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Molecular Relationships in the *Veronica hederifolia* Complex (Plantaginaceae, Veroniceae)

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ABSTRACT

Objective: *Veronica* is a species-rich genus of the Plantaginaceae, comprising over 500 annual and perennial herbs distributed across both hemispheres. Relationships within the *Veronica hederifolia* complex (*V. hederifolia*, *V. cymbalaria*, *V. panormitana*, and *V. triloba*), all belonging to *V.* subg. *Cochlidiosperma*, is unclear due to overlapping morphological features that complicate taxonomic delimitation. This study aimed to clarify species boundaries in this complex.

Method: We analysed 28 sequences from three regions: the nuclear low-copy CYCLOIDEA2 region, the nuclear ribosomal internal transcribed spacer (ITS), and the plastid DNA (*trn*L–*trn*F), in combination with morphological characters. Phylogenetic analyses were performed using Bayesian inference.

Results: Morphological variation was observed in leaf shape, calyx and pedicel orientation, corolla diameter and colour, sepal form, and seed features. The CYC2 marker provided the highest resolution, confirming the monophyly of the complex and distinguishing *V. triloba* as a separate species. *V. cymbalaria* and *V. panormitana* formed sister lineages, with *V. panormitana* as the earliest-diverging taxon. Plastid *trn*L–*trn*F sequences showed limited variability, and ITS data displayed partial incongruence, likely due to hybridization, introgression, and polyploidy.

Conclusions: Integrating molecular and morphological evidence highlights the utility of low-copy nuclear markers in resolving recently diverged and reticulate lineages. The results support distinct evolutionary trajectories within the *V. hederifolia* complex and confirm the importance of CYC2 in species delimitation.

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Introduction

Veronica subg. *Cochlidiosperma* (Rchb.) M.M. Mart. Ort, Albach, and M.A. Fisch. is the smallest subgenus of *Veronica* L. (Plantaginaceae sensu APG IV 2016; formerly part of Scrophulariaceae), belonging to tribe Veroniceae. The subgenus comprises annual herbs distributed across Europe, the Mediterranean, Southwest Asia, and Northern Africa (Albach et al., 2004a; Albach et al., 2008). They are highly similar in appearance and have a complex taxonomy (Albach & Chase, 2001; Albach & Greilhuber, 2004). Based on the phylogenetic analyses of the internal transcribed spacer (ITS) and plastid DNA sequence data, this subgenus is considered monophyletic (Albach et al., 2004a, 2004b). Morphological features in this subgenus include stems usually branched, ranging from glabrous to densely pubescent or glandular; leaves ovate to suborbicular or cordate with serrate or lobed margins; foliose bracts; terminal, mostly racemose inflorescences; a quadripartite calyx sometimes exceeding the corolla; a rotate corolla varying white to pink, violet, or dark blue; tricolpate pollen with perforate to irregularly rugulose tectum; distinctly inflated, globose or broad-elliptic capsules; and 1–4 ovoid, cymbiform seeds with an elaiosome, bearing cristate, corrugate, or alveolate-reticulate coats (Albach et al., 2004a, 2004b; Garnock-Jones et al., 2007).

Parallel evolution of morphological features, such as leaf shape and seed morphology, has occurred repeatedly within *Veronica*, particularly among annual species (Albach et al., 2004d; Hassemer et al., 2021). Such convergent patterns obscure phylogenetic signals and hinder species delimitation in morphologically similar subgenera, such as subgenus *Cochlidiosperm*a. Accurate species identification is critical for taxonomic work, particularly for distinguishing newly evolved or closely related species (Rieseberg et al., 2006; Fazekas et al., 2009). Four species—*V. hederifolia* L., *V. cymbalaria* Bodard, *V. panormitana* Tineo ex Guss., and *V. triloba* Opiz— exemplify these challenges, as they share overlapping morphological features that complicate their delimitation (Chater, 1978; Fischer, 1981; Saeidi-Mehrvarz, 2011). Hereafter, these species are referred to as the *Veronica hederifolia* complex. The Flora of Turkey (Chater, 1978), Flora Iranica (Fischer, 1981), and Flora of Iran (Saeidi-Mehrvarz, 2011) have shown that these species share overlapping morphological characteristics (Table 1).

