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Investigating pollination strategies in disturbed habitats: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae) on mountain screes

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Abstract Plant mating systems may reflect an adaptation to a habitat type, with self-pollination being potentially common in unstable and disturbed conditions. We investigated the reproductive ecology of an Alpine, narrow-range toadflax, *Linaria tonzigii* Lona (Plantaginaceae), occurring in steep and dynamic mountain screes. We explored self-compatibility and spontaneous autogamy, seed viability, daily nectar production, pollinator behaviour and pollen transfer in wild populations, using hand pollination treatments, quantification of nectar volume, viability Tetrazolium assay, active pollinator sampling and video recordings, and UV-bright dust for pollen substitution. After *ex novo* sequencing of several genetic regions of *L. tonzigii*, we performed a multi-marker phylogenetic analysis of 140 *Linaria* species and tracked the occurrence of the self-compatibility trait. Our results showed that this species is self-compatible, pollinated mostly via spontaneous autogamy and pollinator-mediated geitonogamy, and self-pollinated seeds are as viable as cross-pollinated ones. Selfing could be due to pollinator rarity because, despite the studied species providing a high nectar volume, wild bees, moths and small beetles infrequently visited its flowers in the sparsely vegetated

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scree slopes. In addition, a preliminary survey showed a low genetic haplotype diversity in the study plant. Moreover, the phylogeny shows that self-compatibility is scattered in the tree, suggesting the adaptive nature of this reproductive trait in the genus *Linaria*. This study supports for theories that high prevalence of selfing is an adaptation to environments unfavourable for cross-pollination, and particularly where pollinators are rare, such as in perturbed, poorly vegetated high-elevation habitats.

Keywords Pollination rare plants · Plant reproduction · Genetic diversity · Pollen limitation · Outcrossing · Molecular marker

Introduction

Plants can reproduce in several ways, either asexually through vegetative propagation, or through sexual reproduction by means of self- or cross-pollination (Yang and Kim 2016). On one hand, self-pollination is expected to ensure progeny in condition of low pollinator activity, i.e. the hypothesis of "reproductive assurance" (Baker 1955; Stebbins, 1957). It is theorized that selfing holds a higher transmission rate compared to outcrossing, i.e. the "automatic selection" or "transmission advantage of selfing" hypothesis, (Fisher 1941; Stone et al. 2014) and it would assure the preservation of successful phenotypes that are particularly adapted to a local environment (Schmitt and Gamble 1990; Massol and Cheptou 2011). On the other hand, cross-pollination increases genetic diversity and therefore allows for a high potential of adaptation from a long-term perspective (Busch and Delph 2011; Cheptou 2019).

Although selfing bears all the risks related to inbreeding, theories state that it can occur when the benefits of transferring two copies of the maternal genes outweigh the risks of inbreeding depression (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Busch and Delph 2011). By selfing, the incidence of deleterious alleles is expected to rise, but this can be overcome through DNA purging, for example due to bottlenecks after extreme environmental events (Cooper and Cooper 1995). Furthermore, plant phylogenies clearly showed that plants transitioned from self-incompatibility to self-compatibility multiple times, independently (Igic et al. 2008; Goldberg et al. 2010), and this is even confirmed by cases of recent acquisition of the selfing (Voillemot et al. 2018). These aspects indicate the adaptive nature of the selfing trait, that could be advantageous at least in the short term (Cheptou 2019).

Despite the theories presented above describing how selfing could be advantageous for plant fitness, it seems less clear if selfing can be triggered by the type of habitat a plant lives in (Barrett et al. 2014). In general, it is believed that stable habitats are inhabited by highly outcrossing species, whilst unstable and disturbed habitats are usually colonized by selfing or even clonal plants (Lloyd 1980). Plausibly, this observation could be explained by associating self-pollination to environments where pollinators and/or mates are rare (Baker 1955). However, numerous exceptions to that observation have been noted and convincing experimental evidence is still needed (Barrett et al. 2014). In particular, when considering the incidence of selfing within speciose genera, there seems to be less consistency between reproductive strategy and habitat type. For example, both selfing and out crossing species of the genus *Senecio* occur in Australian unstable habitats (Lawrence 1985). Likewise, instances of selfing have been recorded in *Linaria* species both in stable habitats (e.g., grasslands and rock cliffs) and in unstable habitats (e.g., sand dunes and arable land) (Carrió et al. 2013). When comparing selfing rates between different populations, it was found that outcrossing predominates in areas where populations are frequently visited by pollinators, whilst in populations where visitation to flowers does not occur, selfing is the most common way to reproduce (Ness et al. 2010). This phenomenon shows that "reproductive assurance" could often interplay with "automatic transmission" in determining the presence and maintenance of the selfing trait in a given species (Barrett et al. 2014). In addition, it was shown that selfing carries an advantage where populations are fragmented and/or have low density, and when repeated colonization/extinction events have occurred (Good-Avila et al. 2008). These conditions link the selfing trait to *taxon* history, and not only to the type of habitat. Therefore, understanding how the plant reproductive strategies relate to the habitat is still a key question for understanding the reproductive biology of plants.

A wide variety of environments are colonized by species of the genus *Linaria* (Plantaginaceae), therefore it is a suitable model for studying mating systems. Whist some *Linaria* species have a large distribution range, a number of *taxa* occur in small areas, and many species grow in peculiar environmental conditions, e.g. rocks, cliffs or sand dunes (Segarra-Moragues and Mateu-Andrés 2007). A remarkable case is *Linaria tonzigii* Lona, calcicole toadflax with a narrow biogeographical range, that is endangered at the global scale according to the IUCN, and occurs in an endangered habitat listed in the EU Habitats Directive (European Commission 2009; Mangili and Rinaldi 2011). In addition, *L. tonzigii* has a high local cultural value and forms the logo of the botanical association Flora Alpina Bergamasca (F.A.B.). In addition, its reproductive biology is unknown and this can jeopardize even the simplest conservation measures (Falk 1992; Janečková et al. 2019). The species occurs on mountainous screes, a peculiar, disturbed habitat characterized by a dynamic and incoherent substrate with low vegetation cover (Fisher 1952; Cannone and Gerdol 2003). The ground instability is mainly caused by the frequent debris supply from the neighbouring cliffs, high material flow on the scree, absence of cementification in the rocky debris, and the instability is correlated with slope angle and other topological factors (Statham 1973; Cannone and Gerdol 2003). As a result of the low vegetation cover, plants occurring on the screes are exposed to wind and other harsh weather conditions that are typical for high altitudes (Fisher 1952). Such extreme conditions impact not only plant life (Fragnière et al. 2020) but also pollinator abundance and consequently also outcrossing rates (Mathews and Collins 2014).

