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The Linaria amethystea complex: convergence, divergence and radiation in the western Mediterranean

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Table 1 to 2 are available in the Supplementary Files section.

The *Linaria amethystea* complex: convergence, divergence and radiation in the western Mediterranean

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ABSTRACT

The Mediterranean Region is one of the world's major hotspots of floristic diversity, characterized by a high number of endemic taxa. An example is *Linaria amethystea* (Vent.) Hoffmanns. & Link (Antirrhineae, Plantaginaceae), a species endemic to the western Mediterranean Region, including the Iberian Peninsula and northwestern Africa, in which a wide morphological diversity has been recognized. This variability has complicated its taxonomic classification, leading to the current recognition of seven subspecies, as well as two recently described, morphologically similar species (*L. pseudamethystea* and *L. semialata*). In this study, a phylogenetic analysis of this taxonomic complex was conducted to determine whether the current classification is supported by phylogenetic relationships and investigate its main evolutionary patterns. Phylogenetic trees were reconstructed using plastid (*rpl*32-*trn*L, *trn*S-*trn*G and *trn*L-*trn*F) and nuclear (ITS) DNA regions, complemented by a plastid haplotype network and phylogenetic dating based on plastid DNA sequences. The results indicated that *L. pseudamethystea* is phylogenetically distant from the rest of the group, suggesting that its morphological resemblance to *L. amethystea* is the result of evolutionary convergence. Furthermore, the grouping of the northwestern African taxon *L. amethystea* subsp. *broussonetii* with *L. munbyana* indicates that *L. amethystea* may not form a monophyletic group. Although phylogenetic resolution for the remainder of the complex was limited, results point towards a recent Quaternary radiation of a clade comprising the Iberian subspecies of *L. amethystea* and other closely related taxa, resulting in a considerable morphological and ecological diversity despite limited genetic differentiation.

Keywords

Antirrhineae, convergence, *Linaria*, Mediterranean plant radiation, phylogenetics, Sanger sequencing

INTRODUCTION

The Iberian Peninsula is located in the Mediterranean region, a hotspot of vascular plant diversity ranked as the third richest in the world (Myers et al., 2000; Fady & Concord, 2010). This exceptional floristic diversity, including a high number of endemic taxa (Buira et al., 2020), is a result of the interaction of a variety of climatic, geological and topographic factors (Aedo et al., 2017; Buira et al., 2017), together with biogeographic connections to surrounding regions, particularly to northwestern Africa (Molina-Venegas et al., 2013). The onset of mediterranean-type climatic seasonality during the Pliocene played a major role in shaping this diversity by promoting the establishment of the current Mediterranean flora and the decline of the paleotropical and arctotertiary floras, which were unable to adapt to the new environmental conditions (Suc, 1984; Barrón et al., 2010). Following the establishment of the mediterranean-type climate, plant lineages that were pre-adapted to these climatic conditions expanded and diversified, and the subsequent glacial and interglacial periods of the Quaternary further contributed to plant diversification (Barrón et al., 2010). Numerous Mediterranean plant lineages have undergone recent radiations, particularly during the Quaternary, as documented in genera such as *Cistus* L., *Dianthus* L., or *Linaria* Mill. (Guzmán et al., 2009; Valente et al., 2010; Fernández-Mazuecos & Vargas, 2015). In such cases, the taxa resulting from radiation are very closely related genetically, which may blur the line between taxonomic categories such as species and subspecies. This issue is especially pronounced in *Linaria* (Blanco-Pastor et al., 2012; Fernández-Mazuecos & Vargas, 2015; Fernández-Mazuecos et al., 2019), a genus with a high frequency of hybrid individuals and intermediate morphologies (Sáez & Bernal, 2009).

The genus *Linaria*, comprising c. 200 species, stands out as the most diverse within the tribe Antirrhineae (Plantaginaceae) (Sutton, 1988; POWO, 2026). Its distribution range extends across most of the European continent (excluding the highest latitudes), North Africa, much of Asia (except for Southeast Asia) and North and South America (Sutton, 1988; Fernández-Mazuecos et al., 2013). The genus is particularly diversified in the Iberian Peninsula and the Balearic Islands, where 92 native taxa (species and subspecies) are currently accepted (Sutton, 1988; Sáez & Bernal, 2009; Sáez et al., in prep.), 74 of which are endemic. This places *Linaria* fifth among Iberian genera in terms of the number of endemic species (Buira et al., 2017). The exceptional richness of *Linaria* in the Iberian Peninsula appears to be the result of an ancient crown age (around the early Miocene) in the proto-Mediterranean region, together with the presence of a key evolutionary innovation promoting speciation, the nectar spur, among other factors (Fernández-Mazuecos et al., 2019; Gorospe et al., 2020). The

taxonomy of *Linaria* in the Iberian Peninsula remains dynamic, with multiple taxa described in recent years (Blanca et al., 2018; Juan et al., 2018; Luceño et al., 2019; Jiménez et al., 2020; Blanca et al., 2023; Farminhão et al., 2026).

Sutton (1988) established an infrageneric classification of *Linaria* comprising seven sections. Recent advances in DNA-based phylogenetics have enabled the refinement of infrageneric classification and provided a better understanding of evolutionary relationships and patterns within the genus (Blanco-Pastor et al., 2012; Fernández-Mazuecos et al., 2013; Yousefi et al., 2017; Fernández-Mazuecos et al., 2019). The present study is framed within *Linaria* sect. *Supinae* (Benth.) Wettst., a monophyletic group comprising three subsections, as suggested by phylogenetic studies: subsect. *Supinae*, subsect. *Saxatile* Valdés and subsect. *Arvenses* (equivalent to sect. *Arvenses* (Benth.) Wettst., not formally published at the subsection level) (Blanco-Pastor et al., 2012; Blanco-Pastor & Vargas, 2013; Yousefi et al., 2017). In particular, this study focuses on the "*Linaria amethystea* (Vent.) Hoffmanns. & Link complex", a polymorphic group of taxa distributed across the Iberian Peninsula and northwestern Africa, and currently included in subsect. *Saxatile* (Yousefi et al., 2017). The group has experienced substantial taxonomic rearrangements over time, summarised in Table 1. Valdés (1970) treated this complex as its own subsection within sect. *Supinae*, named subsect. *Amethystea* Valdés, which included all taxa displaying winged seeds with a disc bearing prominent tubercles and a thickened wing. Within this group, Valdés recognised four taxa: *L. amethystea* var. *amethystea* f. *amethystea*, *L. amethystea* var. *amethystea* f. *broussonetii* (Poir.) Valdés, *L. amethystea* var. *albiflora* Boiss. and *L. multipunctata* (Brot.) Hoffmanns. & Link. Subsequently, Sutton (1988) proposed a revised classification with four subspecies of *L. amethystea* and included the species in subsect. *Supinae*. This author subordinated *L. multipunctata* as a subspecies of *L. amethystea* and treated var. *albiflora* as a synonym of subsp. *amethystea* (see Table 1). The four subspecies recognized by Sutton were *L. amethystea* subsp. *amethystea*, *L. amethystea* subsp. *multipunctata* (Brot.) Chater & D.A.Webb, *L. amethystea* subsp. *broussonetii* (Poir.) Malato-Beliz and *L. amethystea* subsp. *ignescens* (Kunze) D.A.Sutton. The latter was not recognized by Valdés (1970), as he considered it lacked enough distinctive characters to be regarded as separate from *broussonetii*. Sutton's (1988) treatment was adopted by *Flora iberica* (Sáez & Bernal, 2009). Later studies have notably complicated this classification. In addition to the four subspecies of *L. amethystea* accepted by Sutton (1988), three others have been described: *L. amethystea* subsp. *albiflora* (Boiss.) Casim.-Sor.Solanas & Cabezudo, *L. amethystea* subsp. *toloxense* Casim.-Sor.Solanas & Cabezudo and *L. amethystea* subsp. *aedoi* J.F.Jiménez et al.

