



Low genetic diversity, high inbreeding and limited gene flow in the endangered species *Linaria flava* subsp. *sardoa* revealed by genome-wide analysis of polymorphisms

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Abstract

Linaria flava subsp. *sardoa* is a critically endangered plant endemic to the coastal sand dunes of Sardinia (Italy) and Corsica (France), and is recognized under European frameworks for biodiversity conservation and strict protection actions. However, despite its conservation status, little is known about its genomic landscape. Investigating its population genetic structure is essential to assess levels of genetic diversity, and detect potential inbreeding or isolation among populations. Such information provides a critical basis for the development of effective management and conservation strategies. In this study a genotyping-by-sequencing approach was used to identify 24,312 Single Nucleotide Polymorphisms and to investigate genomic diversity and population structure in 96 samples of *L. sardoa*, belonging to eight populations. Our results evidenced high levels of inbreeding (mean inbreeding coefficient=0.87), low levels of genetic diversity within population (mean H_e =0.05), coupled with high genetic differentiation between populations of *L. sardoa* (mean pairwise F_{ST} =0.57). These patterns suggest a lack of genetic diversity, long-term isolation and limited gene flow, raising concerns about the species' resilience to environmental change and stochastic events, and calling for immediate conservation actions. Only some individuals from the MOL population showed significantly lower levels of inbreeding and higher levels of heterozygosity. All these findings provide valuable insights to inform both current and future conservation strategies for the species. A possible conservation action for *L. sardoa* could therefore be the reinforcement of existing populations including in the donor pool at least a small proportion of individuals from other populations, in particular from populations, like MOL, showing higher genetic diversity.

Keywords *Linaria* · ddRAD · Population genetics · Conservation · Endangered plants · Mediterranean vascular flora

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Introduction

Mediterranean islands are recognized as biodiversity hotspots and are considered one of the most vulnerable areas to the impacts of climate change (Vogiatzakis et al. 2016; Solomou 2025). Human activities have contributed to habitat degradation or fragmentation leading to a reduction in the size of many plant populations and increasing their spatial isolation (Kolb 2005; Fois et al. 2020). Climate change and invasive alien species (IAS) are significantly contributing to this decline representing an additional threat (Dukes and Mooney 1999). These changes can result in genetic erosion and accelerate genetic divergence among populations due to random genetic drift, higher levels of inbreeding, decreased gene flow and disrupted pollination (Kwak et al. 1998; Kolb 2005). Endangered plant species with small, isolated populations have been the focus of conservation actions over the past decade (Bacchetta et al. 2011; Gentili et al. 2019; Di Iorio et al. 2024; Perez-Bello et al. 2025). These efforts aimed to prevent biodiversity loss and try to understand survival and extinction mechanisms within an evolutionary framework (Beissinger 2000; Abeli et al. 2009). Genomic data offer valuable tools to address these challenges by enabling the assessment and ongoing monitoring of genetic variation (Theissinger et al. 2023). In this context, the LIFE SEEDFORCE program was established to protect 29 critically endangered native Italian plant species and restore their habitats. The initiative integrates genetic diversity assessments with both ex-situ conservation (seed banks) and in-situ restoration, including population reinforcement and reintroduction.

The Mediterranean Basin encompasses portions of Europe, Africa, and Asia and despite being considered a crucial center of plant diversity (Medail and Quezel 1997; Solomou 2025) and a hotspot for biodiversity and habitats (Cañadas et al. 2014), the diversity and ecological functioning of these systems are increasingly threatened by global environmental change, particularly due to anthropogenic pressures, climate and sea level fluctuations, and the spread of IAS (Medail 2017; Whittaker et al. 2017; Fois et al. 2020). Coastal dune ecosystems support a highly specialized vascular flora (Maun 2009), including numerous endemic plants, like species belonging to the *Linaria* genus (Blanco-Pastor et al. 2012; Pinna et al. 2012). Although the genus *Linaria* is distributed across Europe, Asia, and North Africa (Sutton 1988; Cheriet et al. 2015), its center of diversity is the Mediterranean Basin, which hosts approximately 90 *taxa* (Sutton 1988), several of which are associated with coastal habitats.

Linaria flava (Poir) subsp. *sardoa* (Sommier) A. Terrac. (hereafter *L. sardoa*) is an endangered species exclusive to Sardinia (Italy) and Corsica (France) (Jeanmonod et al.

2009; Fois et al. 2020; <https://www.gbif.org/taxon/6QGL6>). *Linaria sardoa* belongs to the large genus *Linaria* and was traditionally placed in the Scrophulariaceae family (Pignatti 1982). However, advances in DNA analysis and molecular phylogenetic studies have led to a reclassification, and the genus is currently included in the Plantaginaceae family (Albach et al. 2005). The genus *Linaria* comprises more than 200 species (Sutton 1988) with several of them known for having active compounds with potential medical applications (Cheriet et al. 2015). For this reason, effective conservation actions are needed not only to prevent biodiversity loss, but also to prevent the extinction of species with potential medical relevance.

Effective restoration and conservation strategies frequently rely on a combination of conservation approaches, including assisted gene flow and translocation to enhance the adaptability and resilience of at-risk populations (Médail and Baumel 2018; Gargiulo et al. 2019; Zimmer et al. 2019; Van Rossum et al. 2022). The outcome of translocation can be affected by several factors, such as habitat suitability (Bellis et al. 2020) and traits of the introduced material, such as the mating system type of the studied species, quantity, type, and developmental stage (Menges 2008) of the translocated material. Research has shown that greater genetic diversity can boost short-term survival in translocated populations, highlighting the importance of using plant material with high genetic variability (Menges 2008; Schäfer et al. 2020).

Although genetic diversity plays a crucial role in the success of translocation programs, and the IUCN (International Union for Conservation of Nature) guidelines for translocation strongly suggest increasing the genetic diversity of small and isolated populations (IUCN/SSC, 2013), its significance has often been underestimated. Whether for habitat restoration, conservation or species reintroduction, translocations are more likely to succeed when the introduced populations maintain high levels of genetic variation. Several studies have demonstrated that a greater genetic diversity improves the short-term survival and adaptability of translocated populations in their new environment (Weeks et al. 2011; Frankham 2015; Van Rossum et al. 2022).