Morphological delimitation within the *V. hederifolia* complex is hindered by overlapping features, phenotypic plasticity, and the frequent occurrence of polyploidy. Different taxonomic treatments have resulted in inconsistent classifications. For example, although Chater treated *V. triloba* as synonymous with *V. hederifolia* var. *triloba* Opiz, biometric analyses by Cieślak and Mirek demonstrated distinct differences, supporting its species status (Chater, 1978; Cieślak & Mirek, 1996). Polyploidy has played a central role in the evolutionary diversification of *Veronica*, particularly within subgenus *Cochlidiosperma*. Previous cytological and molecular evidence indicates that *V. hederifolia* (2n = 6x = 54) likely originated via allopolyploidy from the tetraploid *V. sublobata* and diploid *V. triloba* (Fischer, 1975a; Albach et al., 2008). Likewise, *V. cymbalaria* appears to have multiple independent origins from different parental lineages, including *V. panormitana* and *V. trichadena* Jord. & Fourr (Hofelich, 1935; Fischer, 1975b; Albach, 2007; Meudt et al., 2015). Such reticulate evolutionary processes, combined with incomplete reproductive isolation, increase the difficulty of resolving species boundaries in this group (Soltis & Soltis, 2009; Meudt et al., 2015).

Earlier phylogenetic studies on *V.* subg. *Cochlidiosperma* have employed nuclear ribosomal internal transcribed spacer (ITS), plastid DNA (cpDNA), the nuclear low-copy CYCLOIDEA2 region (Albach et al., 2004a, 2004b; Albach, 2007; Albach & Meudt, 2010; Meudt et al., 2015). The chloroplast genome is widely used in plant phylogenetics due to its uniparental inheritance

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and generally conserved structure, yet its slow evolutionary rate limits resolution at lower taxonomic levels, especially in groups with recent divergence or hybridization histories (Shaw et al., 2014; Li et al., 2019). Nuclear ribosomal DNA, particularly the ITS region, has also been a standard phylogenetic marker; however, concerted evolution can homogenize paralogous sequences, obscuring historical hybridization events and providing insufficient resolution among recently diverged taxa (Álvarez & Wendel, 2003; Nieto Feliner & Rosselló, 2007). By contrast, the nuclear low-copy genes are biparentally inherited, often exhibit higher sequence variability, and are especially informative for elucidating relationships at both interspecific and intraspecific levels within polyploid complexes (Sang, 2002; Small et al., 2004; Zimmer & Wen, 2015). Contrary to plastid DNA and ITS markers, the use of low-copy nuclear (LCN) markers is particularly useful at the interspecific and intraspecific levels since they define relationships and phylogenetic reconstruction within the species, specifically for elucidating the evolutionary history of polyploids (Sang, 2002; Small et al., 2004). The LCN markers, as biparental nuclear markers, are expected to effectively reveal relationships in polyploid *Veronica* (Mayland-Quellhorst et al., 2016).

This study used the nuclear low-copy CYCLOIDEA2 (CYC2), cpDNA (*trn*L–*trn*F) and ITS markers for phylogenetic analyses. The CYC2 gene plays a significant role in floral asymmetry and has been successfully applied in phylogenetic studies of Veroniceae and other angiosperms (Preston et al., 2009; Albach & Meudt, 2010), offering higher discriminatory power than ITS or cpDNA in closely related or morphologically cryptic taxa. Phylogenetic trees based on *trn*L–*trn*F and ITS markers were constructed and compared with the tree generated from CYC2 data. For the first time, *V. hederifolia*, *V. cymbalaria*, and *V. panormitana* were analysed based on CYC2, while *trn*L–*trn*F and ITS sequences were sourced from GenBank (Hoggard et al., 2003; Albach & Greilhuber, 2004; Albach et al., 2004a; Albach, 2007; Albach & Meudt, 2010). In addition to the four species of the *V. hederifolia* complex, three closely related species, *V. reuterana* Boiss., *V. acinifolia* L., and *V. serpylifolia* L. were included in the phylogenetic analyses to provide a broader context for evaluating species boundaries and to confirm the monophyly of the complex. The goal of this study is to clarify phylogenetic relationships within the *V. hederifolia* complex through the analysis of nuclear and plastid markers, including CYC2, ITS and *trn*L–*trn*F.

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Table 1. The studied species of *V. hederifolia* complex and their overlapping morphological characteristics (Chater, 1978; Fischer, 1981; Saeidi-Mehrvarz, 2011).