In this study, we investigated the pollination biology of *L. tonzigii*, with reference to its habitat (mountain screes) and the evolutionary history of the genus. As *L. tonzigii* displays conspicuous zygomorphic flowers and a long nectar spur, we hypothesize a major contribution by long-tongued pollinators in both visitation and pollination (e.g. Vlašánková et al. 2017). In addition, the timing of daily nectar production could reveal the plant preference for a specific pollinator group (Zimmerman 1988), and thus we hypothesize that nectar production prior to dusk time would be connected to a preference for nocturnal pollination, whilst a production prior to and during the daylight could indicate a preference for diurnal ones (Zimmerman 1988). Therefore, in order to shed light on the pollination system of the plant, we focused on (a) its self-compatibility, spontaneous autogamy and cross-pollination; and on (b) the daily pattern of nectar allocation, the pollinator fauna and foraging behaviour. Furthermore, we aimed to (c) explore the genetic identity of the study species and place it in the phylogeny of the genus *Linaria*, information that was not previously available. The latter will be helpful for comparing the species' mating systems with that of related species.

Methods

The plant and the study area

Linaria tonzigii is a perennial hemicryptophyte plant that is endemic to a small area within the Bergamasque Orobie mountains, in the south-central part of the Alpine chain (area number 68 in the Alpenvereinseinteilung der Ostalpen classification and 29 in the SOUISA system). It occurs in an extremely restricted range (convex hull of about 10 km^2) and is found in unstable and dynamic habitats characterized by incoherent accumulations of coarse gravel in particularly steep mountain screes (Giupponi and Giorgi 2019). It usually occurs in the alpine and subalpine altitudinal planes and exclusively on calcareous substrates (Orsenigo et al. 2016). The plant is about 6–12 cm tall, each stalk usually hosts 3–6 (rarely 10) single flowers, and the flowering phenology lasts from around mid-June to mid-July, with some individuals flowering earlier or later in the season.

Most of the field work for this study took place in two locations on Mt. Arera, with little to no isolation, that can be considered as a unique population (N 45.933, E 9.804 at 2100 m a.s.l.; N 45.929, E 9.807 at 2000 m a.s.l., 500 m apart). An additional population located in Mt. Cavallo was included in the pollinator survey (N 46.034, E 9.695 at 2100 m a.s.l., 12 km from Mt. Arera). These populations occur on screes with a slope range of 53.5–60.4% (calculated as difference in elevation between top and bottom of the scree divided by their distance).

Pollination biology

In the field, the study plants in pre-flowering stage were covered with bags of fine mesh to exclude pollinators during the time of the investigation. Twenty plant individuals received four pollination treatments each, as follows: (1) Cross-pollination by hand (with pollen from various randomly chosen individuals), (2) Self-pollination by hand (with a flower own pollen), (3) Autogamy (autonomous self-pollination), (4) Emasculation (test of agamospermy). These treatments were carried out during the peak flowering period of the population and were applied during two consecutive days in the field. Single flowers were deprived of the anthers during the pre-flowering stage (except those of the autogamy treatment) and the flowers of a given individual plant were treated at the same time. To avoid an effect of the maternal plant on the results, all four treatments were applied to the flowers of each selected individual simultaneously, as in Hamilton and Mitchell-Olds (1994).

The seed set for each of these treatments was calculated as the number of seeds per capsule and compared to the seed set of plants exposed to open pollination. The seeds in each capsule of these treatments were weighed and the total weight was divided by the number of seeds relative to each fruit (Kern ABT 120-5DNM, 0.00001 g). The seed viability in open-, self- and cross- pollination treatments was tested with a tetrazolium assay at 1% concentration for 24 h after cutting the seeds in half for exposing the embryo to the solution.

The number and mean weight of seeds per capsule were analysed as response variables in linear mixed effect models, pollination treatment was a predictor and plant individual identity was a random intercept. The assumptions of normality were checked visually. The percentage of viable seeds per capsule was tested as a proportion of the total number of seeds (viable + non-viable seeds) in a generalized mixed effect model with binomial error distribution, with the pollination treatment as a predictor variable and the plant individual identity as a random intercept.

To measure the difference and significance of seed number, weight, or viability of each treatment in relation to open pollination, the regression slopes and the Z-statistic were used. For these and the above detailed regressions, the package *glmmTMB* in R was used (Brooks et al. 2017; R Core Team 2017). A post hoc test comparing the seed number associated to cross- and self- pollination was performed with the package *emmeans* (Lenth 2020).

Nectar production and sugar concentration

Flowers were emptied in each location and the sugar concentration in the nectar was measured with a field refractometer (Eclipse Handheld Refractometer − Sugar, 0–50% − Bellingham + Stanley, London), one flower per plant from randomly chosen individuals (different from those who received pollination treatment, see above). For recording the pattern of nectar production during the day, the nectar volume was measured with microcapillaries (0.5 µL microcapillary tube Drummond Microcaps, Sigma-Aldrich) by comparing the volume of 24 h with that produced after night and every 6 h during the day (i.e., collections at 9:00 A.M, 3:00 P.M., 9:00 P.M. from the same flowers, with the dusk time and the sun set usually happening before 9 PM in the sampling area). All flowers were covered in bags of fine mesh to exclude flower visitors, and corolla spurs were previously emptied to assure nectar production during the time frame indicated above. The nectar volume of a total of 48 flowers was measured, half of which were used for the 24 h-volume assessment, and the others for the 6-h production.