Comprehensive knowledge of the genetic structure and diversity of *L. sardoa* populations is still lacking. To address this gap, we performed ddRAD-sequencing on 96 individuals sampled from eight populations across Sardinia and Corsica, and we propose a reproducible workflow for the analysis of ddRAD-seq data and the extraction of genotype information for population genetic structure analyses. The aim of the present study is to determine the genetic structure of *L. sardoa*, and to use this newly acquired knowledge to guide conservation actions in the framework of the LIFE SEEDFORCE project and beyond.

Materials and methods

Study species

Linaria sardoa is an annual herbaceous plant belonging to the family Plantaginaceae that is exclusively found in Corsica (France) and Sardinia (Italy), where it occurs in 20 and 28 sites, respectively (Pinna et al. 2012). It is considered a neoendemic *taxon* originating through geographical isolation from other endemic *taxa* of North Africa and the eastern Mediterranean (*L. flava* subsp. *flava*) and the Iberian Peninsula (*L. flava* subsp. *oligantha*) (Arrigoni 1980). In Corsica it blooms from the end of February to the beginning of April and bears fruit between April and May (Paradis & Piazza, 2021); in Sardinia it flowers from early January to the first half of May and bears fruit between the end of March and June (Pinna et al. 2012). The reproductive biology of *L. sardoa* remains poorly studied, with no available information on its pollination, germination capacity, or the optimal temperatures required for germination (Pinna et al. 2012).

Regarding breeding system, several studies have shown that many species of *Linaria* are self-incompatible (Docherty 1982; Fernández-Mazuecos et al. 2013). However, studies in *L. cavanillesii* and *L. tonzigii* demonstrated that some species within the genus *Linaria* can shift from self-incompatibility to self-compatibility when pollinators are scarce (Voillemot and Pannell 2016; Voillemot et al. 2019; Biella et al. 2021).

The seeds are small and ovoid, and the dispersion occurs via barochory and anemochory (Paradis & Piazza, 2021). It is mainly found on coastal siliceous sands (Pinna et al. 2012) and, more rarely, in inland sand deposits along rivulets and streams. The reduction of its populations is due to the impact of human pressures mainly related to tourism, urbanization and recreation activities, which have profoundly changed the areas in which it grows naturally, the presence of IAS, and the coastal erosion (Paradis & Piazza, 2021; Pinna et al. 2024). It is included in the IUCN Red List category as Endangered (EN) (Orsenigo et al. 2018) and in the Annex II and IV of the Habitats Directive (92/43/EEC).

Sample collection

The populations of *L. sardoa* are generally small, spatially restricted, and usually occupy just a few square meters, with the number of individuals varying considerably depending on the year. The sampling strategy was designed to capture representative genetic variation while minimizing disturbance to these restricted and vulnerable populations.

A total of ninety-six individuals of *L. sardoa* were collected from five populations in Sardinia (Italy), and three

populations in Corsica (France), as detailed in Fig. 1 and Supplementary Table 1.

Individuals within each population were collected randomly by sampling individuals at least 2–3 meters apart, and ensuring a spatially representative coverage of the population. From each individual young healthy leaves were selected, avoiding leaves with visible signs of senescence or pathogen infection. Approximately 2 g of fresh material per sample were collected, dried in silica gel and transported to the laboratory of the University of Udine (Italy) for subsequent DNA extraction, library preparation and sequencing. Further details about the samples are available in Supplementary Table 1.

DNA extraction, library preparation and sequencing

Total genomic DNA was extracted from dried leaves using the kit NucleoSpin Plant II (Thermo Fisher Scientific), according to manufacturer's instructions. All the libraries were prepared by IGA Technology services s.r.l. (Udine, Italy), with minor custom modifications from the protocol for ddRAD-seq (Peterson et al. 2012). Briefly, genomic DNA was quantified fluorometrically, normalized to a uniform concentration and double digested with *SphI* and *EcoRI* endonucleases (New England BioLabs, Ipswich, MA). DNA fragments were purified using AMPureXP beads (Beckman Coulter, Brea) and ligated with barcode adapters. Targeted fragments were collected using BluePippin instrument (Sage Science Inc.), by setting the range 400–600 bp. Final libraries were checked for concentration and size distribution with qubit 2.0 Fluoremeter (Invitrogen, Carlsbad, CA) and with Bioanalyzer High sensitivity DNA assay (Agilent technologies, Santa Clara, CA) respectively. Libraries were sequenced with 150 cycles in paired-end mode on NovaSeq 6000 instrument following the manufacturer's instructions (Illumina, San Diego, CA).

De novo ddRAD genotyping pipeline

Samples were demultiplexed based on their barcode sequence using the `process_radtags` package included in the Stacks v2.61 software (Catchen et al. 2013) by IGA technology services s.r.l. (Udine, Italy). A catalog with consensus loci across all samples was created for *L. sardoa* following the de-novo pipeline for paired-end sequencing mode from Stacks v2.0, allowing a maximum of 2 nucleotide mismatches (-M 2) between stacks (loci) when building a consensus locus for each individual.

The software populations, included in Stacks v2.0, was used to call genotypes following an approach previously used by our group (Magris et al. 2022; Perez-Bello et al. 2025). To avoid biases due to mapping of repetitive