Characters	*V. hederifolia*	*V. triloba*	*V. cymbalaria*	*V. panormitana*
Petiole length	up to 12 mm	N/A	5–18 mm	2–12 mm
Leaf shape	Palmatilobate broadly elliptic	Palmatilobate almost Transverse-rectangular	Palmatilobate almost circular ellipsoidal or ovate	Palmatilobate ellipsoidal or ovate
The number of leaves lobes	3–5	3–5	5–9	5–9
Leaf size	8–15×5–10 mm	4–9(-12) ×6–12 (-17) mm	10–15×6–11 mm	9–14 ×5–10 mm
Leaf base	truncate or almost Cordate	truncate or almost Cordate	Cordate, truncate or Cuneiform	Cordate or truncate
Number of leaves	2–4 pairs	N/A	2–4 pairs	1–3 pairs
Pedicle length	4–15 mm	N/A	12–20 mm	up to17 mm
Calyx lengthin fruit state	5–8.5 mm	4–5 (-6) mm	4–6 mm	2.5–4 mm
Corolla diameter	4–7 (-9) mm	4–6 mm	6–12 mm	2–5 mm
Style length	0.6–1.2 mm	0.4–0.8 mm	0.9–1.7 mm	0.4–0.9 mm
Sepal shape	Triangular-subcordate	Triangular-Cordate	ellipsoidal to almost circular, complete	ellipsoidal, often indented at the base
Capsule base	Truncate, subcordate	almost rectangular, subcordate	Truncate, subcordate	truncate subcordate
Capsule size	3–4 ×4.5–6 mm	2.5–3 (-3.5) ×4–5mm	2.5–3.5 × 3.5–4.5mm	2–3 × 3–4 mm
Number of lateral racemes	(5-) 10–15 (-20)	N/A	(5-) 10–20 (30)	(5-) 10–20 (-30)
Seed shape	Ellipsoidal to almost orbicular, corrugated cymbiform	ellipsoidal rectangular	Ellipsoidal.to semiorbicular cymbiform, more or less corrugated	globose cymbiform , almost corrugated
Seed size	1.4–2.9×1.2–2.5 mm	2.2–2.8×1.5–2.2 mm	1.4–2.9×1.2–2.5 mm	2–2.5×2 mm
Number of seeds	2–4	4	2–4	4

* N/A not applicable

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Method

Plant materials

Morphological analyses were performed on nine species from distinct populations of (*V. hederifolia*, *V. triloba*, *V. cymbalaria*, *V. panormitana*, *V. reuterana*, *V. acinifolia*, and *V. serpylifolia*). Voucher specimens and precise collection localities for all species are detailed (Table 2). For the molecular analyses, fresh specimens of *V. hederifolia* were collected and deposited in the IAUM herbarium. Leaf tissues were also obtained from dried specimens preserved in the herbaria of IRAN, MPU, and OHN. Species identification was conducted using authoritative floras, including Flora Europaea (Webb et al., 1972), Flora of Turkey (Chater, 1978), Flora Iranica (Fischer, 1981), and Flora of Iran (Saeidi-Mehrvarz, 2011). *Veronica filiformis* Sm. and *V. polita* Fr. belong to the *V.* subg. *Pocilla* (Dumort.) M.M. Mart. Ort., Albach & M.A. Fisch., were selected as the outgroups in this study based on prior phylogenetic frameworks (Albach et al., 2004c; Meudt & Albach, 2005a; Garnock-Jones et al., 2007). These studies consistently indicate that *V.* subg. *Cochlidiosperma*, which comprises the *V. hederifolia* complex, is phylogenetically distinct from *V.* subg. *Pocilla*. Consequently, these species serve as suitable outgroups for rooting the phylogenetic trees and for polarizing character states within the ingroup species. GenBank accession numbers for all studied species are listed (Table 2).

Table 2. Voucher data for species investigated in the present study, including GenBank accession numbers.

Species	Accession No.			Locality	Voucher
	ITS	trnL-trnF	CYC2		
V. hederifolia	AJ548981	AF510425	in Req.	N/A (ITS) N/A (trnL-trnF) Iran,Khoras, Bojnord, Baba Aman Forest parak 3333.3 FT, N37˚29´4.48˝ E57˚26´ 6.18˝ Ahmadian Yazid, 2019. (CYC2)	Hoggard, RK #508. (ITS) Albach ,589, WU. (trnL-trnF) Ahmadian Yazid, 10322IAUM. (CYC2)
V. triloba	AF509804	AF513333	FJ848290	Turkey, Aphrodisias	Albach ,242WU
V. cymbalaria	AY850104	AY850068	in Req.	N/A (ITS) N/A (trnL-trnF) Iran, Kermanshah, Rijab, Iranshahr. (CYC2)	Fischer ,85-16-28, WU. (ITS) Albach, 235, WU. (trnL-trnF) Iranshahr, 39856/1, IRAN. (CYC2)
V. panormitana	AY850100	AY850080	in Req.	Greece, Olympia. (ITS) Spain, Minoa. (trnL-trnF) N/A (CYC2)	Albach 402, WU. (ITS) Delgado 662, SALA. (trnL-trnF) 1008389, MPU. (CYC2)
V . serpyllifolia	AF313017	AF486400	FJ848284	Germany, Bonn.(ITS) N/A (trnL-trnF), N/A (CYC2)	Albach64, WU. (ITS) N/A (trnL-trnF), N/A (CYC2)
V. acinifolia	AF509798	AF486399	FJ848282	Greece, Lefkas. (ITS) Turkey, Van. (trnL-trnF). N/A(CYC2)	M. Fischer s.n., WU. (ITS) Rechinger 30780,WU. (trnL-trnF). N/A CYC2
V. reuterana	AY540866	AY486447	FJ848283	N/A	
Outgroups					
V. polita	AF509818	PV868881	FJ848301	N/A	N/A
V. filiformis	AF486363	AF486368	FJ848299	N/A	Albach ,858 WU
V. filiformis	N/A	N/A	in Req.	Seweden,Andersson,Ulla-Britt, 2016 (CYC2)	Andersson ,248335, OHN. (CYC2)

