%. This value is in line with that obtained in the review by Christie *et al.* (2022), confirming the frequent importance of ecological barriers in maintaining species boundaries in plants.

In sympatry, all prezygotic barriers contributed to RI between the two species of our study. The flowering phenology showed wide variation among populations, but, in most cases, LL flowered earlier and had a markedly longer flowering period than LA. This phenological difference, which had previously been described for glasshouse-grown plants (Arista *et al.*, 2013; Jiménez-López *et al.*, 2020b), implies that, at the beginning of flowering, pollen flow occurs only among LL plants as a result of strongly assortative mating, as reported in other species (Franks & Weis, 2009). Assortative mating among LL flowers was also favoured by the longer period over which they remained open at the beginning and the end of the day. Taken together, these patterns suggest that LA was more temporally isolated from LL than vice versa. Differences in the opening pattern between *Lysimachia* species may be related to the petal colour of their flowers, as darker petals absorb long wavelengths more efficiently than lighter (Mu *et al.*, 2010) and may thus be warmer (Jewell *et al.*, 1994; Seymour, 2001; Seymour *et al.*, 2009). In fact, differences in opening and closing patterns due to distinct floral pigments are relatively frequent in species with floral colour polymorphism (Mølgaard, 1989; Mu *et al.*, 2010).

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Table 2 Cumulative strength and relative contribution values of each prezygotic and postzygotic reproductive isolation (RI) barrier for *Lysimachia loeflingii* and *L. arvensis*.

	L. loeflingii as maternal parents				*L. loeflingii* as paternal parents				*L. loeflingii* mean			
	Backcrosses		F_2 hybrids		Backcrosses		F_2 hybrids		Backcrosses		F_2 hybrids	
Barriers	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution
Geographic	0.2545	0.2582	0.2545	0.2618	0.2545	0.2643	0.2545	0.2922	0.2545	0.2613	0.2545	0.2723
Seasonal phenology	0.3272	0.0737	0.3272	0.0747	0.3272	0.0754	0.3272	0.0834	0.3272	0.0746	0.3272	0.0777
Daily phenology	0.3807	0.0543	0.3807	0.0550	0.3807	0.0556	0.3807	0.0614	0.3807	0.0549	0.3807	0.0572
Pollinators	0.7118	0.3358	0.7118	0.3405	0.7040	0.3356	0.7040	0.3710	0.7079	0.3359	0.7079	0.3500
Pollen precedence	0.8632	0.1536	0.8632	0.1557	0.8256	0.1263	0.8256	0.1397	0.8450	0.1408	0.8450	0.1467
Seeds per fruit of maternal parents	0.8632	0.0000	0.8632	0.0000	0.7411	−0.0877	0.7411	−0.0970	0.7977	−0.0486	0.7977	−0.0507
Germination of F_1 seeds	0.7227	−0.1425	0.7227	−0.1445	0.4467	−0.3057	0.4467	−0.3380	0.5786	−0.2249	0.5786	−0.2344
Survival to flowering of F_1 daughters	0.7227	0.0000	0.7227	0.0000	0.4467	0.0000	0.4467	0.0000	0.5786	0.0000	0.5786	0.0000
Pollen production of F_1 daughters	0.8443	0.1233	0.8443	0.1251	0.3819	−0.0672	0.3819	−0.0743	0.6514	0.0748	0.6514	0.0779
Fruit-set by F_1 daughters	0.8921	0.0485	0.8921	0.0491	0.5116	0.1347	0.5116	0.1489	0.7430	0.0940	0.7430	0.0979
Seeds per fruit of F_1 daughters	0.9345	0.0430	0.9345	0.0436	0.7139	0.2101	0.7139	0.2322	0.8489	0.1087	0.8489	0.1133
Fertility of F_2 plants	0.9387	0.0043	0.9369	0.0025	0.7330	0.0198	0.7235	0.0110	0.8590	0.0104	0.8543	0.0058
Fruit-set by F_2 plants	0.9666	0.0282	0.9621	0.0259	0.9331	0.2078	0.8262	0.1179	0.9463	0.0897	0.9110	0.0606
Seeds per fruit of F_2 plants	0.9860	0.0197	0.9724	0.0106	0.9631	0.0312	0.8712	0.0516	0.9741	0.0285	0.9348	0.0255
Total RI	0.9860	1.0000	0.9724	1.0000	0.9631	1.0000	0.8712	1.0000	0.9741	1.0000	0.9348	1.0000