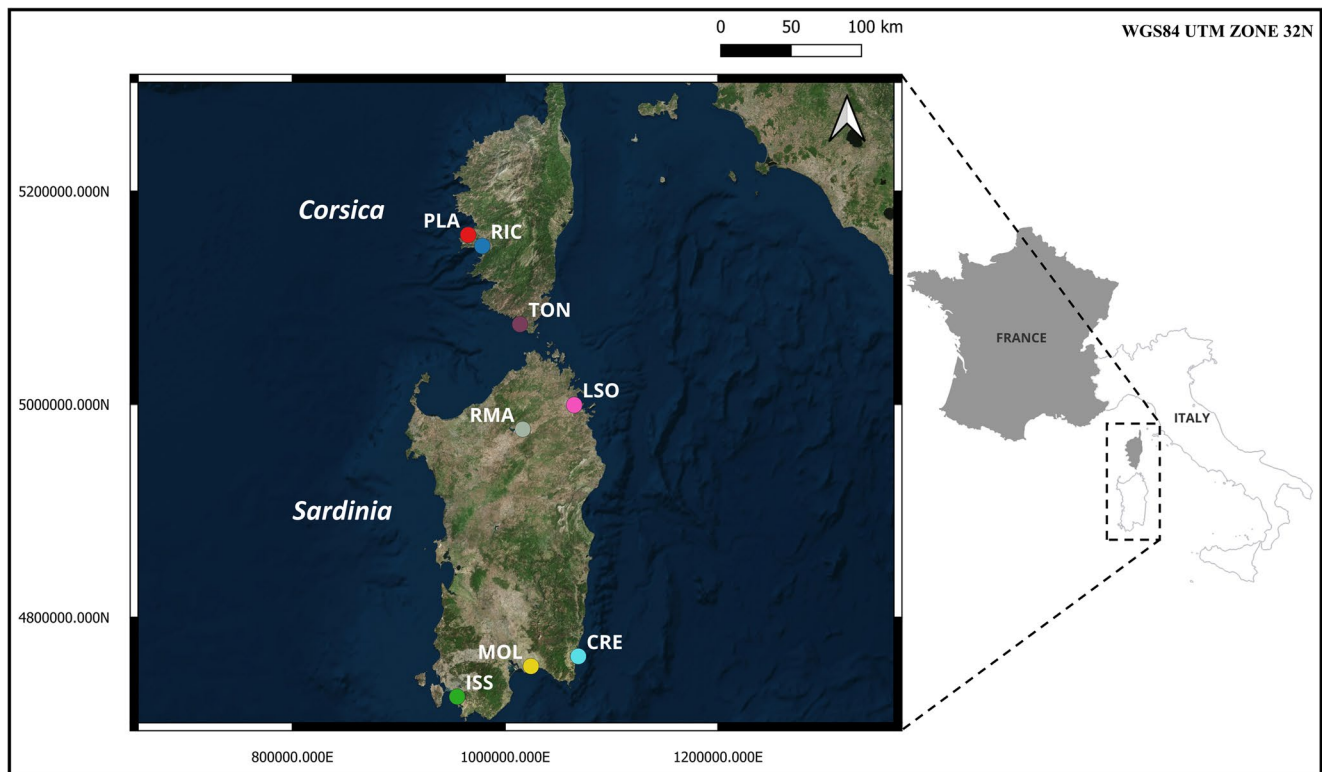


Fig. 1 Geographic location of the studied samples of *Linaria flava* subsp. *sardoa* in Corsica (France) and Sardinia (Italy). Collection sites (Municipalities are indicated between brackets): PLA, Paese di Lava (Alata); RIC, Ricantu (Ajaccio); TON, Tonnara (Bonifacio); LSO,

Lido del Sole (Olbia); RMA, Riu Mannu (Berchidda); CRE, Costa Rei (Muravera); MOL, Stagno di Molentargius (Quartu Sant'Elena); ISS, Is Solinas (Masainas)

sequences only Single Nucleotide Polymorphisms (SNPs) with a maximum observed heterozygosity of 0.8 ($-\text{max-obs-het } 0.8$) were retained. To avoid biases due to missing genotypes only SNPs present in at least 75% of the individuals ($-R \ 0.75$) were retained. In addition, the parameter $-\text{fstats}$ was included to enable SNP and haplotype-based F statistics.

Polymorphisms that were covered by less than 5 reads were removed from the rest of the analyses using *vcftools* (Danecek et al. 2011) with the parameter $-\text{minDP } 5$, and only genotyped positions that were informative in at least 50% of the samples were retained for further analyses.

Genomic diversity and population structure

Expected (H_e) and observed (H_o) heterozygosity, as well as the number of private alleles per population were calculated using the software *Stacks v2.0* (Catchen et al. 2013) as follows: for each population, observed heterozygosity was determined as the proportion of heterozygous individuals at each locus, expected heterozygosity under Hardy-Weinberg equilibrium was determined as $2 * p * (1 - p)$, with p being the frequency of one of the two alleles, and private alleles were determined as the alleles having frequency greater than zero

only in one population. Fixation index F_{IS} was calculated using the formula $1 - H_o/H_e$.

The kinship coefficient between individuals (within and between populations) was assessed by calculating the maximum likelihood estimator of identity-by-descent (IBD) from multilocus SNP genotype datasets with the R package *SNPRelate* (Zheng et al. 2012). Kinship and Inbreeding coefficients were calculated using a minimum threshold for the Minor Allele frequency (MAF) set to 0.05 and pruning SNP pairs showing a linkage disequilibrium LD (measured as r^2) higher than 0.2. Tajima's D was calculated using the software *vcftools* (Danecek et al. 2011) with the option $-\text{TajimaD } 1000$, where 1000 bp is the dimension of the windows in which to perform Tajima's D analysis and was chosen to ensure that each window comprises one and only one stack.

To assess the robustness of population-specific estimates across different sample sizes, down-sampling analyses were conducted by randomly selecting eight individuals (the size of the smallest population) from each population and calculating the distribution of H_o , IBD and kinship across ten replicates. In addition, to evaluate whether differences in sequencing depth among individuals influenced our results, we performed a correlation test between mapped reads and

two genetic parameters: observed heterozygosity (H_o) and Inbreeding coefficient (IBD).

To investigate population structure, a Principal Component Analysis (PCA) was performed using the R library SNPRelate (Zheng et al. 2012). Using the same SNP dataset, admixture analysis (Alexander et al. 2009) was performed with default parameters, adding the flag `--cv=10` to perform 10-fold cross-validation to determine the relative probability of different numbers of ancestral populations (K). Admixture analysis was performed with K values ranging from 2 to 10. For each individual, the admixture algorithm calculates the probability Q ($Q_1, Q_2, \dots, Q_K$) that the individual belongs to each of the K populations. The highest probability for each individual is defined as the maximum Q ($\max Q$). Individuals with a $\max Q$ below 0.95 were considered admixed.