(Casimiro-Soriguer Solanas & Cabezudo, 2019; Jiménez et al., 2020). Additionally, two other species morphologically similar to *L. amethystea* have been recently separated from it: *L. pseudamethystea* Blanca et al. (Blanca et al., 2023) and *L. semialata* D.López et al. (Jiménez et al., 2020).

Thus, in this study the "*L. amethystea* complex" is considered to be composed of nine taxa in total (Fig. 1), including seven taxa currently considered subspecies of *L. amethystea* (subsp. *amethystea*, subsp. *aedoi*, subsp. *albiflora*, subsp. *toloxense*, subsp. *multipunctata*, subsp. *ignescens* and subsp. *broussonetii*) and two treated as separate species (*L. pseudamethystea* and *L. semialata*). Table 2 summarizes the main morphological differences between these taxa, based on the available literature (Valdés, 1970; Sutton, 1988; Sáez & Bernal, 2009; Casimiro-Soriguer Solanas & Cabezudo, 2019; Jiménez et al., 2020; Blanca et al., 2023), while their geographic distributions are illustrated in Fig. 2.

The type subspecies, *L. amethystea* subsp. *amethystea*, is widely distributed throughout much of the Iberian Peninsula, from sea level to 2500 m a.s.l., occupying a broad range of habitats, such as therophytic grasslands, shrub clearings, forests, cultivated fields and coastal sandy areas (Fig. 2; Sáez & Bernal, 2009). Other subspecies have more restricted distributions (Fig. 2). The subsp. *albiflora* is considered endemic to the southern Iberian Peninsula, ranging from 0 to 1000 (1300) m. It is commonly found to the south of the Guadalquivir river, in the Baetic Mountains and adjacent regions. It inhabits therophytic grasslands, from shrub clearings to cultivated fields, with an affinity for calcareous substrates, similar to subsp. *aedoi*. The latter is endemic to the Southern Iberian Plateau, between 500 and 1050 m in elevation (Jiménez et al., 2020; Blanca et al., 2023). The subsp. *toloxense*, also associated with therophytic grasslands, can be found at much higher altitudes (1650 to 1800 m) on soils derived from the decalcification of limestones, in open formations dominated by *Quercus faginea* Lam. This subspecies has a much more restricted distribution, being endemic to Sierra de las Nieves, in the province of Málaga (Casimiro-Soriguer Solanas & Cabezudo, 2019). The subsp. *ignescens* occurs in the southernmost part of the Peninsula, in the province of Cádiz, in calcareous sands from sea level to 1000 m a.s.l. The subsp. *multipunctata* is distributed in Portugal, in the provinces of Beira Litoral and Estremadura (Sáez & Bernal, 2009). Finally, the subsp. *broussonetii* is the only present in northern Africa, occurring across a wide range of altitudes in northern, central and western Morocco (Sutton, 1988). One of the most prominent variable characters among subspecies of *L. amethystea* is corolla colour (Fig. 1): violet in subsp. *amethystea*; white with a violet spur in *albiflora*, *aedoi* and *toloxense*; and

yellow with a violet or orange spur in *multipunctata*, *ignescens* and *broussonetii*. Other distinctive characters are listed in Table 2.

The two morphologically similar taxa currently considered distinct species occur in the southern Iberian Peninsula (Fig. 2). *Linaria semialata* is endemic to the provinces of Murcia and Almería, in southeastern Spain, at altitudes between 750 and 1200 m a.s.l. It is found on volcanic soils containing dolorite, in therophytic grasslands intermixed with steppes, shrublands and holm oak clearings (Jiménez et al*.,* 2020). *Linaria pseudamethystea* is considered endemic to the southwestern Iberian Peninsula, from eastern Sevilla to southern Portugal. It grows in therophytic grasslands with poorly developed siliceous soils between 50 and 300 m a.s.l. (Blanca et al*.,* 2023). Both species exhibit violet corollas similar to those of *L. amethystea* subsp. *amethystea*, but can be distinguished by seed and indumentum characters (Table 2).

The main aim of this study was to carry out a phylogenetic and evolutionary analysis of the "*Linaria amethystea* complex", with the following specific objectives: (1) to determine whether the complex and the species *L. amethystea* form monophyletic groups; (2) to verify whether the current taxonomy is supported by phylogenetic relationships; and (3) to conduct a first evaluation of evolutionary patterns of the group. DNA sequencing methods were used to estimate phylogenetic trees based on plastid and nuclear sequences, along with a haplotype network derived from plastid sequences. The working hypothesis was that the complex and the species do not form a monophyletic group, and therefore, the taxonomy requires a revision integrating morphological characters and phylogenetic relationships.

MATERIALS AND METHODS

Sampling

All nine taxa considered part of the *L. amethystea* complex were included in this study. We sampled 32 individuals representing the full distribution range of the eight Iberian taxa (Online Resource 1). For 30 of these, green tissue was collected in the field and preserved in silica gel, whereas for the remaining two, samples were provided by the MA herbarium (Real Jardín Botánico, CSIC, Madrid). For the North African taxon *L. amethystea* subsp. *broussonetii*, sequences from one individual were already available in GenBank (see below). Additionally, we sampled 6 individuals representing 5 taxa from subsect. *Saxatile* considered closely related to the *L. amethystea* complex (Gorospe et al., 2020): *L. diffusa* Hoffmanns. & Link, *L. glauca* (L.) Chaz., *L. intricata* Coincy, *L. munbyana* Boiss. & Reut. and *L. saxatilis* (L.) Chaz. For these, leaf samples were collected in the field, except for the *L. intricata*

sample, which was obtained from the MA herbarium. Voucher specimens were deposited in the MA herbarium.