	L. arvensis as maternal parents				*L. arvensis* as paternal parents				*L. arvensis* mean			
	Backcrosses		F_2 hybrids		Backcrosses		F_2 hybrids		Backcrosses		F_2 hybrids	
Barriers	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution
Geographic	0.5747	0.6019	0.5747	0.6363	0.5747	0.5816	0.5747	0.5899	0.5747	0.5877	0.5747	0.6034
Seasonal phenology	0.6516	0.0805	0.6516	0.0851	0.6516	0.0778	0.6516	0.0789	0.6516	0.0786	0.6516	0.0807
Daily phenology	0.6932	0.0436	0.6932	0.0461	0.6932	0.0421	0.6932	0.0427	0.6932	0.0425	0.6932	0.0437
Pollinators	0.7529	0.0626	0.7529	0.0662	0.7515	0.0590	0.7515	0.0599	0.7522	0.0604	0.7522	0.0620
Pollen precedence	0.8263	0.0768	0.8263	0.0812	0.8576	0.1074	0.8576	0.1090	0.8421	0.0919	0.8421	0.0944
Seeds per fruit of maternal parents	0.8263	0.0000	0.8263	0.0000	0.8576	0.0000	0.8576	0.0000	0.8421	0.0000	0.8421	0.0000
Germination of F_1 seeds	0.7351	−0.0955	0.7351	−0.1010	0.8106	−0.0476	0.8106	−0.0483	0.7749	−0.0687	0.7749	−0.0705
Survival to flowering of F_1 daughters	0.6691	−0.0691	0.6691	−0.0730	0.8106	0.0000	0.8106	0.0000	0.7749	0.0000	0.7749	0.0000
Pollen production of F_1 daughters	0.5683	−0.1056	0.5683	−0.1116	0.8770	0.0672	0.8770	0.0682	0.7749	0.0000	0.7749	0.0000

Table 2 (Continued)

Barriers	*L. arvensis* as maternal parents				*L. arvensis* as paternal parents				*L. arvensis* mean			
	Backcrosses		F_2 hybrids		Backcrosses		F_2 hybrids		Backcrosses		F_2 hybrids	
	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution	Cumulative strength	Relative contribution
Fruit-set by F_1 daughters	0.6683	0.1047	0.6683	0.1107	0.9152	0.0387	0.9152	0.0392	0.8372	0.0637	0.8372	0.0654
Seeds per fruit of F_1 daughters	0.7934	0.1310	0.7934	0.1385	0.9426	0.0277	0.9426	0.0281	0.8955	0.0596	0.8955	0.0612
Fertility of F_2 plants	0.8114	0.0189	0.8006	0.0080	0.9507	0.0082	0.9448	0.0022	0.9075	0.0123	0.8994	0.0041
Fruit-set by F_2 plants	0.9377	0.1323	0.8767	0.0843	0.9813	0.0310	0.9669	0.0227	0.9676	0.0614	0.9391	0.0417
Seeds per fruit of F_2 plants	0.9547	0.0179	0.9031	0.0292	0.9880	0.0068	0.9742	0.0075	0.9779	0.0106	0.9524	0.0140
Total RI	0.9547	1.0000	0.9031	1.0000	0.9880	1.0000	0.9742	1.0000	0.9779	1.0000	0.9524	1.0000

For each species, values recorded for the respective species acting as maternal or paternal parents are presented, as well as the mean between cross directions, and two parallel calculations are shown (either considering backcrosses or F_2 hybrids).

Our results indicate that pollinator behaviour had the greatest impact on preventing gene flow between study species. When *Lasioglossum* and *Halictus* bees visit flowers of either species, they usually continued visiting flowers of the same species, ensuring strong floral constancy (Waser, 1986) and assortative mating. It has been suggested that the contrasting colours of LL and LA flowers allow pollinators to discriminate species and undoubtedly mediate floral constancy (Ortiz *et al.*, 2015; Jiménez-López *et al.*, 2020a). The role of pollinator preference as a component of RI was asymmetric between the two species, being more effective in isolating LL from LA than vice versa, due to the lower number of pollinator visits received by LA. The role of pollinators in isolating LL and LA is probably even stronger than measured in this study, as pollinators have also been shown to select against F_1 hybrids when they appear in populations (Jiménez-López *et al.*, 2020a), thus minimizing backcrossing with either of the parental species.