A neighbor-joining (NJ) analysis (Nei 1973) was performed to create a genetic distance tree using the R libraries `vcfR` (Knaus and Grünwald 2017) and `ape` (Paradis et al. 2024) by converting the genotype matrix to a distance matrix to construct the neighbor-joining distance tree using the function `plot.phylo`. Pairwise F_{ST} was calculated using `Stacks v2.0` (Catchen et al. 2013) and plotted using the `heatmap` function in R.

Migration rates between populations were calculated using the software `migrate-n` (Beerli et al. 2019) and `PGD-Spider` (Lischer and Excoffier 2012) was used to generate the required input file. To determine the number of immigrants per generation (N_m), we applied the following formula: $N_m = \Theta * M / 4$, where Θ is the estimated mutation-scaled effective population size and M the mutation-scaled immigration rate.

A Mantel test was performed using the R `vegan` package (Oksanen et al. 2025) to assess the correlation between genetic distances, measured by pairwise F_{ST} and geographic distances, with 9999 permutations. Geographic distances

were calculated using the `dism` function from the `geosphere` R package (Hijmans 2024). This function uses the Haversine formula to determine the distance in kilometers between two locations based on their latitude and longitude.

Using the software ‘`metapop2`’ (López-Cortegano et al. 2019), three components were calculated for allelic and gene diversity: total (AT and HT, respectively), within populations (AS and HS, respectively), and between populations (DA and DG, respectively). In addition, the percentage of allelic diversity loss (+) or gain (-) after the removal of each population was estimated.

The workflow used for de novo genotyping from ddRAD-seq data is summarized in Supplementary Figure S1.

Results

A total of 477,631,194 paired-end reads were obtained from 96 samples, corresponding to an average of 4.97 M reads per individual. The average number of mapped reads was 3 M, ranging from 306 K to 6.37 M, for an average coverage of 67.52x ranging from 15.74x to 105.65x. The number of reads, mean coverage, mapped reads, $\max Q$ and observed heterozygosity for each sample are listed in Supplementary Table 1.

For each population, the average coverage, H_e , H_o , F_{IS} , number of admixed individuals, number of private alleles, nucleotide diversity (π), kinship and inbreeding coefficients are reported in Table 1. MOL and PLA were the populations showing respectively the higher and lower values of heterozygosity and nucleotide diversity, and the lower and higher levels of inbreeding and kinship, respectively.

Figure 2A shows the distribution of H_o across the populations of *L. sardoa*. A subset of individuals from the MOL population shows a higher heterozygosity compared to other individuals and populations. Figure 2B shows the

Table 1 Genetic diversity parameters for *Linaria flava* subsp. *sardoa*. Code: Three letter population code used for indicating populations. Collection sites: PLA, Paese di Lava (Alata); RIC, Ricantu (Ajaccio); TON, Tonnara (Bonifacio); LSO, Lido del Sole (Olbia); RMA, Riu Mannu (Berchidda); CRE, Costa Rei (Muravera); MOL, Stagno di Molentargius (Quartu Sant’Elena); ISS, Is Solinas (Masainas). “ALL” refers to statistics computed considering the whole dataset as a single population. *N* Inds: refers to the number of individuals that passed quality control. *Cov*: mean sequencing coverage per population; *He*: mean expected heterozygosity; *Ho*: mean observed heterozygosity; F_{IS} : inbreeding coefficient; *N* admixed: indicates the number of individuals considered admixed ($\max Q < 0.95$) for a number of clusters $k=8$; *P. alleles*: private alleles per population; π : nucleotide diversity; *Kinship*: kinship coefficient; *IBD*: inbreeding coefficient measured by Identity by Descent

Code	<i>N</i> Inds	<i>Cov</i>	<i>He</i>	<i>Ho</i>	F_{IS}	($K=8$) <i>N</i> admixed	<i>P. alleles</i>	π	<i>Kinship</i>	<i>IBD</i>
PLA	12	89.33	0.011	0.015	-0.32	0	514	1.00E-04	0.49	0.96
RIC	15	83.54	0.032	0.027	0.16	1	1098	3.00E-04	0.46	0.91
TON	15	78.00	0.013	0.018	-0.35	0	1699	1.20E-04	0.49	0.96
LSO	8	35.54	0.053	0.028	0.48	0	1471	5.30E-04	0.43	0.93
RMA	8	49.82	0.053	0.023	0.56	1	1232	5.10E-04	0.44	0.95
CRE	15	72.56	0.027	0.026	0.03	2	646	2.50E-04	0.47	0.93
MOL	15	65.19	0.094	0.119	-0.26	7	1259	8.70E-04	0.36	0.53
ISS	8	29.72	0.058	0.041	0.31	0	2769	5.70E-04	0.43	0.91
ALL	96	62.96	0.043	0.040	0.08	11	10,688	4.06E-04	0.45	0.89