DNA extraction, amplification and sequencing

DNA extraction was conducted following a modified CTAB protocol (Doyle & Doyle, 1987; Cullings, 1992) and using between 10 and 20 mg of green tissue per sample. The plastid DNA (ptDNA) regions *rpl*32-*trn*L, *trn*S-*trn*G and *trn*L-*trn*F, along with the nuclear region ITS, were targeted for amplification and sequencing using standard primers (see Blanco-Pastor & Vargas, 2013 for details). These regions have been proven useful in previous phylogenetic studies of the genus *Linaria*, and particularly in sect. *Supinae* (Blanco-Pastor et al., 2012; Blanco-Pastor & Vargas, 2013; Fernández-Mazuecos et al., 2013). DNA regions were PCR-amplified in 25-µl reactions containing 2.5 µl of buffer (10x), 1.4 µl of $MgCl_2$ (50 mM), 1.6 µl of dNTPs (2.5 mM each), 0.5 µl of each primer (10 µM), 0.3 µl of BIOTAQ polymerase (5 U/µl; Bioline, London, UK), 1 µl of BSA (1 mg/ml), 1 µl of DNA, and 16.2 µl of Milli-Q water. PCR reactions were run on a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using standard PCR conditions used in previous studies (Fernández-Mazuecos & Vargas, 2011; Fernández-Mazuecos et al., 2013), with 30 cycles and annealing temperatures of 52-56ºC. Gel electrophoresis was used to confirm successful DNA amplification. The amplified products were sent to Macrogen Inc. (Madrid, Spain) for Sanger sequencing using both primers.

Sequence assembly

The chromatograms were reviewed, edited and assembled using Geneious Prime 2025.1.1 (Kearse et al., 2012). Forward and reverse reads were assembled into consensus sequences using the built-in assembler with the highest sensitivity settings. In addition, sequences from the same DNA regions were retrieved from GenBank (National Center for Biotechnology Information, USA), representing almost all species in sect. *Supinae*, as well as the outgroup species *L. purpurea* (L.) Mill. from *Linaria* sect. *Speciosae* (Benth.) Wettst., in agreement with previous phylogenetic studies (Gorospe et al., 2020). The sequences used, together with their GenBank accession numbers, are listed in Online Resource 2. Newly generated sequences were also deposited in GenBank (see Online Resource 1 for accession numbers). Sequences for each DNA region were independently aligned using the MAFFT 7.490 algorithm (Katoh & Standley, 2013). Then, the three ptDNA alignments (*rpl32-trn*L*, trn*S-*trn*G and *trn*L-*trn*F) were concatenated into a single matrix.

Phylogenetic trees

Phylogenetic trees were generated for the concatenated ptDNA matrix (*rpl32*-*trn*L*, trn*S-*trn*G and *trn*L-*trn*F) and for the nuclear ITS region. Trees resulting from the three ptDNA regions separately were consistent with each other in preliminary analyses, albeit with different levels of resolution (results not shown), supporting their concatenation. Phylogenetic analyses were conducted using both maximum likelihood (ML) and Bayesian inference (BI). ML was implemented in RAxML 8.2.11 (Stamatakis, 2014) within Geneious Prime, employing the 'Rapid bootstrapping and search for the best-scoring ML tree' option. For the concatenated matrix, partitions corresponding to the three DNA regions were defined. A thousand bootstrap replicates were run, and the GTRGAMMA nucleotide substitution model was used (given that this software does not allow different models across partitions). BI was implemented in MrBayes 3.2.7a (Huelsenbeck & Ronquist, 2001). The best-fitting nucleotide substitution model was estimated for each DNA region using jModelTest 2.1.10 (Darriba et al., 2012) under the Akaike information criterion (AIC), which assigned the models GTR+G, GTR, HKY and SYM+I+G to *rpl32*-*trn*L*, trn*S-*trn*G, *trn*L-*trn*F and ITS, respectively. For each analysis, two independent runs with four Markov Chain Monte Carlo (MCMC) chains each (three heated and one cold) were conducted for 10 million generations, sampling every 1,000 generations, with a burn-in of 10%. Tracer v.1.7 (Rambaut et al., 2018) was used to confirm that effective sample sizes (ESS) for all parameters were above the standard threshold of 200. Fifty-percent majority-rule consensus trees with posterior probabilities at nodes were inferred. All trees were rooted using *L. purpurea* as the outgroup and edited using FigTree 1.4.4 (Rambaut, 2018).

Phylogenetic dating of the plastid phylogeny

Given the higher support values of the concatenated ptDNA phylogeny in the maximum likelihood analyses (see Results), phylogenetic dating was performed using only the concatenated ptDNA sequences. To this end, a single specimen of each taxon was selected. A single secondary calibration point with a uniform distribution was used, based on the divergence times estimated by Fernández-Mazuecos et al. (2019) for the tribe Antirhineae, specifically the divergence between sect. *Supinae* and the outgroup (10.07–16.85 Ma). The best-fitting substitution models were estimated based on AIC values in jModelTest. The GTR+G model was selected for the *rpl*32-*trn*L region, GTR for *trn*S-*trnG*, and HKY for *trn*L-*trn*F. Subsequently, two Bayesian analyses were conducted using BEAST 1.10.5

(Suchard et al., 2018), each consisting of 10 million generations. A birth-death tree model and an uncorrelated lognormal relaxed molecular clock were applied. Samples were recorded every 1,000 generations, with a 10% burn-in. Tracer 1.7.2 (Rambaut et al., 2018) was used to ensure ESS values above 200 for all parameters. The two runs were then combined with LogCombiner v1.10.4, and a maximum clade credibility tree was calculated using TreeAnnotator v.1.10.4, both part of the BEAST package. Finally, the resulting tree was visualised and edited using FigTree.

Haplotype network

To further investigate the evolutionary relationships within the clade in subsect. *Saxatile* that included most samples of the *L. amethystea* complex (see Results), a haplotype network was inferred using the concatenated ptDNA matrix. This clade received high bootstrap support in previous analyses but showed very poor internal resolution (see Results). The analysis included most of the subsect. *Saxatile*, except for early-diverging lineages (*L. oligantha* Lange, *L. alpina* (L.) Mill., and *L. alpina* subsp. *filicaulis* (Boiss. ex Leresche & Levier) M.Laínz). The network was generated using TCS 1.21 (Templeton et al., 1992; Clement et al., 2000) under the statistical parsimony method, with a connection limit of 95%, and gaps treated as missing data; thus, only nucleotide substitutions were considered.