* N/A not applicable

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DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from leaf tissues using the CTAB method, followed by a modification (Doyle & Doyle,1987; Joly et al., 2006). The nuclear low-copy CYC2 region was amplified using specific primers adopted from (Albach & Meudt, 2010). The primer sequences are listed (Table 3). The PCR program used for the amplification of CYC2 is detailed (Figure 1). Following amplification, the PCR products were purified using polyethylene glycol (PEG) precipitation (Joly et al. 2006). Direct sequencing of purified PCR products was performed by Niagen Inc. (IRAN).

In addition to generating new CYC2 sequences, chloroplast *trn*L–*trn*F intergenic spacer and nuclear ribosomal Internal Transcribed Spacer (ITS) sequences were retrieved from GenBank (https://www.ncbi.nlm.nih.gov) to complete the dataset. These sequences were selected due to their extensive use and proven reliability in phylogenetic studies of *Veronica* and related taxa. Both ITS and *trn*L–*trn*F regions exhibit considerable sequence variability at the species level, offering complementary perspectives from nuclear and plastid genomes. The suitability and effectiveness of these markers have been demonstrated in several prior studies (Albach et al., 2004c; Meudt et al., 2015; Ulas et al., 2020).

Phylogenetic analyses

Molecular analyses of 2^ sequences employed the nuclear low-copy CYCLOIDEA2 region, the nuclear ribosomal internal transcribed spacer (ITS), and the plastid DNA (*trn*L–*trn*F). The CYC2 sequences were edited by Sequencher (Gene Code version 4.1, Inc., Ann Arbour, Michigan). The edited CYC2, Gene-Bank *trn*L–*trn*F, and ITS sequences were separately aligned using Clustal W (Thompson et al., 1994) as implemented in BioEdit Sequence Alignment Editor (Hall, 1999), followed by more manual modifications. Afterwards, Gap programmer coder ver.6.00 was employed to code the gaps using the "simple indel coding" method (Simmons & Ochoterena, 2000). The best-fitted model of DNA substitution for each partition was determined using MrModeltest, ver. 2.2 (Nylander, 2004), considering the Akaike information criterion (AIC; Akaike, 1974) with an executable MrModelblock file implemented in PAUP* version 4.10 b (Swofford, 2002). The substitution models for CYC2 (HKY+I), the chloroplastic region (GTR+I), and ITS (HKY+I) were identified as the best-fitting models. Phylogenetic analyses were performed for each marker separately using Bayesian inference in MrBayes v3.1.2, beyond the combined alignment (Huelsenbeck & Ronquist, 2001). Three parallel runs were carried out (each with three heated and one cold chain) with a random starting tree. Trees were sampled every 100 generations. The chains reached stationarity, and the average standard deviation of the split frequencies stabilized in less than 1000000 generations. The first 25% sampled trees were excluded ("burn-in") based on an assessment of convergence of log-likelihoods and stationarity of runs in Tracer v1.5 (Rambaut & Drummond, 2009) and a 50% majority-rule consensus tree was drawn by TreeView 1.66 (Page, 2001). Nucleotide distances were obtained using BioEdit Sequence Alignment Editor (Hall, 1999).

Table 3. Primer sequences used for amplification of CYC2.

Primer names	Sequences	Refrences
CYC2-F (Forward)	DYTVTCCATCGGCATTGC	(Albach & Meudt, 2010)
CYC2-R (Revers)	GATGAAYTTRTGCTGATCCAAAATG	(Albach & Meudt, 2010)

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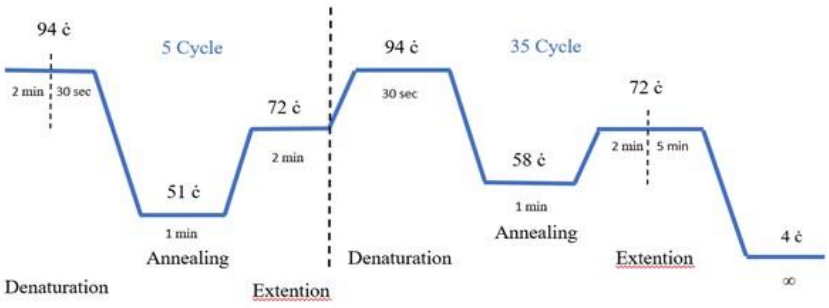

Figure 1. PCR amplification program used for CYC2.