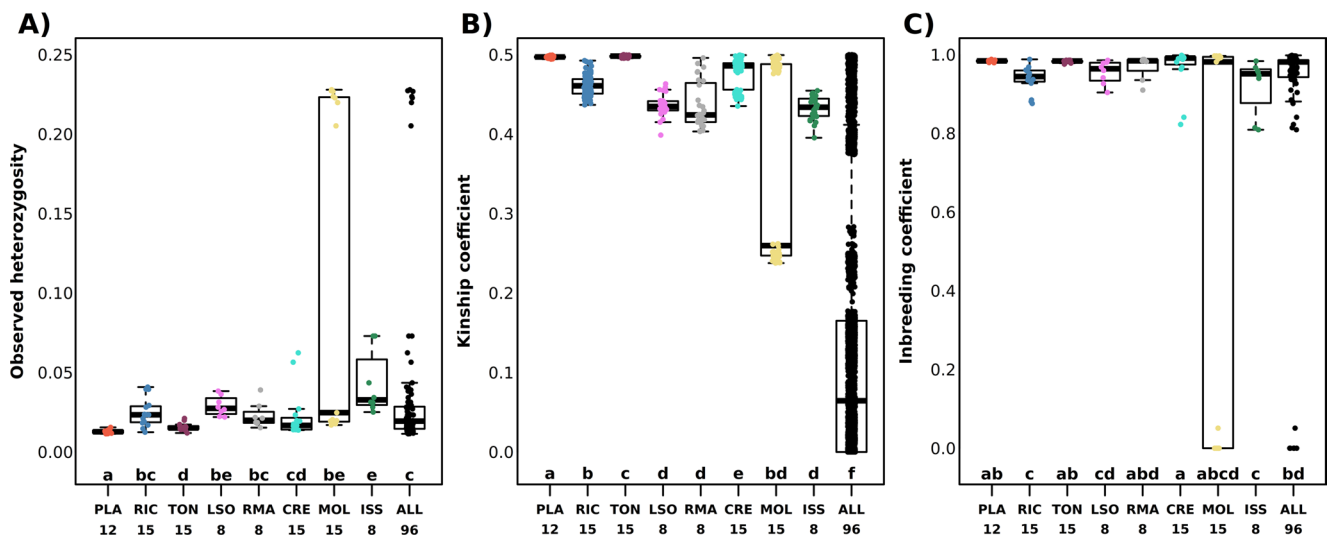


Fig. 2 Distribution of population genetic parameters in *Linaria flava* subsp. *sardoa*. (A) Observed heterozygosity. (B) Kinship coefficient. (C) Inbreeding coefficient. “ALL” refers to statistics computed considering the whole dataset as a single population. Populations with same

letter are not significantly different from each other (Wilcoxon test). The number below each population code indicates the number of individuals sequenced from that population

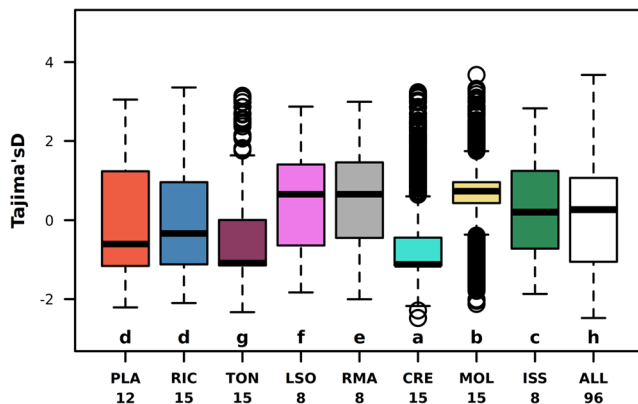


Fig. 3 Boxplot showing the distribution of Tajima's D values in *Linaria flava* subsp. *sardoa*. “ALL” refers to statistics computed considering the whole dataset as a single population. Populations with same letter are not significantly different from each other (Wilcoxon test). The number below each population code indicates the number of individuals sequenced from that population. Information regarding collection sites is available in Supplementary Table 1

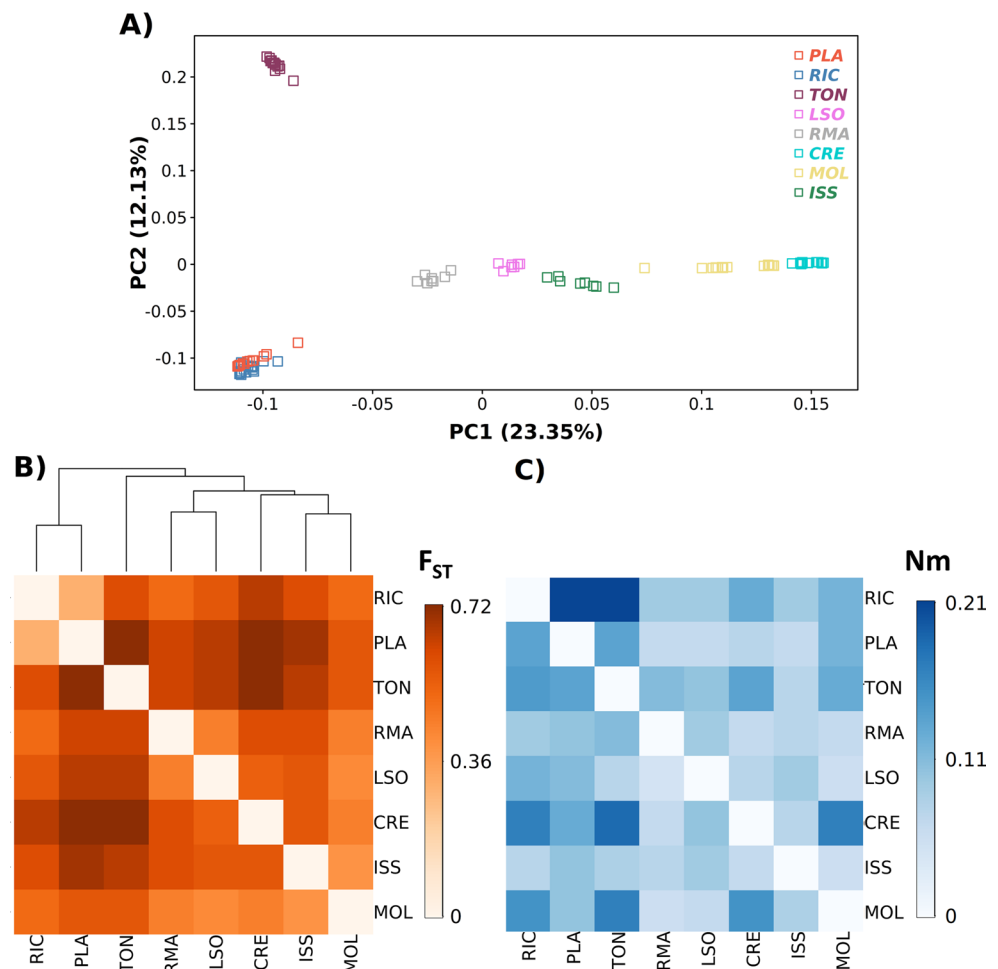
distribution of kinship coefficients for all pairwise comparisons between individuals of the same population (colored dots) and for all pairwise comparisons regardless of their population (black dots). Overall, the kinship coefficient between individuals was high, with only some individual pairs from MOL showing values lower than 0.3. The kinship coefficient for all pairwise comparisons regardless of population of origin is low, suggesting low levels of relatedness between populations. Figure 2C shows high levels of inbreeding for all the populations, except for a subset of seven individuals from MOL population. Measures of H_o , IBD and kinship coefficients remained consistent across

all down-sampling replicates; indicating that the variation in sample size among populations did not compromise the robustness of population specific estimates (Supplementary Table 2). No significant correlations were detected between the number of mapped fragments and either H_o (Supplementary Figure S2A) or IBD (Supplementary Figure S2B), suggesting that variation in read counts did not bias these estimates.