The volume of nectar was analysed as a response variable in generalized mixed effect models with Gamma error distribution and log-link function. Categorical predictor variables were whether nectar was taken after 24 h or during a phase of the day. Plant individual identity was the random intercept. The difference in estimated coefficients associated to the predictors and its significance was tested with the delta beta and the associated Z-statistic, using the package *glmmTMB* in R (Brooks et al. 2017; R Core Team 2017).

Pollinator monitoring, pollinator behaviour and pollen analysis

Flower visitors found inside or entering the corollas and touching the reproductive structures of the flowers were sampled with a mouth aspirator or by hand netting. Surveys consisted in walking repeatedly across the screes during day and evening,

approximately between 8:00 A.M. and 11:00 P.M. for 20 days distributed in the two populations of Mt. Arera in 2018 and 2019 and a population of Mt. Cavallo in 2019. Additionally, video cameras with infrared (IR) night vision were placed both during day and night with continuous recording (SuperEye RJ0090-UK, OneThingCam™).

Pollen from the insects body was removed using fuchsin jelly (i.e., from the head of large bees and from the body of small beetles), which was subsequently melted onto glass slides and inspected with an optic microscope (Leitz Laborulux K). These slides were compared to a reference slide of pollen of *L. tonzigii* created by touching freshly opened *L. tonzigii* flowers with fuchsin gel (this reference slide is stored in MIB:ZPL herbarium Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy).

To quantify self-pollination and geitonogamy in wild populations, we tracked the flow of the pollen with pollen-substitutes. Flowers of 10 randomly chosen individuals were dusted with UV-reflective dust (RadGlo RC 33, Radiant Color, Europark 1046 B 3530 Houthalen) on their inner surface of the corolla near to and on the anthers, similarly to Wanderley et al. (2020). At night, a UV emitting torch (MorPilot, 51 Led) was used for checking the inner corolla of all flowers on the same stalk as the source UV flower and on all individual plants occurring within one meter radius from the dusted, source flower.

DNA extraction and phylogenetic analysis

One to five plants of *L. tonzigii, L. vulgaris* and *L. alpina* (the three *Linaria* species occurring in the same administrative province where the study was conducted) were sampled in several populations for subsequent laboratory analysis (Table S1 in Online Resource Appendix 1). These samples were vouchered following the protocol specified by the Global Registry of Biodiversity Repositories (http://grbio.org/) and the data standards for DNA barcode records (http://www.boldsystems.org/docs/dwg_data_standards-final.pdf). Young leaves were used for genetic screening (1–3 per plant) and they were stored immediately at − 20 °C until laboratory analysis. The specimens were stored in herbarium at the Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy (herbarium code MIB:ZPL).

One hundred milligrams of each sample were used for DNA extraction using Plant DNeasy Isolation and Purification kit (Qiagen, Hilden, Germany). Purified DNA concentration and quality of each sample were estimated fluorometrically with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA), by measuring the absorbance (Abs) at 260, 280, and 230 nm. DNA-based characterization of *L. tonzigii* was performed by amplification and sequencing of four DNA barcode markers (i.e., the plastidial *rbcL*, *matK*, the intergenic spacer *trnH-psbA*, and the nuclear ITS) and other three genomic regions that showed variation in studies of other plant species of a range size similar to *L. tonzigii* (i.e., *agt1*, *rpl32-trnL* and *trnL-trnF*; Blanco-Pastor and Vargas 2013; Zecca et al. 2017). For each locus, PCR amplification was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Freiburg, Germany) in a 25 µL reaction according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation step for 7 min at 94 °C, 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C for *rbcL* and *trnL-trnF*, 53 °C for *matK* and *trnH-psbA*, 55 °C for ITS, *agt1* and *rpl32-trnL*), and extension (1 min at 72 °C), and a final extension at 72 °C for 7 min. The primer pairs used were 1F/724R for *rbcL* (Fay et al. 1998), 390F/1326R for *matK* (Cuénoud et al. 2002), psbA/trnH for *trnH-psbA* (Newmaster et al. 2008), p5/u4 for ITS (Cheng et al. 2016), AGT1_F1/AGT1_R1 for *agt1* (Kovacova et al. 2014), rpL32-F/trnL for *rpl32-trnL* (Shaw et al. 2007) and trnT-F_cF/trnT-F_fR for *trnL-trnF* (Taberlet et al. 1991).

PCR products were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea. Sequence editing and alignment were performed using Bioedit (Hall 1999) and the sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI, see Table S1 in Online Resource Appendix S1). The variability among *L. tonzigii* samples was tested with the regions *trnH-psbA*, *agt1*, *rpl32-trnL* and *trnL-trnF* as in Blanco-Pastor and Vargas (2013) and Zecca et al. (2017). The genetic variation at these markers was described by calculating the number of haplotypes, the haplotype diversity and the nucleotide diversity with DnaSP v5.10.1 software (Librado and Rozas 2009).

To analyse the phylogeny, we downloaded sequences for nuclear (ITS and *agt1*) and plastidial (*rbcL*, *matK*, *trnH-psbA*, *rpl32-trnL*, and *trnL-trnF*) markers in all the *Linaria* species and subspecies available in Genbank using the *rentrez* package version 1.2.1 (Winter 2017) in R (R Core Team 2017). We also obtained sequences for these markers in *Maurandya scandens*, *Chaenorhinum macropodum*, *Antirrhinum majus*, and *A. graniticum* to be used as outgroups. Since the genetic identity of the analysed *L. tonzigii* specimens were very similar to each other (see section 'Results'), only the sequence of the Mt. Arera haplotype was used in the phylogenetic analysis. Downloaded and newly sequenced markers were aligned using MAFFT version 7.307 (Katoh and Standley 2013) and the best evolutionary model and partition scheme was estimated using PartitionFinder version 2.1 (Lanfear et al. 2017). Phylogenetic trees were performed using both, Maximum Likelihood as implemented in RaxML version 8.2.9 (Stamatakis 2014) and Bayesian Inference as implemented in MrBayes version 3.2.6 (Ronquist and Huelsenbeck 2003) and visualized using the ape 5.1 library (Paradis and Schliep 2018) in R. Bayesian analyses were performed using 4 chains, 20 million generations sampled every 1000 generations each with 0.25 as relative burning value. For maximum likelihood analyses, branch support was estimated after 100 bootstrap replicates.