RESULTS

Phylogenetic trees

The *rpl*32-*trn*L, *trn*S-*trn*G, *trn*L-*trn*F, and ITS sequence matrices (Online Resource 3) had aligned lengths of 755, 575, 307, and 616 bp, respectively. The ML and BI analyses yielded consistent results. The ML tree obtained from the concatenated ptDNA matrix is shown in Fig. 3, with both bootstrap support (BS) values from the ML analysis (above branches) and Bayesian posterior probabilities (PP) from the BI analysis (below branches). In this tree, the three subsections of sect. *Supinae* (*Saxatile*, *Supinae* and *Arvenses*) were recovered as supported clades. Subsect. *Saxatile* received very high support (BS = 100%; PP = 1), whereas the clade grouping the subsections *Arvenses* and *Supinae* received lower support in the ML than in the BI analysis (BS = 68%; PP = 1). Within this clade, the clades corresponding to each subsection were strongly supported, with BS = 98% / PP = 1 for *Arvenses* and BS = 100% / PP = 1 for *Supinae*.

Both samples of *L. pseudamethystea* were found to be phylogenetically distant from all samples of *L. amethystea*, forming what we refer to as clade A (BS = 92%; PP = 1; Fig. 3),

within subsect. *Supinae*. These samples were grouped with two North African subspecies of *L. trisitis* (L.) Mill. (*L. tristis* subsp. *mesatlantica* D.A.Sutton and *L. tristis* subsp. *pectinata* (Pau & Font Quer) Maire) in a well supported clade (BS = 97%; PP = 1).

In contrast, most of the taxa of the *L. amethystea* complex formed a poorly resolved clade C within subsect. *Saxatile* (BS = 86%; PP = 1; Fig. 3). These taxa include *L. semialata* and most of the subspecies of *L. amethystea*, except for *L. amethystea* subsp. *broussonetii*, which was grouped (still within subsect. *Saxatile*) with *L. munbyana* in clade B (BS = 70%; PP = 0.99). Other taxa besides those of the *L. amethystea* complex were also included in clade C, specifically *L. saxatilis*, *L. intricata*, *L. coutinhoi* Valdés, *L. diffusa*, *L. huteri* Lange*, L. bipunctata* (L.) Chaz. and *L. glauca* subsp. *olcadium* Valdés & D.A.Webb, while the other two individuals of *L. glauca* displayed an earlier divergence within subsect. *Saxatile* (BS = 67%; PP = 0.97). The Clade C polytomy comprised 24 individual samples and two subclades: a clade supported by BI (BS = 63%; PP = 0.99) including all individuals of *L. amethystea* subsp. *multipunctata*, *L. saxatilis*, *L. diffusa* and *L. bipunctata*, together with some individuals of *L. amethystea* subsp. *amethystea*; and the other, with high support in both ML and BI analyses (BS = 95%; PP = 1), comprising most of the individuals of *L. amethystea* subsp. *aedoi*, with the exception of one sample.

The nuclear ITS tree (Online Resource 4) exhibited very low resolution and low support values, but broadly recovered the three subsections in sect. *Supinae*, albeit with a slightly different composition. Two of the three distinct clades of the *L. amethystea* complex were recovered with good statistical support: Clade A, which grouped the two samples of *L. pseudamethystea* (BS = 100%; PP = 1); and clade B, which grouped *L. amethystea* subsp. *broussonetii* with *L. munbyana*, BS = 80%; PP = 1). Clade C was not recovered in this tree.

Divergence times

The time-calibrated phylogenetic tree based on ptDNA sequences (Fig. 4) showed high statistical support (posterior probability, PP = 1) for the three subsections of *Linaria* subsect. *Supinae*, and their relationships are consistent with those obtained in the maximum likelihood phylogenetic tree.

The stem age of subsect. *Saxatile* was estimated to date back to the Miocene (8–15.8 Ma), whereas the divergence of extant taxa of this subsection (crown age) appears to have started between the late Miocene and the early Pleistocene (1.8–8 Ma; PP = 1). Similarly, the divergence between subsect. *Supinae* and subsect. *Arvenses* would have also occurred during the Miocene (5.4–13.8 Ma; PP = 0.9), while their crown ages are distributed between the

Miocene and the Pleistocene, 2.1–8.8 Ma (PP = 1) for subsect. *Arvenses*, and 1–6.1 Ma (PP = 1) for subsect. *Supinae*.

Most sampled taxa appear to have diverged from their closest relatives during the Quaternary, which, in combination with the low statistical support of terminal nodes, indicates a very recent divergence. The estimated age for the most recent common ancestor of clade C (including *L. semialata* and all subspecies of *L. amethystea* except for *L. amethystea* subsp. *broussonetii*, together with 7 other taxa) clearly fell within the Pleistocene (0.24–1.67 Ma; PP = 1).

Plastid haplotype network

In the haplotype network analysis of subsect. *Saxatile* (Fig. 5), 18 haplotypes were detected, numbered 1-18. For most of the taxa in the *L. amethystea* complex, haplotype 10 was obtained in one or more individuals, more specifically for *L. amethystea* subsp. *albiflora*, *L. amethystea* subsp. *amethystea*, *L. amethystea* subsp. *ignescens* and *L. amethystea* subsp. *toloxense*, *L. amethystea* subsp. *aedoi* and *L. semialata*. Additionally, haplotype 10 was found in *L. intricata*, *L. diffusa* and *L. huteri.* This haplotype occupied a central position in the network, connecting to six other haplotypes.

Haplotype 14 was obtained for all individuals of *L. amethystea* subsp. *multipunctata* and *L. saxatilis*, and some individuals of *L. amethystea* subsp. *albiflora* and *L. amethystea* subsp. *amethystea*. This haplotype formed a lineage with haplotypes 15 and 16 (found in *L. amethystea* subsp. *amethystea* and *L. bipunctata*, respectively), connecting to central haplotype 10.

All samples of *L. amethystea* subsp. *aedoi* were grouped in haplotypes 17 and 18 (except for one individual in which haplotype 10 was found), forming a lineage separated by at least three nucleotide substitutions from the central haplotype 10. *Linaria amethystea* subsp. *broussonetii* was the only taxon in the complex in which haplotype 7 was found, forming a lineage with haplotypes 8 and 9 (both corresponding to *L. munbyana*). In *L. amethystea* subsp. *ignescens*, haplotypes 10 and 13 were found, separated from haplotype 10 by a single substitution. Finally, *L. amethystea* subsp. *amethystea* was found scattered across the network in four haplotypes (9, 10, 11 and 15).