Tajima's D values varied among populations of *L. sardoa* (Fig. 3). Median values differed across populations: CRE and TON exhibited more negative medians; MOL, RMA, and LSO showed slightly positive medians, and the remaining populations generally centered around zero. The distribution of values also varied among populations, and several outliers—particularly in MOL and CRE—were observed, indicating substantial heterogeneity across loci and populations.

Figure 4A shows the differentiation among populations based on PCA. PC1 separates samples from Corsica (left side of the plot) from Sardinian samples (center and right side of the plot). All populations show moderate to high degree of separation, except for individuals collected in PLA and RIC, which appear to cluster together. A closer inspection for individuals collected from PLA and RIC (Supplementary Figure S3) revealed that these two Corsican populations also show some degree of differentiation, and they can be separated when other samples are removed from analysis. Similar to the results observed in the PCA, F_{ST} pairwise analysis shown in Fig. 4B also revealed high degree of differentiation between populations with the lowest differentiation observed between PLA and RIC. The average pairwise F_{ST} was 0.57. Similarly, the gene flow

Fig. 4 Visualization of genetic structure of *Linaria flava* subsp. *sardoa* populations. **(A)** Principal component analysis. **(B)** Fixation index analysis (F_{ST}). **(C)** Gene flow estimation by calculating the number of migrants (N_m). Values below the diagonal represent migration rates from the population in the column to the population in the row. Values above the diagonal represent migration rates from the population in the row to the population in the column



(N_m) analysis shown in Fig. 4C indicated restricted gene flow between populations, with an average value of 0.10. The highest gene flow was detected between RIC and TON (0.212) and between RIC and PLA (0.211), i.e. the three Corsican populations, occupying the left side of the PCA representation. Pairwise comparisons of F_{ST} and N_m values for each population pairs are provided in Supplementary Tables 3 and Supplementary Table 4, respectively.

Pairwise genetic distance (F_{ST}) as a function of pairwise geographic distance is reported in Supplementary Figure S4. Mantel test showed a moderate but statistically significant relationship between genetic and geographic distances ($r=0.4401$ and $p=0.013$).

Cross-validation showed that numbers of populations greater than seven were the most probable number of ancestral populations, with $K=10$ being slightly lower (Supplementary Figure S5). We report results for $K=8$ because this is the number of sampling populations and the cross-validation error is similar to the one measured for $K=10$. Results for $K=7$, $K=9$ and $K=10$ are shown in Supplementary Figures S6, S7 and S8, respectively.

Admixture analysis assuming the presence of eight ancestral populations (Fig. 5A) shows that most populations are clearly separated, except for LSO and RMA (represented with pink color). The southern Sardinian population MOL was instead split into two distinct groups, with seven individuals exhibiting mixed ancestry with CRE. Additionally, some evidence for admixture was observed for two individuals from CRE, one individual from the Corsican RIC and one individual from the Sardinian RMA.

The genetic distance tree conducted at the individual level using Nei's distance (Nei 1973) grouped individuals belonging to the same populations together except for MOL, where two different groups were observed (Fig. 5B).

Analyses performed with metapop2 showed on average, low within-population allelic ($AS=0.109$) and gene ($HS=0.034$) diversity. Between populations allelic ($DA=0.233$) and gene ($DG=0.191$) diversity was moderately high and were the major contributors to total diversity ($AT=0.342$ and $HT=0.225$, respectively).

Interestingly, the exclusion of populations PLA and RIC, led to an increase in allelic and gene diversity.

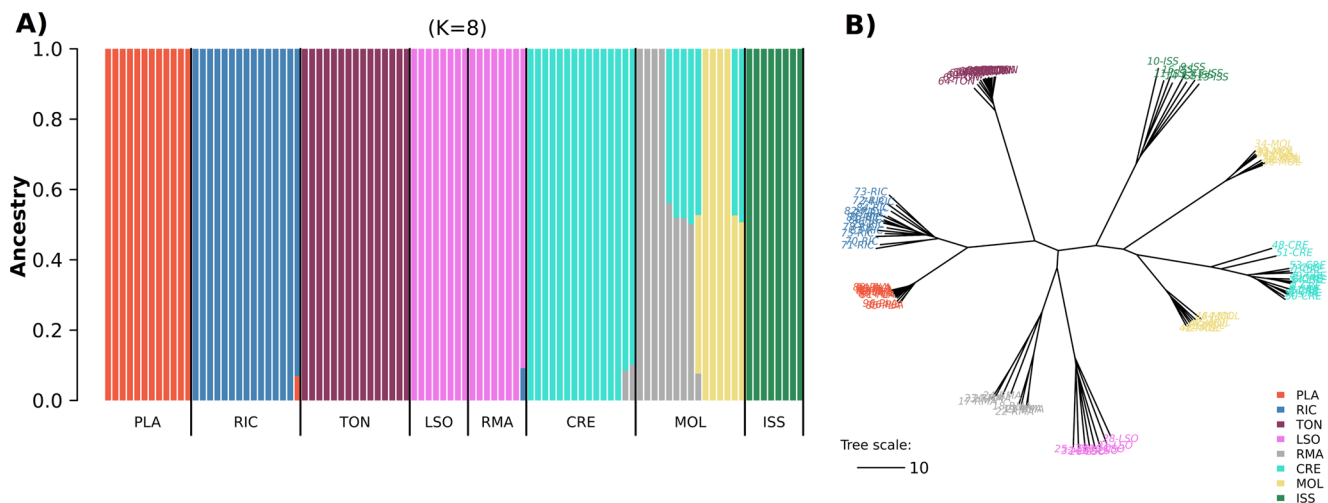


Fig. 5 Visualization of genetic structure of *Linaria flava* subsp. *sardoa* populations. (A) Admixture analysis in the studied individuals of *Linaria flava* subsp. *sardoa*. Each barplot denotes an individual. The elevation of each colored bar represents the probability of assignment

to the corresponding population when assuming the presence of eight ancestral populations ($K=8$). (B) Genetic neighbor-joining unrooted tree based on Nei's distance