Data on self-compatibility for *Linaria* species were taken from Valdés (1970), Docherty (1982), Carrió et al. (2013), Voillemot et al. (2018).

Results

Pollination biology

Pollination treatment was a significant categorical predictor of the seed set (χ^2 = 38.893, *df* = 1, *P* $\leq$ 0.001; Fig. 1a). Flowers exposed to open pollination produced less seeds than the hand cross- or self-pollinated ones, and open pollination produced a similar number of seeds as spontaneous autogamy (Table 1). The numbers of cross- and self-pollinated seeds were not significantly different in the post hoc test (estimated difference cross-self = − 6.35, SE = − 3.38, *P* = 0.07). Testing for agamospermy did not yield any seeds.

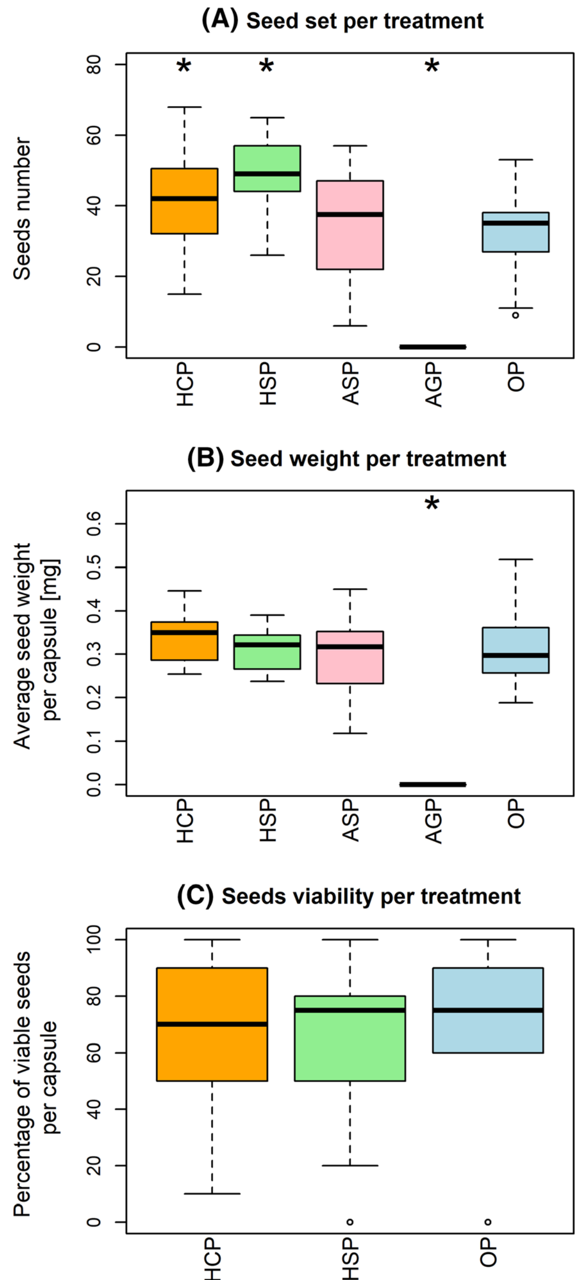

Fig. 1 Boxplots of seed set (**a**), seed weight (**b**) and seed viability (**c**) of *L. tonzigii* from the pollination treatments of hand cross-pollination (HCP), hand self-pollination (HSP), autonomous self-pollination (ASP), agamospermy (AGP) and control open pollination (OP). Significant differences to open pollination are indicated with "*" and statistical details are reported in Table 1

The seed weight was significantly related to the pollination treatment (χ^2 = 47.669, *df* = 1, *P* $\leq$ 0.001; Fig. 1b), however, there was no significant

Table 1 Statistical comparison of fitness data such as seed set per capsule, seed weight (mg) and viability (% of seeds)

Response variable	Pollination treatment	β_0	
Seed set	Open pollination	33.086	
Weight per seed	Open pollination	0.306	
Seed viability	Open pollination	0.78	
		$\beta_i - \beta_{\text{open pollination}}$	Significance of the difference to open pollination
Seed set	Self-pollination	15.125	**< 0.001**
Seed set	Cross-pollination	8.771	**< 0.05**
Seed set	Autogamy	0.973	0.8
Seed set	Agamospermy	− 33.45	**< 0.001**
Weight per seed	Self-pollination	0.022	0.695
Weight per seed	Cross-pollination	0.021	0.335
Weight per seed	Autogamy	− 0.009	0.663
Weight per seed	Agamospermy	− 0.303	**< 0.001**
Seed viability	Cross-pollination	0.028	0.891
Seed viability	Self-pollination	− 0.015	0.944

The upper part of the table reports the statistical estimation of plant fitness of open pollinated flowers, against which each pollination treatments was compared with (in the lower part of the table); Significance below 0.05 is indicated with bold and other statistical details are in 'Methods'. Treatments were hand self-pollination, hand cross-pollination, spontaneous self-pollination ("Autogamy") and emasculation ("Agamospermy")

difference between open pollination and either hand cross- or self-pollination or spontaneous autogamy (Table 1). The rate of seed viability was not significantly predicted by the pollination treatment ($\chi^2 = 0.296$, $df = 1$, $P = 0.862$; Table 1, Fig. 1c).

Daily nectar production and sugar concentration

Sugar concentration in the nectar averaged around 35.4%. The nectar volume produced varied during the day: the volume produced in 24 h was similar to that produced in the morning, but it was significantly higher than that produced around noon and evening (Table 2).

Table 2 Statistical comparison of nectar volume (µl) in 24 h (upper part of the table) and in different time phases of the day relative to the production during 24 h (second part of the table); Significance below 0.05 is indicated with bold and other statistical details are in 'Methods'

Time phase	Real mean (SD)	β_0	
24 h	1.04 (0.39)	0.708	
		$\beta_i - \beta_{24h}$	Significance of the difference to open pollination
Before 9:00 AM	1.18 (1.03)	− 0.078	0.279
9:00 AM–3:00 PM	0.75 (0.48)	− 0.175	**< 0.01**
3:00 PM–9:00 PM	0.23 (0.21)	− 0.889	**< 0.001**

Pollinator monitoring and behaviour

In total, 141 h of video were recorded and about 240 h of active sampling carried out. Despite the high amount of time spent in sampling (actively or with videocameras), few insects were found.