DISCUSSION

Polyphyly and convergence in the *Linaria amethystea* complex

This study provides the first detailed phylogenetic analyses of the *Linaria amethystea* complex within *Linaria* sect. *Supinae*, allowing the evaluation of its current taxonomic classification and, in particular, the monophyly of the complex and its constituent species. We focus on the phylogenetic relationships inferred from ptDNA sequences (Figs. 3-5), as they show higher resolution and support values than those obtained from nuclear ITS sequences (Online Resource 4). The ptDNA analyses, including new sequences from 38 individuals, are consistent with previous studies regarding the major clades within sect. *Supinae*, as the three subsections (*Saxatile*, *Arvenses* and *Supinae*) are recovered as strongly supported clades (Blanco-Pastor et al*.,* 2012; Blanco-Pastor & Vargas, 2013; Yousefi et al., 2017). Most of the *L. amethystea* complex, as initially defined here, falls within subsect. *Saxatile*, which agrees with previous phylogenetic analyses based on a limited number of samples from the group (Blanco-Pastor & Vargas, 2013; Fernández-Mazuecos et al., 2013; Fernández-Mazuecos et al., 2019; Gorospe et al., 2020). Our study expands these analyses by incorporating eight additional taxa that had not been previously sequenced.

The exception to the general pattern is *L. pseudamethystea*, a recently-described species that is morphologically similar to *L. amethystea* (Blanca et al., 2023). Remarkably, our results reveal that this species is phylogenetically distant from the remaining taxa of the complex, as it is consistently recovered in a different subsection (*Supinae*) (Fig. 3). *Linaria pseudamethystea* and the remaining taxa of the complex belong to clades that appear to have diverged during the Miocene (Fig. 4). Although unexpected, this separation is strongly supported in the ptDNA tree and confirmed by the sequencing of two individuals from geographically distant populations (Fig. 3). The nuclear ITS tree (Online Resource 4) revealed a similar pattern, albeit with lower statistical support.

Morphologically, *L. pseudamethystea* resembles *L. amethystea* subsp. *amethystea*, differing mainly in the absence of two characters considered diagnostic of the *Amethystea* group by Valdés (1970): seed-wing thickening and tubercles on the seed disc (Blanca et al., 2023). Additional differences include the calyx indumentum (glabrous in *L. pseudamethystea*) and the curvature of the nectar spur (more pronounced in *L. pseudamethystea*). Prior to the description of *L. pseudamethystea*, these traits had apparently been overlooked, resulting in the frequent identification of these plants as *L. amethystea* subsp. *amethystea*. Most of the similarities between the two taxa concern floral morphology, including violet corollas of similar size, with darker violet veins and a yellow spot on the palate. These similarities could be explained by evolutionary convergence, whereby different lineages of sect. *Supinae* have independently evolved similar floral traits, possibly in response to similar selective pressures.

Comparable convergence patterns have been inferred in other Iberian *Linaria* species, such as those in sect. *Versicolores* (Fernández-Mazuecos et al., 2018). Notably, *L. algarviana* Chav. is a species of sect. *Versicolores* also endemic to southwestern Iberia (Algarve) that exhibits a similar corolla colour pattern to that of *L. amethystea* subsp. *amethystea* and *L. pseudamethystea* (Sáez & Bernal, 2009). The similarities in corolla coloration among these three phylogenetically distant taxa could represent evidence of convergent floral evolution within different clades of *Linaria* in the same geographical region.

All remaining species of the *L. amethystea* complex are clearly recovered within the subsect. *Saxatile* clade in the ptDNA tree, and most of them are part of an unresolved polytomy. The only exception is the North African *L. amethystea* subsp. *broussonetii*, which appears to be closely related to *L. munbyana*, a species distributed across the southern Iberian Peninsula and North Africa (Fig. 3). The sister relationship between these two taxa is also recovered in the nuclear ITS tree (Online Resource 4). In the ptDNA haplotype network (Fig. 5), they form a distinct haplotype lineage. These results suggest a certain evolutionary distance between subsp. *broussonetii* and the remaining subspecies of *L. amethystea*. In addition, it is worth noting the similarity in seed-wing morphology between *L. amethystea* subsp. *broussonetii* and *L. munbyana*, both of which have a slightly thickened wing (Sutton, 1988; Sáez & Bernal, 2009).

Overall, these results indicate that both the *L. amethystea* complex (as defined here) and the species *L. amethystea* are polyphyletic, reflecting evolutionary convergence. The treatment of *L. pseudamethystea* as a distinct species is clearly supported, in agreement with Blanca et al. (2023). Similarly, the North African taxon *L. amethystea* subsp. *broussonetii* could potentially be recognized as a distinct species, for which a name already exists, *L. broussonetii* (Poir.) Steud. However, further sampling of this taxon would be desirable to confirm this treatment.

Radiation and divergence in *Linaria* subsect. *Saxatile*

Phylogenetic dating based on ptDNA sequences (Fig. 4) estimated that most speciation events leading to the extant species of subsections *Supinae* and *Saxatile* occurred during the Quaternary. Both clades have undergone rapid diversification in recent times and can therefore be considered radiations, consistent with previous studies (Blanco-Pastor & Vargas, 2013; Fernández-Mazuecos et al., 2019; Gorospe et al., 2020). In particular, Blanco-Pastor & Vargas (2013) estimated that diversification within sect. *Supinae* occurred mostly in the Iberian Peninsula during the Quaternary, following the establishment of the Mediterranean

climate, which is consistent with the divergence times estimated in this study (Fig. 4). The Quaternary is characterized by alternating cold and warm episodes, which may have played an important role in the parallel radiations of this group. Many taxa belonging to subsections *Supinae* and *Saxatile* are narrow endemics (Blanco-Pastor & Vargas, 2013) and, given that their divergence postdates the Pliocene-Pleistocene boundary, can be considered neoendemics (Médail & Baumel, 2018).

The low phylogenetic resolution within subsect. *Saxatile*, as evidenced by the large clade C polytomy in the ptDNA tree (Fig. 3) and the low support values in the time-calibrated tree (Fig. 4), may be partly explained by recent diversification. Given the Quaternary diversification of clade C, its constituent taxa may not yet have accumulated sufficient changes in the DNA regions analysed to resolve their relationships.