Table 2 Percentage of gain (-) or loss (+) of allelic diversity parameters after removal of each population of *Linaria flava* subsp. *sardoa*. Code: three letter population code used for indicating populations. AS: Average allelic diversity within populations; DA: Average allelic diversity between populations; AT: Total allelic diversity. HS: Average gene diversity within populations; DG: Average gene diversity between populations; HT: Total gene diversity. The highest value of each column is shown in bold

Population removed	Allele diversity			Gene diversity		
	AS within	DA between	AT total	HS within	DG between	HT total
PLA	-4.080	-0.957	-5.037	-1.750	-0.544	-2.293
RIC	-1.400	-0.413	-1.813	-0.831	-1.336	-2.167
TON	-3.989	1.742	-2.247	-2.431	4.544	2.112
LSO	2.001	2.118	4.119	0.690	2.091	2.780
RMA	0.823	0.439	1.262	0.542	0.373	0.916
CRE	-2.473	1.438	-1.035	-1.782	5.637	3.855
MOL	5.161	0.940	6.101	4.436	0.676	5.112
ISS	3.958	5.272	9.230	1.231	4.143	5.374

The removal of CRE and TON resulted in increased within-population diversity but a loss in between-population diversity.

Table 2 reports the percentage of gain (-) or loss (+) of allelic and gene diversity after removal of each population. Sardinian populations are in general contributing to allelic and gene diversity more than Corsica populations with ISS and MOL being the major contributors to total allelic and gene diversity.

Discussion

The common expectation for endemic plant species is that they are genetically depauperate, and this poses specific challenges in conservation (Ellstrand and Elam 1993). Endangered species additionally tend to show low levels of

heterozygosity, low levels of intra-population differentiation and high levels of inter-population differentiation (Islam et al. 2023; Perez-Bello et al. 2025). However, in some cases, endemic species maintain high levels of genetic variability relative to their widespread congeners (Gitzendanner and Soltis 2000; Abeli et al. 2014), and this has been observed for some species endemic to Sardinia (Italy) (Bacchetta et al. 2011). Our results indicate that the genetic structure of *L. sardoa* is more in line with expected patterns, characterized by low heterozygosity and nucleotide diversity, elevated intra-population relatedness, high inbreeding, high levels inter-population differentiation and low levels of gene flow between populations.

The observed situation is compatible with high levels of self-pollination or bi-parental inbreeding, which may result in low levels of heterozygosity and high differentiation between populations (Cisternas-Fuentes et al. 2022). Plant mating systems can represent adaptations to specific habitat

types, with self-pollination being potentially favoured in environments that are unstable or frequently disturbed (Yang and Kim 2016). Previous work showed that *Linaria tonzigii* (Biella et al. 2021), and *Linaria cavanillesii* (Voillemot and Pannell 2016) are self-compatible. Although no study specifically addressed the exact pollination strategy of *L. sardoa*, it has been reported that several *Linaria*, including *Linaria flava* itself, are self-compatible (Biella et al. 2021), thus suggesting that selfing may be at least partially responsible for the observed genetic structure.

In addition, the relatively high levels of isolation between populations coupled with the low levels of gene flow may be attributable to physical or reproductive barriers (Sexton et al. 2014; Kulmuni et al. 2020).

Our results suggest that geographic distance is at least partially a determinant of genetic differentiation between populations. The Mantel test showed a significant correlation between genetic and geographic distance, and the PCA and the genetic distance tree are compatible with the presence of three major clusters: one corresponding to Corsica, one to Northern Sardinia, and one with Southern Sardinia. Estimates of migration rates also suggest that at least the Corsican populations form a cluster with a higher migration rate than the average.

Estimates of Tajima's *D* suggest that the populations from the two islands have different demographic histories. The three Corsican populations show negative *D* values, suggesting a recent expansion. Three Sardinian populations (RMA, LSO and ISS), show a moderately positive value of *D*, suggesting population contraction. On the other hand, the distribution of *D* in MOL and CRE is at the same time interesting and puzzling. MOL and CRE are genetically similar according to PCA, genetic distance tree, F_{ST} , N_m and admixture. Yet, they show contrasting patterns of Tajima's *D* values, with MOL showing a positive value and CRE, a negative one. While we cannot exclude that the difference reflects population expansions, it is also possible that the two contrasting patterns are due to the fact that both populations are structured. Structured populations can show an excess of rare or common alleles depending on the structure itself, and this may turn result in negative or positive values of Tajima's *D*, respectively (Tajima 1993).

In terms of genetic diversity, the picture emerging from our results is alarming, even if compared to the expectations for an endangered species. Compared to other endangered species of the Mediterranean Basin, *L. sardoa* shows higher levels of inbreeding and lower levels of nucleotide diversity and gene flow (Szczecińska et al. 2016; Perez-Bello et al. 2025). One of the populations investigated in the present study (MOL) slightly deviates from the described pattern, as it includes a few individuals with high heterozygosity, and showing evidence of admixture, possibly with CRE.

Individuals with high heterozygosity and low inbreeding coefficient are particularly interesting as potential donors for reinforcement and/or reintroduction.

The MOL population is particularly vulnerable due to the high urbanization in the psammophilus belt between the coast and the pond of Molentargius (Quartu Sant'Elena) where *L. sardoa* is still present in a small population of approximately 150 individuals. In addition, according to admixture analysis, individuals belonging to MOL that do not show admixture, belong to two different genetic backgrounds, suggesting that the population itself was formed from two different populations, with some additional contribution from CRE (Costa Rei, Muravera); this is consistent with the observation that the migration rate between MOL and CRE is relatively high, compared to the average.