The active sampling of pollinators yielded insects of different functional groups and taxonomical orders that were either inside the flowers or foraging on flower resources, and scattered in different flowers along the surveyed screes (total sums indicated): 32 small thrips (Thysanoptera), 41 small beetles of Coleoptera: Staphylinidae (Staphylinidae: *Eusphalerum* sp.), a large moth of Lepidoptera: Noctuidae, three large wild bees of Hymenoptera: Apidae that were two queens of the bumblebee *Bombus monticola* and a female bee of *Anthophora quadrimaculata*. All these insects are stored as voucher specimens in PB collection. Video recordings yielded additional flower visitors and their behaviour, namely: a large moth of Lepidoptera: Noctuidae and a butterfly of *Erebia* sp. (Lepidoptera: Nymphalidae) collecting nectar, a queen of the bumblebee *Bombus monticola* collecting nectar and a worker of *Bombus* sp. collecting pollen. In addition, an *Andrena* cf. *rogenhoferi* bee was recorded and landed on the corolla of a *L. tonzigii* flowers carrying a lot of light-yellow pollen, which is a colour very similar to that of *L. tonzigii*, but it was not seen entering the flower. Videos showed very clearly that whilst foraging, the bumblebees visited in fast succession a relatively high number of *L. tonzigii*

flowers (i.e., 19, 7, 6 flowers), whilst the moth and the butterfly visited only a few flowers (1 and 2 flowers, respectively). Instead, the vegetated meadows growing at the sides of the screes host a high activity of pollinating insects both during the day and during the night. For instance, eight moths were found visiting flowers of different families during a transect walk of one hour, but none on *L. tonzigii* during that time.

The captured specimens were examined in the laboratory and pollen of very similar morphology to *L. tonzigii* was found on the body of both small beetles and of the large bees, and the latter carried a higher quantity of pollen on their body (i.e. their head) than the small beetles. 99% of the pollen taken with the fuchsin jelly was identified as belonging to *L. tonzigii*. Pollen was not found on the one moth collected.

UV-reflective dust indicated that most of the investigated flowers did not receive dust (47.92%), whilst flowers of the dust-treated stalks received more dust than those of surrounding stalks (29.86% and 22.22%, respectively).

Some flowers had a hole in the spur, a particular feature compatible with nectar robbing, especially in those flowers growing closer to the highly vegetated meadows.

Phylogenetic analysis and genetic identity

The genetic diversity among the analysed specimens was very low, with three poorly differentiated haplotypes at *agt1*, *trnH-psbA* and *rpl32-trnL* (see Table S2 in Online Resource Appendix S1).

A total of 140 species and subspecies of the genus *Linaria* and *Nuttallanthus* (*Linaria* s.l.) were included in the phylogenetic analysis. The best models for the different partitions were SYM + I + G (ITS), GTR + G (*matK*), JC (*rbcL*) and HKY (*psbA-trnH*). *L. tonzigii* clustered within the Clade E (sensu Fernández-Mazuecos et al. 2013b), together with species of the Section *Speciosae*: *L. purpurea*, *L. repens*, and *L. capraria* (Fig. 2). This position in the tree is consistently observed when nuclear and plastidial markers are used separately (see Figs. S1–S4 in Online Resource Appendix S2).

Discussion

In this study, we investigated several aspects of the pollination ecology and genetic identity of a toadflax species, *L. tonzigii,* inhabiting Mediterranean screes, a threatened habitat according to the IUCN (European Commission 2009). The target species is a rare and steno-endemic plant, threatened of extinction ("Endangered" category in the IUCN Red List, Mangili and Rinaldi 2011). Based on the literature records of other *Linaria* species and on the flower morphology (i.e., zygomorphic flowers with a nectar spur), we previously hypothesized that the pollinating fauna would be composed uniquely of long-tongued insects. In detail, other *Linaria* species are visited by large bees, bee flies of the Bombyliidae family and both diurnal and nocturnal Lepidopterans (Sutton 1988; Stout et al. 2000; Fernández-Mazuecos et al. 2013a), which are insects with long mouthparts. In addition, since the flowers of *L. tonzigii* have a very long spur, we hypothesised that if nectar is produced in the evening, a preference for nocturnal pollination could be expected (Zimmerman 1988).

This field study of *L. tonzigii* only partly confirmed these expectations. Our data showed a prevalence of diurnal pollinators, which also matched the higher production of nectar volume during morning hours. This result hints that the study plant allocates resources to diurnal pollinators (Zimmerman 1988), and this conclusion could potentially be strengthened in future by recording the daily dynamics of the scent production. This is in congruence with a general pattern that pollinators usually visit flowers when the nectar resources are the highest (Real and Rathcke 1991; Aguiar and Pansarin 2013; Biella et al. 2019). In addition, our survey indicated that not all flower visitors were long-tongued insects, and not all the pollinators were equal in terms of abundance and visitation behaviour. Large bees with long mouthparts were rarely found in the flowers, although they visited several flowers per foraging bout, whilst small beetles with short mouthparts were more abundant visitors, but apparently very sedentary in the flowers. Moreover, the fact that open pollinated flowers produced similar seeds number as the autogamic flowers, together with the pollinator behaviour recorded in the videos and the pollen tracking suggest that the majority of the pollen flow is within the same flower (spontaneous autogamy) or between flowers of the