Results

Taxonomic relationships within the *V. hederifolia* complex (*V. hederifolia*, *V. cymbalaria*, *V. panormitana*, and *V. triloba*) were assessed using both morphological characters and molecular data. In addition to the members of the *V. hederifolia* complex, three closely related species (*V. reuterana*, *V. acinifolia*, and *V. serpylifolia* were included in the phylogenetic analyses. Their placement in the consensus trees indicated that they form sister lineages to the main clade, providing additional context for relationships within the genus.

The aligned datasets for CYC2, *trn*L–*trn*F, and ITS markers included 561, 966, and 807 nucleotide sites, respectively. Bayesian 50% majority-rule phylogenetic trees of the studied species, based on each marker, are shown with posterior probability (PP) values (Figures 2-4). Pairwise nucleotide distances are shown among *V. hederifolia*, *V. triloba*, *V. panormitana*, and *V. cymbalaria* (Tables 4–6). Morphological analyses revealed consistent differences among taxa in several diagnostic features, including leaf shape, calyx and pedicel orientation, corolla size and color, sepal morphology and indumentum, and seed characteristics, which are largely congruent with molecular evidence.

The Bayesian phylogenetic tree derived from the CYC2 provided strong resolution of relationships within the *V. hederifolia* complex (Figure 2). Two main clades were identified with high posterior probabilities. Clade 1 encompassed the three taxa (*V. acinifolia*, *V. serpyllifolia*, and *V. reuterana*) with robust support (PP = 0.99), distinctly separated from members of the *V. hederifolia* complex. Clade 2 contained all representatives of the target complex. Within this clade, *V. cymbalaria* was placed as sister to a subclade comprising *V. hederifolia* and *V. triloba*, both of which were strongly supported as distinct lineages (PP = 1.0) and within clade 2, *V. hederifolia* and *V. triloba* clustered as sister taxa (PP = 1.0), with a very low but detectable divergence (pairwise distances = 0.0054–0.0086). *V. cymbalaria* was more distantly related to this pair (0.0435–0.0452 from *V. hederifolia*; 0.0435 from *V. triloba*). The largest distances were observed between *V. panormitana* and the other taxa (0.1080–0.1155). In the CYC2 tree, *V. panormitana* was resolved as the earliest-diverging lineage of clade 2 (PP = 1.0), whereas *V. cymbalaria* formed a closer sister relationship to the *V. hederifolia–V. triloba* subclade. The combined morphological and CYC2 data support the recognition of *V. triloba* as a distinct species rather than a variety or subspecies of *V. hederifolia*. The CYC2 phylogeny also suggests that *V. hederifolia* may represent a hybrid taxon, consistent with its intermediate morphological features. *Veronica panormitana* was resolved as an early diverging lineage within clade 2, distinct from the *V. hederifolia–V. triloba–V. cymbalaria* assemblage, with (PP = 1.0),

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indicating that *V. panormitana* represents an evolutionarily independent lineage within the complex.

The *trn*L–*trn*F showed lower resolution, with several internal polytomies (Figure 3). Clade 1 comprised the three non-complex species, clearly separated from the *V.* hederifolia complex. Two strongly supported subclades were identified within clade 2: (clade 2-1) *V. hederifolia* and *V. triloba* grouped (PP = 1.0), reflecting their high plastid similarity, with very low divergence (≤0.0086); (clade 2-2) *V. cymbalaria* and *V. panormitana* formed a distinct lineage with moderate divergence (0.0563). The overall plastid divergence among the four taxa was lower than that observed in nuclear datasets, consistent with the reduced substitution rate of plastid DNA.

The ITS phylogeny revealed two well-supported clades (Figure 4). Within clade 1, *V. cymbalaria* and *V. panormitana* formed a strongly supported sister pair (PP = 1.0), with minimal nucleotide distance (0.012). *Veronica triloba* was moderately divergent from this pair (0.0838–0.0853) and resolved as their sister lineage (PP ≈ 0.90). *Veronica hederifolia* showed the greatest divergence (0.155–0.160) and was consistently recovered as the earliest-diverging lineage of the complex. Clade 2 also comprised the three non-complex species, clearly separated from the *V. hederifolia* complex.

Overall, the phylogenetic analyses based on the three molecular markers (CYC2, *trn*L–*trn*F, and ITS) consistently confirmed the close relationships within the *V. hederifolia* complex and its related taxa. Despite some minor topological differences among the markers, the overall pattern of species grouping remained largely consistent. The nuclear markers (CYC2 and ITS) offered greater resolution within the complex, while the plastid marker (*trn*L–*trn*F) exhibited lower variation and consequently weaker support for some internal nodes. Observed incongruences among markers, such as the close similarity of ITS sequences between *V. panormitana* and *V. cymbalaria* despite greater divergence in CYC2, likely reflect a complex evolutionary history involving historical hybridization and introgression, polyploidy, locus-specific evolutionary rates, or limited sampling. Nevertheless, the combined evidence from all three datasets supports the monophyly of the *V. hederifolia* complex and emphasises its distinct position among closely related groups.