Pollen precedence also played an important role in isolating the two species of *Lysimachia*. When both types of pollen were deposited simultaneously on stigmas, heterospecific pollen showed reduced fertilization success than pollen from the same species, as has been found for other pairs of closely related species (Howard, 1999). LL produced a lower rate of hybrid offspring when acting as the maternal parent, indicating a lower competitive capacity particularly for LA pollen. Such asymmetry has also been found in *Iris fulva*/*I. brevicaulis*/*I. hexagone* (Carney *et al.*, 1996), *Centaurium littorale*/*C. erythraea* (Brys *et al.*, 2014) and *Mimulus guttatus*/*M. nasutus* (Martin & Willis, 2007). Interestingly, in both *Centaurium* and *Mimulus*, less competitive pollen was found for the species more prone to selfing, as was the case for LA (Jiménez-López *et al.*, 2020b).

Postzygotic RI barriers in sympatric populations

An important result of our study was the finding of heterotic effects of hybridization for early-stage components of fitness and deleterious effects at later stages of the life cycle. Importantly, the germination rate of hybrid F_1 seeds was markedly and consistently higher than that of pure seeds, interspecific crosses tended to yield more seeds than intraspecies ones, and hybrid seedling survival was greater than pure seedling survival. By contrast, the fitness components that affect the fertility (fruit-set and seeds per fruit) were significantly reduced for F_1 hybrids. Such variability in F_1 hybrid fitness has been commonly reported, with different fitness components showing contrasting patterns (e.g. Valentine, 1947; Stebbins, 1959; Ramsey *et al.*, 2003; Grundt *et al.*, 2006; Lowry *et al.*, 2008; Karrenberg *et al.*, 2019; Sandstedt *et al.*, 2021). Clearly, in *Lysimachia*, the deleterious effects of hybridization on the late stages of F_1 performance contributed to RI between the two species.

Another important finding of our study was the enhanced reduction in the fertility of hybrid F_2 and backcross progeny compared with F_1 progeny. Hybrid sterility is considered the most common form of postzygotic RI in plants (Ouyang *et al.*, 2010). Aside from a difference in the ploidy level of the parental species (Grant, 1981; Fishman & Willis, 2001), which

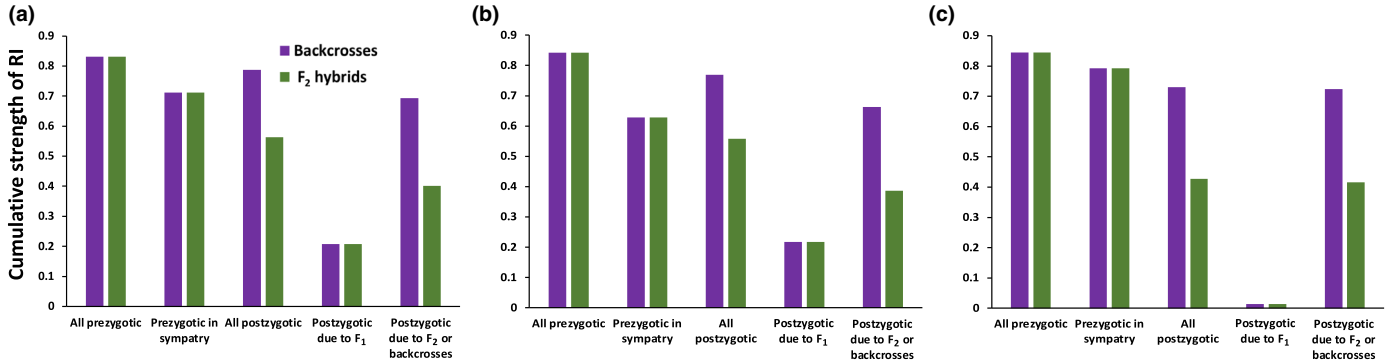

Fig. 5 Cumulative strength of reproductive isolation (RI) barriers for different sets of barriers considering backcrosses and F_2 hybrids: shown are means for (a) both species, (b) *Lysimachia arvensis* and (c) *L. loeflingii*.

is not the case for LL and LA, two genetic mechanisms have been proposed to explain hybrid sterility: chromosomal rearrangements and negative epistatic interactions among loci (Dobzhansky, 1951; Fishman & Willis, 2001; Stathos & Fishman, 2014). Chromosomal rearrangements would cause sterility in F_1 hybrids, but fertility would rebound in F_2 hybrids (Fishman & Willis, 2001; Martin & Willis, 2010). Our F_2 hybrids from LL and LA were as sterile as, or more sterile, than F_1, hinting at a possible role played by epistatic interactions among loci as a cause of reduced fertility (Fishman & Willis, 2001; Martin & Willis, 2010), likely as a result of the accumulation of Dobzhansky–Muller incompatibilities during species divergence (Coyne & Orr, 2004; Gavrilets, 2004; Ouyang *et al.*, 2010; Zuellig & Sweigart, 2018).