A specimen was collected by Moris in 1981 at Fiumini (Quartu Sant'Elena) and is preserved in the Herbarium of Florence (Arrigoni 1980). However, this population was not detected during our most recent monitoring activities, and it is likely extinct due to extensive urban development including housing, parking lots, and tourism infrastructure over the past several decades. It is possible that this extinct population contributed to the genetic background of MOL.

Analysis of allelic and gene diversity (Table 2) revealed that MOL and ISS are the principal contributors to diversity. MOL, sharing some genetic background with CRE and having a high level of heterozygosity contributes more to within population diversity, while ISS, having a high number of private alleles, contributes more to between population diversity. In general, the removal of Sardinian populations led to greater allelic and genetic losses than the removal of populations from Corsica. Among populations from Corsica, TON was the only one contributing to allele and gene diversity and could be used as a source of genetic material.

Overall, our results call for immediate conservation actions to safeguard *L. sardoa*. To address this urgency, several *in situ* and *ex situ* actions are already ongoing, in the framework of the Life SEEDFORCE project-LIFE20/NAT/IT/001468 (<https://lifeseedforce.eu/en/linaria-flava>; <https://lifeseedforce.eu/en/monitoring-in-sardinia>), though further efforts are required. In this regard, *ex situ* conservation via seed banking has been undertaken since 2005 at the Sardinian Germplasm Bank (Porceddu et al. 2017) for several *L. sardoa* seed lots; furthermore, various tests are underway, both in controlled and field conditions, to identify an optimal germination protocol for the species.

In view of our results, we propose some possible options for *L. sardoa* conservation. One common recommendation is to use local germplasm for restoration (McKay et al. 2005) to maintain the genetic integrity of the population. However, given the very low genetic diversity of at least some of the populations, one may wonder if in this specific case, it

would be better to violate the recommendation by introducing at least some external individuals in order to decrease inbreeding and homozygosity. The use of admixed reintroduction material, although possibly reducing the integrity of local populations, proved to be a predictive factor for successful reintroduction actions (Godefroid et al. 2011).

However, we suggest that in addition to these two reasonable general strategies, one could design intermediate approaches, based on detailed knowledge of the genetic status of the population. According to our results, in some of the sampled populations, specifically the PLA and TON populations from Corsica, all individuals appear to have very low genetic diversity and high inbreeding. In this case, the condition may justify reintroducing individuals from other sites; this could be the RIC population from Corsica, characterized by some residual heterozygosity and at the same time lower geographic distance, or by MOL and ISS populations, located further apart, but showing higher diversity. Also, a mix of MOL and ISS is possible, and considering previous works, could be advised for future translocations (Godefroid et al. 2011).

Similar reasoning applies to most of the Sardinian populations. However, for ISS and MOL populations one could reason that the reintroduction of local material is reasonable, in particular if the selection of the most heterozygous genotypes is possible.

While preservation of local genetic distinctiveness is important, conservationists should also keep in mind that the present lack of genetic diversity in *L. sardoa* is probably due to anthropogenic intervention and habitat disruption. Indeed, our results suggest that anthropogenic pressure has already changed the natural connection between populations. For example, this was the case for MOL and CRE, suggesting that a future hypothetical translocation to a suitable site positioned between these two populations could be considered as a restoration of the natural condition rather than the creation of a new population. Such long-term conservation strategies that prioritize enhancing gene flow between fragmented populations, for example, by establishing habitat corridors or implementing managed translocations should be considered for future interventions.

Complementary to these approaches, *ex situ* conservation—storage of seeds in germplasm banks—plays a crucial role in safeguarding genetic diversity. Such measures ensure the availability of material for future reinforcement or reintroduction programs and serve as a genetic reservoir in the context of accelerating environmental change and ongoing habitat loss.

Approaches to protect plant species from extinction should focus on all the aspects of plant biology, and should include limiting the invasive alien species, counteracting habitat loss (Weidlich et al. 2020) and increasing population

size through translocation (Whitehead et al. 2023). In the latter case, depending on population dynamics and the results of genetic diversity assessments, various strategies may be employed to mix propagation material.

Thus, long-term conservation strategies should prioritize enhancing gene flow between fragmented populations, for example, by establishing habitat corridors or implementing managed translocations. The possibility that *L. sardoa* is self-compatible, may increase the difficulty of conservation actions. Under that scenario, high inbreeding coefficients may be caused by self-pollination, and reintroduction of individuals from different populations would not be beneficial if self-pollination is still largely predominant. However, the fact that several individuals from the MOL population show a significantly lower inbreeding coefficient may be attributed to cross-pollination, and under the optimistic hypothesis that cross-pollination is happening, the introduction of diverse individuals would be beneficial. Further investigations on the mating system of *L. sardoa* would help to better interpret the observed patterns of genetic diversity and inbreeding, and to clarify the potential role of self-pollination in shaping the species' genetic structure. Such information could also support the design of more effective conservation and translocation strategies, particularly in the context of managed gene flow between populations.

In conclusion, our work showed that Sardinian and Corsican populations of *L. sardoa* show low levels of heterozygosity, high levels of inbreeding and are genetically isolated. These newly characterized properties will be used to inform conservation actions in the framework of the LIFE project SEEDFORCE. Results of the present study may be used for measuring success of conservation actions, and to guide future conservation efforts. Overall, our results provide a genetic baseline for the development and evaluation of targeted conservation strategies for *L. sardoa*, particularly those aimed at increasing connectivity and reducing inbreeding.

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Author contributions P.P. and F.M. designed and performed the bioinformatic analyses. A.C.-L., L.D., L.P., M.P. and G.B. designed and conducted sampling. L.D., M.P., E.D.P., V.C., C.B., M.E.B., A.C.-L., L.P. and G.B. contributed with results interpretation. P.P. and F.M. wrote the first draft of the manuscript. All authors read and approved the manuscript.

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Data availability The sequencing reads used in this study are available in Sequence Read Archive (SRA), under the accession PRJNA1153768 (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1153768>). Scripts and functions used in the present work are released on github under the MIT License: (https://github.com/genomeud/Seedforce/tree/main/Linaria_flava).

Declarations

Competing interest The authors declare no competing interests.

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