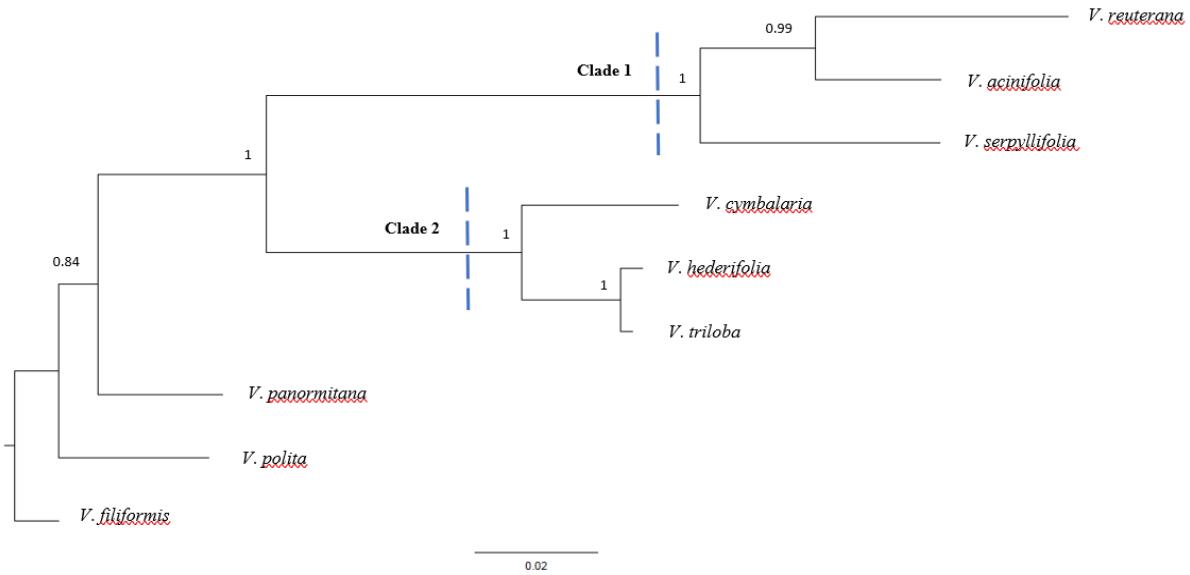

Figure 2. Phylogenetic tree of CYC2 resulting from the Bayesian inference of *V. hederifolia* complex. Numbers at nodes represent posterior probability values.

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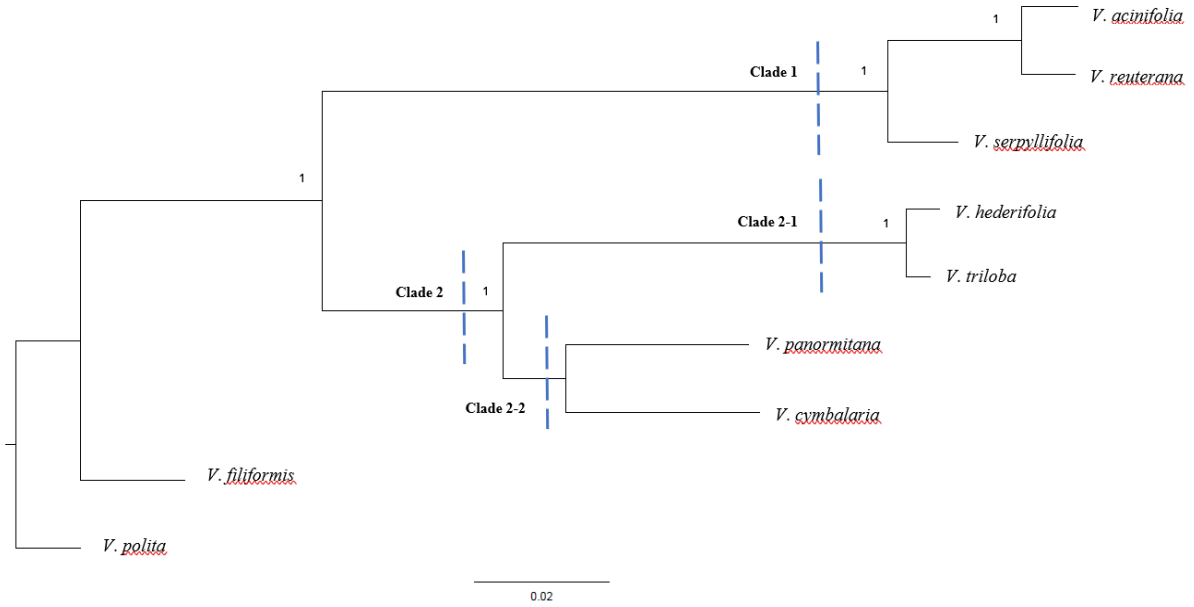

Figure 3. Phylogenetic tree of *trnL–trnF* chloroplastic region resulting from the Bayesian inference of *V. hederifolia* complex. Numbers at nodes represent posterior probability values.