Interestingly, the decline in hybrid fitness was more pronounced for backcross progeny than for F_2 progeny. When backcrossing occurs in nature, the result is the introgression of genes from one species into the other (Anderson & Hubricht, 1938). The sharp fecundity reduction we observed for backcrossed progeny suggests that successful introgression has probably been limited in nature. We do not know the genetic architecture of species divergence for the current species pair. However, barriers to introgression are particularly effective across the genome when many genes contribute to the reduced fertility of hybrids (Whittemore & Schaal, 1991; Rieseberg & Wendel, 1993), we may speculate that this may be the case for LA and LL; if so, our result would be consistent with divergence between the lineages over a long period of 2.5 million yr, as inferred by phylogenetic analysis (Jiménez-López *et al.*, 2022). Given that postzygotic barriers evolve at the same evolutionary rate in sympatry and allopatry (Saldamando *et al.*, 2005), it seems likely that the postzygotic barriers revealed by our study of crosses between sympatric populations of the two species will apply generally also to their allopatric populations.

Cumulative RI and prezygotic vs postzygotic RI

Leaving aside RI by geographical barrier, that is, in sympatry, prezygotic RI, most importantly that resulting from the floral constancy of pollinators, has proven to be very effective in preventing the formation of hybrids between the two *Lysimachia* species, greatly contributing to preserve their integrity. Yet, postzygotic RI revealed by later generation hybrids and at late stages of the life cycle has also proven to be crucial in maintaining the boundaries between these species. It is thus clear from our study that postzygotic barriers play an important role in RI between the two *Lysimachia* species studied here, a conclusion that contrasts with the widespread emphasis on the importance of prezygotic RI in plant speciation (Christie *et al.*, 2022). Significantly, postzygotic isolation between the two species was only fully manifest in reduced hybrid performance at the late stages of the life cycle of F_1 and F_2 progeny and, particularly, in progeny produced by backcrossing. This result is consistent with expectations for the effects of Dobzhansky–Muller incompatibilities and indicates that the relative strength of postzygotic RI may be underestimated when late stages F_1 and F_2 are not considered. Assessing the performance of F_2 and backcross progeny for late stages in the plant life cycle will often be difficult, as noted by Christie *et al.* (2022), especially for perennial species. However, our study clearly demonstrates that failing to do so may grossly underestimate the relative importance of postzygotic RI in maintaining species integrity in plants.

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Competing interests

None declared.

Author contributions

MA and PLOB designed the research. FJJ-L, MA, PLOB and MT collected data in the field. FJJ-L performed field and

glasshouse experiments. PLOB, FJJ-L and JV carried out the data analysis. MA, FJJ-L, PLOB, JRP, JV and LPCM contributed to the interpretation of the results. FJJ-L, MA, PLOB, LPCM and JRP wrote the first versions of the manuscript that was later edited by all authors.

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Data availability

The essential data for the manuscript are included in the manuscript, if any of the readers need more information, they can contact the corresponding author.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Summary of the hand-pollinations conducted in the glasshouse to obtain F_1 hybrid, F_2 hybrid and backcrossed offspring and fitness components measured.

Fig. S2 Seasonal flowering phenology in natural populations.

Fig. S3 Daily flowering phenology in natural populations.

Fig. S4 Percentage of offspring obtained after hand-pollination of *Lysimachia loeflingii* and *Lysimachia arvensis*.

Fig. S5 Fitness components of the first-generation offspring obtained by hand-pollination between *Lysimachia loeflingii* and *Lysimachia arvensis*.

Fig. S6 Fitness components of the second-generation offspring of *Lysimachia loeflingii* and *Lysimachia arvensis*.

Table S1 Considerations in the calculation of reproductive isolation (RI) for each studied RI barrier.

Table S2 Bioclimatic variables to the ENM for *Lysimachia loeflingii* and *Lysimachia arvensis*.

Table S3 Information on the studied natural populations of *Lysimachia loeflingii* and *Lysimachia arvensis*.

Table S4 Excel file with calculations of the strength of RI barriers between *Lysimachia loeflingii* and *Lysimachia arvensis*.

Table S5 Excel file with calculations of the strength of RI barriers between *Lysimachia loeflingii* and *Lysimachia arvensis* in sympatry.

Table S6 Link functions, error distributions and factors considered in GLM for each studied variable.

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