In contrast to the limited DNA sequence variation, the species of clade C (including those of the *L. amethystea* complex) exhibit considerable morphological and ecological diversity that appears to have evolved over a relatively short period. For example, *L. amethystea* subsp. *multipunctata* and *L. saxatilis*, which share the same ptDNA haplotype (Fig. 5), differ markedly in both morphology and ecology. *Linaria saxatilis* is a perennial species with small flowers, generally high pubescence, except at the base of the stem, and no thickened seed wing. In contrast, *L. amethystea* subsp. *multipunctata* is an annual species with larger flowers, pubescence restricted to the inflorescence, and a thickened seed wing (Sáez & Bernal, 2009). This type of morphological and ecological variation is observed across the clade C polytomy of subsect. *Saxatile*, including differences in flower colour, indumentum, seed morphology and habitat (see Table 2). Differences in seed morphology are particularly noteworthy, as these characters have historically been used for the identification of *Linaria* species (Valdés, 1970; Sutton, 1988; Sáez & Bernal, 2009). Indeed, clade C includes taxa with diverse seed wing morphologies: well-developed and thickened (*L. amethystea* subspecies), well-developed and thin (*L. saxatilis*, *L. diffusa*), narrow (*L. semialata*), and reduced to a marginal ridge (*L. huteri*, *L. coutinhoi*, *L. intricata*, *L. bipunctata*). The considerable variability of these traits among taxa so closely related suggests that traditional taxonomic criteria may not adequately reflect the evolutionary relationships among these taxa.

Focusing on corolla colour, no clear evolutionary pattern was found within subsect. *Saxatile* or, more specifically, among taxa of the *L. amethystea* complex. Although the limited resolution does not allow reliable ancestral state reconstruction, corolla colour appears to have evolved several times independently. Based on preliminary field observations, an

ecological pattern may be associated with corolla colour variation. In general, white-flowered taxa tend to occur on basic soils, whereas those with violet corollas appear to occur on more acidic soils (see Introduction; Fig. 2). It is worth noting that some white-flowered populations southeast of Madrid, which mainly occur on gypsum substrates, may be identified as *L. amethystea* subsp. *albiflora*, subsp. *aedoi* or subsp. *amethystea* depending on the authors and identification keys consulted (Casimiro-Soriguer Solanas & Cabezudo, 2019; Jiménez et al., 2020; Blanca et al., 2023). According to our ptDNA phylogenetic tree and haplotype network, most of these populations appear to be referable to *L. amethystea* subsp. *aedoi*, which would be in agreement with the distribution map proposed by Blanca et al. (2023). This example highlights the continued uncertainty surrounding the taxonomic delimitation and distribution of certain taxa.

Discordance between plastid and nuclear patterns

In evolutionary radiations, phylogenetic discordance between nuclear loci, and between organellar and nuclear sequences (Wang et al., 2014), is frequent. In our study, the plastid and nuclear phylogenetic trees displayed discordant topologies (Fig. 3; Online Resource 4), although the low resolution and support values of the ITS tree prevents a robust comparison. Discordance may arise from multiple causes. In *Linaria* sect. *Supinae*, incomplete lineage sorting (ILS) was demonstrated by Banco-Pastor et al. (2012) using coalescent simulations based on three independent loci, including ptDNA sequences from two of the same regions analysed in the present study (*rpl*32-*trn*L and *trn*S-*trn*G). Under ILS, phylogenetic discordance arises from the retention of ancestral polymorphisms through speciation events. In *Linaria* sect. *Supinae*, ILS appears to be associated with rapid speciation rather than large ancestral population sizes (Blanco-Pastor et al., 2012). Hybridization is another potential source of phylogenetic discordance, and it is well documented in *Linaria*, with numerous hybrids described (Valdés, 1970; Sáez & Bernal, 2009). Specifically, Blanco-Pastor et al. (2012) also inferred a widespread pattern of hybridization within sect. *Supinae*, even between geographically non-overlapping species, indicating that reproductive barriers among taxa are weak.

While Blanco-Pastor et al. (2012) provided evidence for both ILS and hybridization during the evolutionary history of *Linaria* sect. *Supinae*, the analyses conducted in the present study did not evaluate these processes for the taxa of the *L. amethystea* complex and their relatives. Further research is needed to resolve the complex taxonomy of *Linaria* sect. *Supinae* (Sáez & Bernal, 2009) and to understand the evolutionary patterns of its diagnostic characters within a

robust phylogenetic framework, while accounting for the discordance associated with recent radiations, including that caused by ILS and hybridization. The use of high-throughput sequencing techniques in future studies may improve phylogenetic resolution and help overcome the limitations encountered here, as demonstrated in *Linaria* sect. *Versicolores* (Fernández-Mazuecos et al., 2018; Farminhão et al., 2026).

CONCLUSIONS

This study has significantly contributed to the phylogenetic knowledge of the taxa included in the *L. amethystea* complex and of *Linaria* sect. *Supinae* as a whole. Although resolution of terminal relationships was limited, the three subsections of sect. *Supinae* (*Saxatile, Arvenses* and *Supinae*) were recovered with good statistical support, and major evolutionary patterns of the study taxa were identified, including some unexpected relationships.

Based on our results, the following conclusions can be drawn: (1) *Linaria pseudamethystea* is part of the *Linaria* subsect. *Supinae* clade and is therefore evolutionarily distant from *L. amethystea* (subsect. *Saxatile*). The morphological similarities between these taxa are likely the result of evolutionary convergence. (2) *L. amehtystea* subsp. *broussonetii* does not appear to be closely related to other subspecies of *L. amethystea*, but rather to *L. munbyana*. This suggests that it should be treated as a different species named *L. broussonetii*, although additional sampling is desirable to confirm this. (3) The Iberian subspecies of *L. amethystea* appear to have undergone recent radiation during the Quaternary, as part of a clade that includes other taxa of *Linaria* subsect. *Saxatile*. This has resulted in limited genetic variability within this clade (which limits phylogenetic resolution) but a remarkable morphological and ecological diversity. These findings highlight the need for future evolutionary studies integrating high-throughput sequencing of subsect. *Saxatile* taxa with a revision of their morphological and ecological traits.

Finally, we highlight the importance of including underrepresented lineages in phylogenetic studies, as they may provide valuable insights into evolutionary patterns and processes, including those shaping the Mediterranean flora.

Information on Electronic Supplementary Material

Online Resource 1. *Linaria* samples sequenced in this study, including collection localities, voucher information, and GenBank accession numbers for plastid and nuclear DNA sequences.

Online Resource 2. *Linaria* sequences obtained from GenBank, including accession numbers for plastid and nuclear DNA sequences.
Online Resource 3. DNA sequence alignments of plastid and nuclear regions.
Online Resource 4. Maximum-likelihood phylogenetic tree of *Linaria* sect. *Supinae* based on the nuclear DNA region ITS.

Supplementary Information The online version contains supplementary material.

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Author contributions Conceptualization: MFM, AA; Data curation: SC; Formal analysis: SC, MFM; Funding acquisition: MFM; Investigation: All authors; Visualization: SC; Writing - original draft: SC; Writing - review and editing: All authors.