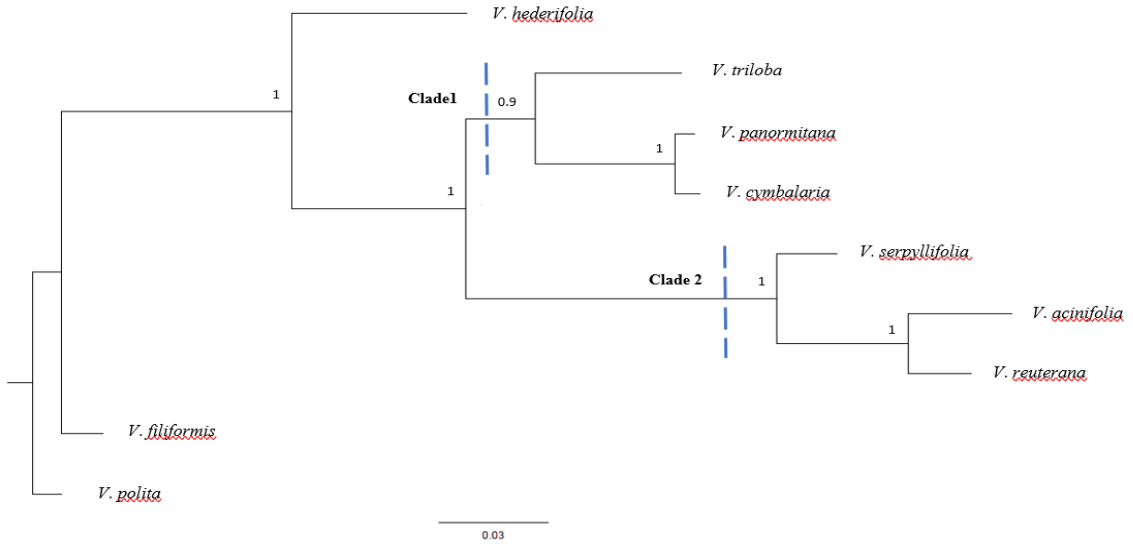

Figure 4. Phylogenetic tree of ITS resulting from the Bayesian inference of *V. hederifolia* complex. Numbers at nodes represent posterior probability values.

Table 4. The nucleotide distance between species in the phylogenetic tree of CYC2.

Species	*V. hederifolia*	*V. triloba*	*V. cymbalaria*	*V. panormitana*
V. hederifolia	-	-	-	-
V. triloba	0.0054	-	-	-
V. cymbalaria	0.0452	0.0435	-	-
V. panormitana	0.1097	0.108	0.1155	-

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Table 5. The nucleotide distance between species in the Phylogenetic tree of *trn*L–*trn*F.

Species	*V. hederifolia*	*V. triloba*	*V. cymbalaria*	*V. panormitana*
V. hederifolia	-	-	-	-
V. triloba	0.0086	-	-	-
V. cymbalaria	0.1034	0.1019	-	-
V. panormitana	0.1017	0.1003	0.0563	-

Table 6. The nucleotide distance between species in the Phylogenetic tree of ITS.

Species	*V. hederifolia*	*V. triloba*	*V. cymbalaria*	*V. panormitana*
V. hederifolia	-	-	-	-
V. triloba	0.155	-	-	-
V. cymbalaria	0.16	0.0853	-	-
V. panormitana	0.1585	0.0838	0.012	-

Discussion

The present work investigated molecular phylogenetic analysis of the taxonomically *V. hederifolia* complex, employing data from the nuclear low-copy CYCLOIDEA2 region, chloroplast *trn*L–*trn*F spacer, and nuclear ribosomal ITS region, and compared these molecular findings with morphological evidence.

Our results reveal that reconstructing the evolutionary history and phylogenetic relationships within *V.* subgenus *Cochlidiosperma* is challenging due to extensive incongruences among phylogenies inferred from different datasets. These discrepancies can arise from various factors, including phylogenetic and sampling errors, recombination, sampling of pseudogenes, unrecognized gene duplication and loss (paralogy), hybridization, gene introgression, ancient polymorphisms, long-branch attraction, unequal rates of molecular evolution, and incomplete lineage sorting (Rieseberg & Soltis, 1991; Wendel & Doyle, 1998).