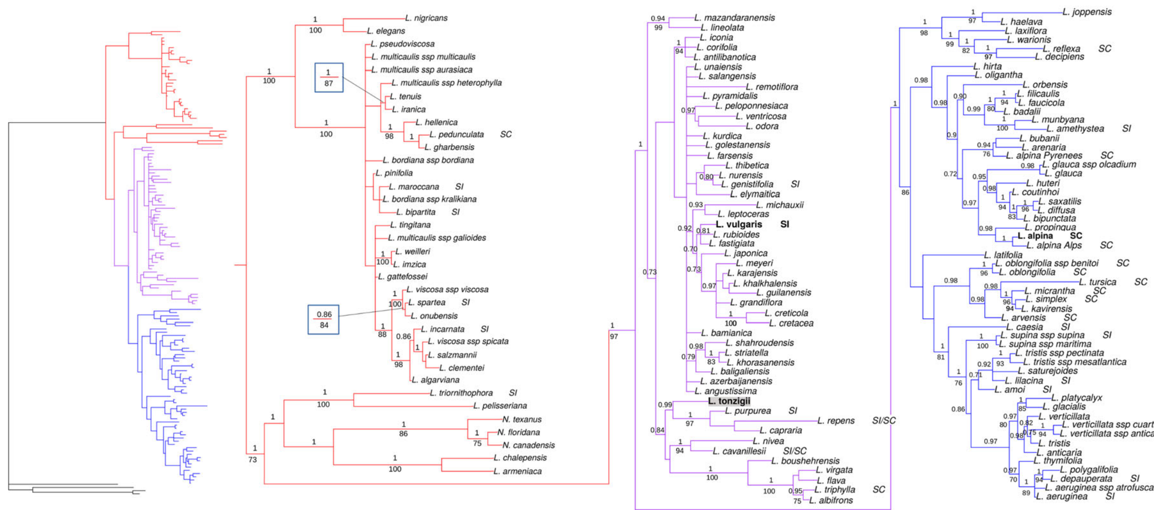

Fig. 2 Phylogenetic analysis of the genus *Linaria* s.l. based on concatenated genetic markers (ITS, *agt1*, *rbcL*, *matK*, *trnH-psbA*, *rpl32-trnL*, *trnL-trnF*). Species sequences newly generated for this study are indicated in bold. Species mating system data compiled from the literature are indicated as SI (self-incompatible), SC (self-compatible) and SI/SC (both self-incompatible and self-compatible depending on the original study), and *L. tonzigii* is SC (this study). The branches of the outgroup species are in black at the bottom of the tree (descending vertically *Maurandya scandens*, *Chaenorhinum macropodum*, *Antirrhinum majus*, *A. graniticum*)

same stalk (geitonogamous pollination). Thus, these evidences suggest for a prevalence of self-pollination in *L. tonzigii.* The reason why large and mobile pollinators rarely visited *L. tonzigii* may be due to low vegetation cover in this habitat (Giupponi and Giorgi 2019), and that highly mobile winged pollinators would prefer foraging in patches with generally higher plant abundance (Redhead et al. 2016). We observed seemingly higher pollinator activity in nearby vegetated meadows than in the screes habitat, although this was not quantified. Rare visits to flowers agrees with the evidence that the plant is pollen limited, because the open pollination flowers produced less seeds than the hand pollination treatments, that is a clear indication of low amount of pollen delivered by pollinators (Kalisz and Vogler 2003). This agrees with the "reproductive assurance" hypothesis. Furthermore, it could be expected that this plant spread also vegetatively, as it grows with underground radicated stalks (see Fig. S5 in Online Resource Appendix S1). These features are compatible with a vegetative reproduction (Pfeiffer et al. 2006), but future studies will need to provide empirical evidence confirming the role of asexual reproduction in this species.

Self- and cross- pollination experiments indicated that the species is self-compatible and that spontaneous autogamy produced as many seeds as open pollinated ones. Moreover, the self-pollinated seeds were equally as viable as cross-pollinated ones. All these results strongly indicate no avoidance of self-pollination and that fruiting is more important than the source of the fecundating pollen (i.e., whether the pollen is from the same flower/individual or from different ones). Self-pollination bears implications for genetic diversity. The hypothesis of automatic advantage of selfing implies that self-pollination is a sustainable strategy if the level of genetic diversity is low, as after DNA purging and bottlenecks (Busch and Delph 2011). We preliminary checked for genetic differences between individuals of *L. tonzigii* collected both in the same population and in different parts of the distribution range using similar genetic markers as other studies focused on geographic ranges comparable to ours (Zecca et al. 2017; Blanco-Pastor et al. 2019). Although the loci used here are less variable than what would be obtainable with microsatellites, DD-Rad or other genomic approaches based on high-troughput sequencing, we still found a very low genetic diversity amongst individuals. Even if preliminary, this result is not contradicting a scenario of severe diversity reduction during the history of the species or due to selfing, similar to reports in other cases of plants with low genetic

diversity (Szczecińska et al. 2016; Blambert et al. 2016; Zecca et al. 2017).

Previous studies supposed that the majority of *Linaria* species are self-incompatible (Bruun 1937; Valdés 1970). However, the species pool considered in those studies was poor. In the multi-marker phylogenetic analysis in our study, the high frequency of the missing information on self-compatibility limits the drawing of clear conclusions on the evolutionary patterns of this character at the level of the entire genus, until additional data are made available. Yet, the fact that the self-compatibility trait occurs in different clades indicate that in *Linaria* spp. the acquisition of this trait arose multiple times and it may be interpreted as an adaptation to local environments, as reported in other plant genera as well (Goodwillie 1999; Goldberg et al. 2010). This seems to be corroborated by another species of this genus that showed different levels of self-compatibility in different parts of its range, varying from self-incompatible to self-compatible populations (Voillemot et al. 2018). Some similar cases are occasionally reported for other plant genera (Busch et al. 2011). The advantage of self-pollination is that it can assure reproduction when pollen flow levels are low (Cheptou 2019), such as in unstable environments with unreliability of pollinator presence and activity, as is the case in dynamic mountain screes inhabited by *L. tonzigii*. Autonomous selfing interplays with pollinator-mediated selfing (geitonogamy), that is considered to assure reproduction in populations where flowering conspecifics are scarce and sparsely distributed (Lloyd 1979; Kropf and Renner 2008). In the dynamic and sparsely vegetated screes of this study, individuals of *L. tonzigii* are often far apart and geitonogamy could benefit reproduction in this challenging environment, in combination with other ways of reproduction. On the long-term, however, it is believed that self-compatibility could become disadvantageous as it could decrease the potential for adaptability and even increase the extinction risk (Goldberg et al. 2010). As indicated by Cheptou (2019), only by tracking the demographic history of the species and by quantifying the roles of inbreeding depression, DNA purging and rearrangement, and the need of reproductive assurance will clarify if *L. tonzigii* populations will be likely to persist over time.