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Data availability All DNA sequences used in this study are available in GenBank (National Center for Biotechnology Information, USA; see Online Resources 1 and 2 for accession numbers). The aligned matrices are available in Online Resource 3.

Statements and declarations

Conflict of interest MFM is an Associate Editor of *Plant Systematics and Evolution*. The authors declare no conflict of interest.

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Figure



Fig. 1 Taxa included in the *Linaria amethystea* complex. (A) *L. amethystea* subsp. *amethystea*, (B) *L. amethystea* subsp. *albiflora*, (C) *L. amethystea* subsp. *aedoi,* (D) *L. amethystea* subsp. *toloxense*, (E) *L. amethystea* subsp. *multipunctata*, (F) *L. amethystea* subsp. *ignescens*, (G) *L. amethystea* subsp. *broussonetii*, (H) *L. pseudamethystea*, (I) *L. semialata*. A-F and H by Alejandro Alonso; G by José Quiles; I by Julián Fuentes

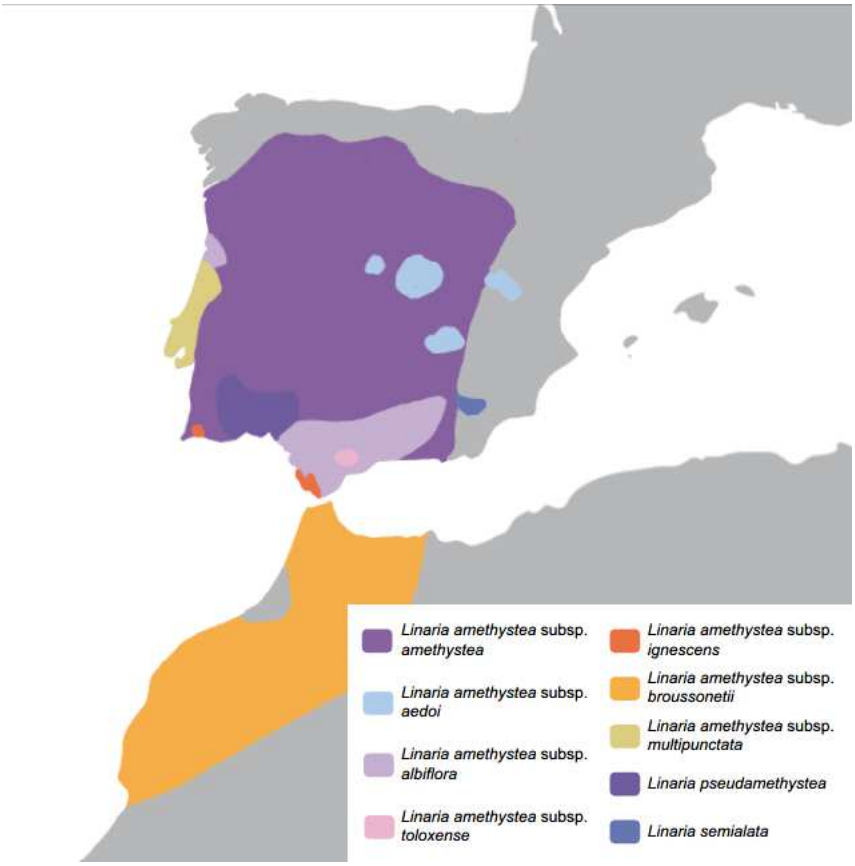


Fig. 2 Geographic distribution of taxa included in the *Linaria amethystea* complex. Based on Blanca et al. (2023) for the Iberian Peninsula and Sutton (1988) for northwestern Africa

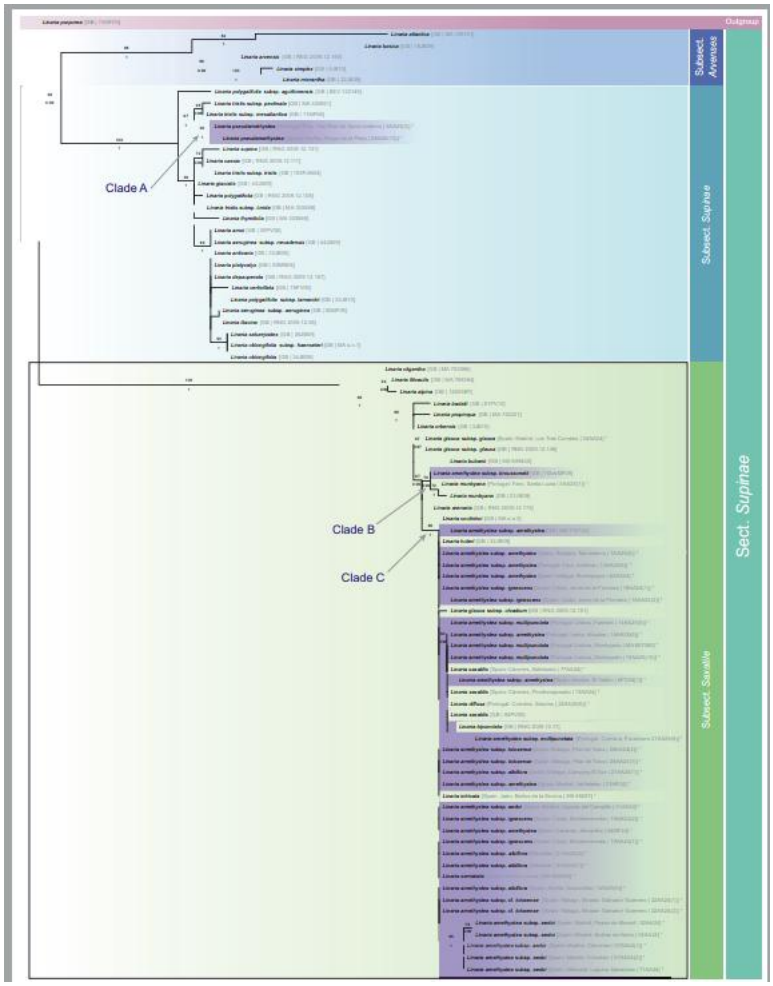


Fig. 3 Maximum-likelihood (ML) phylogenetic tree of *Linaria* sect. *Supinae* (with *L. purpurea* as the outgroup) based on the ptDNA regions *rpl32-trn*L, *trn*S-*trn*G and *trn*L-*trn*F. Statistical support values are shown for nodes with both ML bootstrap support (BS) > 50% and Bayesian posterior probability (PP) > 0.5. BS values are shown above branches, and PP values below them. Taxa included in the *L. amethystea* complex are highlighted in purple, and the three subsections are highlighted in green (*Saxatile*), cyan (*Supinae*) and blue (*Arvenses*). Individuals sequenced in this study are marked with an asterisk, and their locality and voucher information are provided (see Online Resource 1). Individuals with sequences retrieved from GenBank are labelled "GB" followed by their voucher information. Clades containing taxa belonging to the *L. amethystea* complex are labeled A, B and C to facilitate reference to them in the main text