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Authors contributions Conceptualization: PB, JJ, LM; Data collection: PB, AA, NT; Laboratory analyses: PB, AA, NT; Statistical analyses: PB, AJMP, AG; Writing: all authors.

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Availability of data See Online resources and Figshare (https://doi.org/10.6084/m9.figshare.12014574, link: https://figshare.com/articles/dataset/Investigating_pollination_strategies_in_disturbed_habitats_the_case_of_the_narrow-endemic_toadflax_Linaria_tonzigii_Plantaginaceae_on_mountain_screes_/12014574).

Declarations

Conflict of interest The author declare that there is no conflict of interest.

Ethical approval This study was approved by the authorities of the Parco delle Orobie Bergamasche (permits no. 1428 and no. 1283MV/RC).

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Online Resource

Biella P., Akter A., Muñoz Pajares A.J., Federici G., Galimberti G., Jersáková J., Labra M., Mangili F., Tommasi N. and Mangili L. Investigating pollination strategies in disturbed habitats: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae) on mountain screes.

Appendix S1

Genetic identity and variation in *Linaria tonzigii.*

Young leaves (1-3 per plant) from each plant specimen were used for genetic screening (Table S1). The sequences obtained in this study are the very first molecular data regarding *L. tonzigii* ever deposited in public international archives (Table S1). When comparing sequences between populations, the genetic diversity was very low, with three poorly differentiated haplotypes at agt1, trnH-psbA and rpl32-trnL (Table S2). The sample from Mt. Cavallo showed exclusive haplotypes at three out of seven sequenced loci, whereas the population from Mt. Secco showed exclusive haplotypes at two loci. Overall, for each locus, most of the sampled populations shared the same haplotypes (Table S2).

Table S1- Sampling details and haplotypes of DNA sequences produced in this study. GenBank accession numbers are reported within brackets.

Voucher	Field ID	Species	Population	Coordindates	AGT1	ITS	matK	rbcL	trnH-psbA	rpl32-trnL	trnL-trnF
MIB:ZPL:07787	LTA1	*L. tonzigii*	Mt. Arera	45.930611, 9.804611	AG1 (LR746142)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07788	LTA2	*L. tonzigii*	Mt. Arera	45.930611, 9.804611	AG1 (LR746142)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07789	LTA3	*L. tonzigii*	Mt. Arera	45.934194, 9.804472	AG1 (LR746142)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07790	LTC1	*L. tonzigii*	Mt. Cavallo	46.034483, 9.693883	AG2 (LR746143)	IT2 (LR746139)	MK1 (LR746148)	RB1 (LR746145)	TP2 (LR746157)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07791	LTF1	*L. tonzigii*	Mt. Ferrante	45.974583, 10.030139	AG3 (LR746144)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07792	LTM1	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP3 (LR746158)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07793	LTM2	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07794	LTM3	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07795	LTM4	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07796	LTM5	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07797	LTS1	*L. tonzigii*	Mt. Secco	45.924139, 9.882333	AG3 (LR746144)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT2 (LR746152)	LF2 (LR746162)
MIB:ZPL:07798	LTS2	*L. tonzigii*	Mt. Secco	45.924139, 9.882333	AG3 (LR746144)	–	–	–	–	RT3 (LR746153)	LF2 (LR746162)
MIB:ZPL:07799	LA	*L. alpina*	Mt. Triomen	46.021513, 9.592167	–	LR746140	LR746149	LR746146	LR746159	LR746154	LR746163
MIB:ZPL:07800	LV	*L. vulgaris*	Mapello	45.713570, 9.560710	–	LR746141	LR746150	LR746147	LR746160	LR746155	LR746164

Table S2 – Genetic differences among samples of *L. tonzigii*. For each investigated locus, the length of aligned sequences (or the length range in case of indels occurring), the number of segregating sites (S), the number of haplotypes (h), the haplotype diversity (Hd) and the nucleotide diversity and its standard deviation (**π** (SD)) are reported.

	agt1	**trnH-psbA**	**rpl32-trnL**	**trnL-trnF**
length (bp)	846	320-331	804	848-858
S:	2	4	2	0
h:	3	3	3	2
Hd	0.530	0.333	0.318	0.303
π (SD)	0.00106 (0.00027)	0.00417 (0.00269)	0.00041 (0.00023)	0 (0)

Figure S5 – Example of an underground system in *Linaria tonzigii*.

Online Resource

Biella P., Akter A., Muñoz Pajares A.J., Federici G., Galimberti G., Jersáková J., Labra M., Mangili F., Tommasi N. and Mangili L. Investigating pollination strategies in disturbed habitats: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae) on mountain screes.

Appendix S2 – Phylogenetic analyses of nuclear and plastidial genetic markers

The following pages contain phylogenetic analyses of concatenated sequences for nuclear (ITS and agt1) and plastidial (matK, rpl32-trnL and trnL-trnF) genetic markers of the genus *Linaria*, inferred separately with RaxML and MrBayes. The colors indicate the *taxa* in the clades described by Fernández-Mazuecos *et al.*, 2013b, but *taxa* that were not included in that study are in black.

Figure S1 - Phylogenetic analysis of nuclear (ITS and agt1) genetic markers with MrBayes

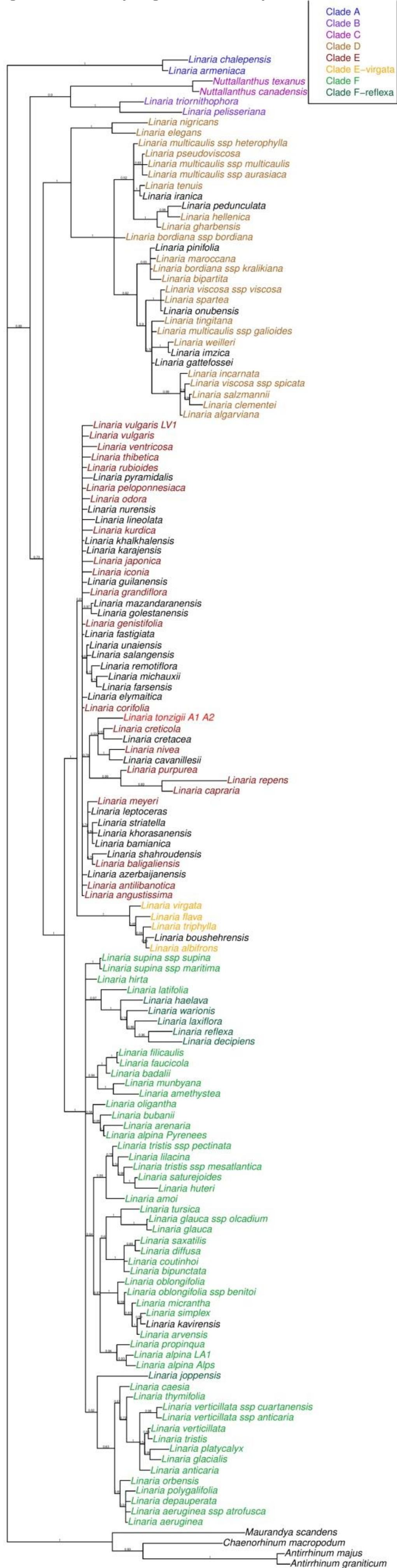

Figure S2 - Phylogenetic analysis of nuclear (ITS and agt1) genetic markers with RaxML

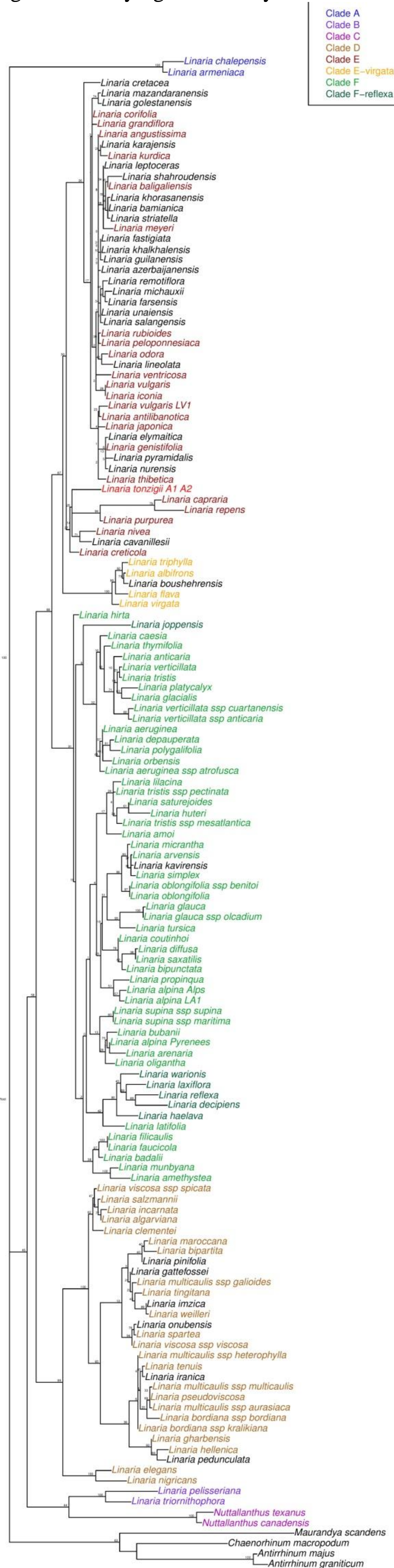

Figure S3 - Phylogenetic analysis of plastidial (matK, rpl32-trnL, and trnL-trnF) genetic markers with MrBayes

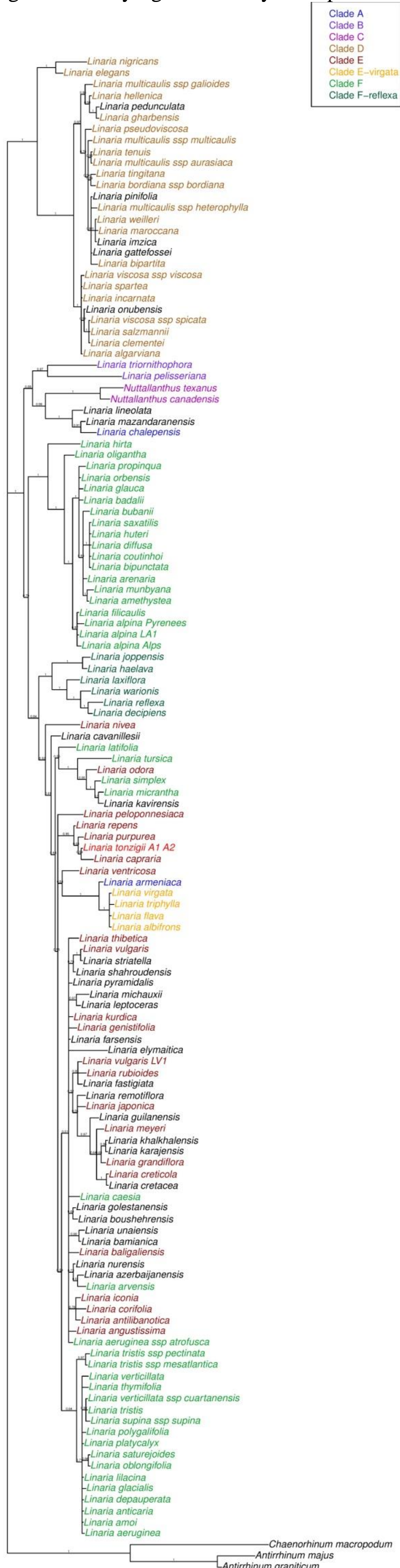

Figure S4 - Phylogenetic analysis of plastidial (matK, rpl32-trnL, and trnL-trnF) genetic markers with RaxML