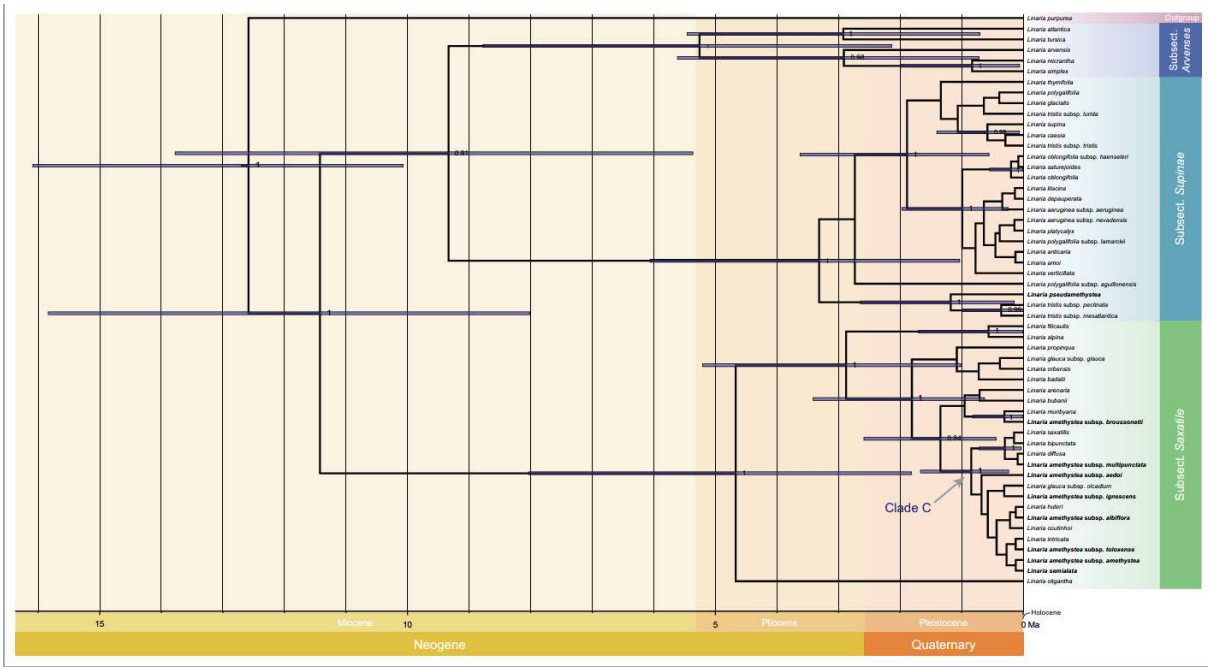


Fig. 4 Phylogenetic dating of sect. *Supinae* based on the ptDNA regions *rpl32-trn*L*, trn*S-*trn*G and *trn*L-*trn*F. The blue bars indicate 95% highest posterior density intervals for divergence times. Bayesian posterior probabilities above 0.9 are shown. The three subsections are indicated in green (*Saxatile*), cyan (*Supinae*) and blue (*Arvenses*) on the right. Taxa included in the *L. amethystea* complex are shown in bold

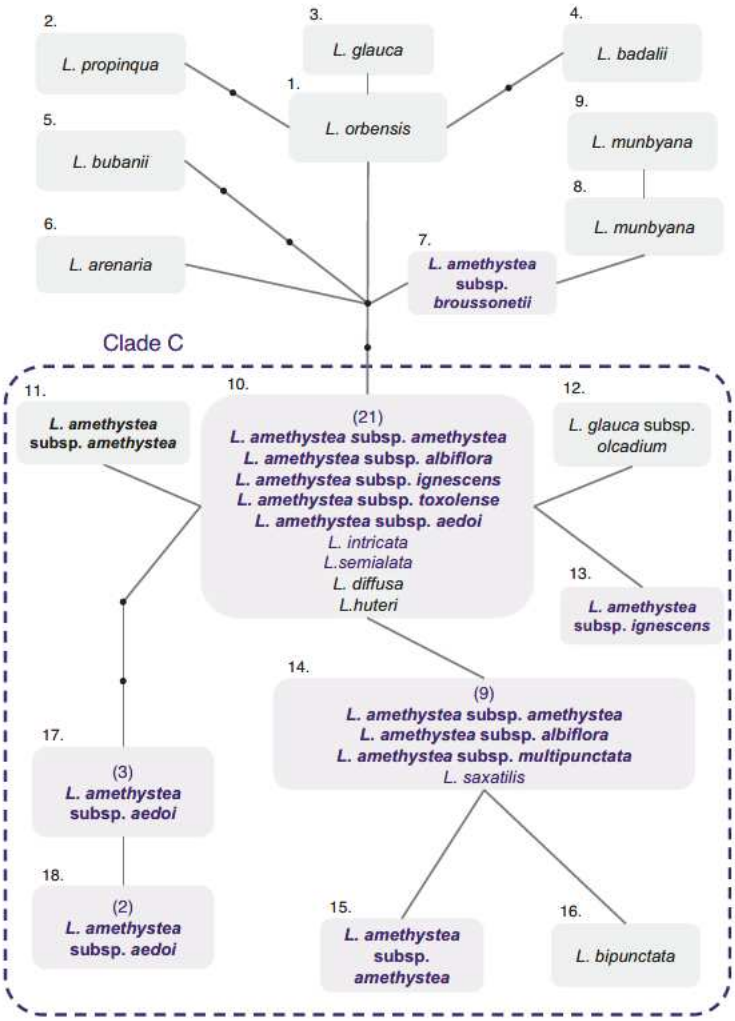


Fig. 5 Haplotype network of *Linaria* subsect. *Saxatile* (excluding early-diverging lineages) based on ptDNA regions *rpl32-trn*L*, trn*S-*trn*G and *trn*L-*trn*F. Each line represents a nucleotide substitution, and each dot represents an extinct or unsampled intermediate haplotype. The haplotypes are numbered 1 to 18 and the taxa sharing each haplotype are indicated. Values in parentheses indicate numbers of sequenced individuals per haplotype (one when no value is given). Taxon names highlighted in purple correspond to samples sequenced in this study, whereas those highlighted in grey correspond to sequences obtained from GenBank. Taxa included in the *L. amethystea* complex are shown in bold. Clade C is delineated by the dotted line

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Table2.docx
- OnlineResource1.docx
- OnlineResource2.docx
- OnlineResource3.zip
- OnlineResource4.docx