Veronica hederifolia closely resembles *V. triloba* in several features, including palmatilobate leaves, truncated or almost cordate leaf bases, pubescent pedicel surfaces, raceme inflorescences, triangular-subcordate or triangular-cordate sepals, ciliate-margined sepals, and reniform capsules (Chater, 1978; Fischer, 1981). Historically, *V. triloba* had been considered a variety or subspecies of *V. hederifolia* (Tacik & Trzcińska-Tacik, 1963; Tacik, 1975). Cytogenetic data suggest that hexaploid *V. hederifolia* (6x) has an allopolyploid origin, with diploid *V. triloba* (2x) and tetraploid *V. sublobata* (4x) as potential parental species (Fischer, 1967, 1975a). Moreover, *V. sublobata* has been treated as a synonym or subspecies of *V. hederifolia*, possibly representing a hybrid between *V. sublobata* and an unknown species (Atha et al., 2021). Biometric studies further distinguish *V. triloba* from *V. hederifolia* and *V. sublobata* based on petal color and shape, seed color and ornamentation, and upper leaf morphology and recognized *V. triloba* as a distinct species (Cieśla & Mirek, 1996; Miriek et al., 2002).

The CYC2 provided the highest resolution, fully resolving all four species without internal polytomies (Figure 2). Nucleotide distances revealed minimal divergence between *V. hederifo-*

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lia and *V. triloba* (0.0054–0.0086), consistent with their morphological similarity, yet sufficient to recognize them as distinct species. Divergence from *V. cymbalaria* (0.0435–0.1034) and *V. panormitana* (0.1017–0.1097).

The *trn*L–*trn*F exhibited several internal polytomies, particularly between *V. hederifolia* and *V. triloba*, reflecting low nucleotide divergence (≤1.1%) and the slow evolutionary rate of plastid DNA (Figure 3). Nonetheless, a close relationship between these two species was maintained. The remaining species, *V. cymbalaria* and *V. panormitana*, displayed poorly resolved relationships in this marker, highlighting the limitations of plastid loci for distinguishing recently diverged taxa.

The ITS also showed partial polytomies, indicative of concerted evolution, incomplete lineage sorting, or historical introgression (Figure 4). ITS distances were minimal between *V. cymbalaria* and *V. panormitana* (0.012), while divergence between *V. hederifolia* and the other taxa was higher (0.155–0.160). The ITS phylogeny recovered *V. cymbalaria* and *V. panormitana* as a strongly supported sister pair, with *V. triloba* as their closest relative, and *V. hederifolia* as the earliest-diverging lineage. This topology illustrates minor cytonuclear discordance relative to CYC2.

The superior resolution of CYC2 in delimiting species within the *V. hederifolia* complex is attributed to its status as a low-copy nuclear (LCN) gene. LCN markers are biparentally inherited and tend to evolve more rapidly than plastid markers, thereby capturing recent diversification events and signatures of hybridization (Zimmer & Wen, 2012; Kates et al., 2017). Consistent with this, CYC2 revealed polymorphisms in *V. hederifolia* aligned with a hybrid origin, whereas ITS and *trn*L–*trn*F showed reduced phylogenetic signals and partial polytomies. These findings underscore the growing recognition that LCN genes provide enhanced resolution for species delimitation in recently diverged or reticulate lineages.

Integrating molecular and morphological evidence reveals clear patterns of differentiation. Despite *V. hederifolia* being morphologically similar to *V. triloba*, they differ notably in corolla color (blue with a white center vs. dark blue) and sepal surface (the margin pubescent vs. the upper surface pubescent). Comparing *V. hederifolia* and *V. cymbalaria*, clear morphological differences are evident in leaf shape (ovate-lanceolate vs. crenulate), corolla color (blue with a white center vs. white with a yellow center), pedicel orientation (erect vs. recurved), calyx orientation (equal with petals vs. superior), and sepal shape (triangular-subcordate vs. ellipsoidal to almost circular). Similarly, notable morphological distinctions separate *V. hederifolia* from *V. panormitana*, including leaf shape (ovate-lanceolate vs. crenulate), corolla color (blue with white center vs. white), pedicel orientation (erect vs. recurved), pedicel surface (pubescent vs. glabrous), and sepal shape (triangular-subcordate vs. ellipsoid). Significant morphological differences also distinguish *V. triloba* from *V. cymbalaria*, particularly in corolla color (dark blue vs. white with a yellow center), and sepal shape (triangular-cordate vs. ellipsoidal to almost circular). *Veronica triloba* and *V. panormitana* differ morphologically in corolla color (dark blue vs. white), pedicel surface (pubescent vs. glabrous), sepal shape (triangular-cordate vs. ellipsoid), and seed shape (ellipsoid-rectangular vs. globose cymbiform, almost corrugated). *Veronica cymbalaria* and *V. panormitana* are morphologically differentiated by corolla diameter (6–12 mm vs. 2–5 mm), corolla color (white with yellow center vs. white), calyx orientation (superior vs. shorter or mostly equal to petals), and pedicel surface (pubescent vs. glabrous).

Overall, the *V. hederifolia* complex forms a monophyletic group, with nuclear CYC2 providing the clearest resolution for species delimitation. Morphological characters and nucleo-

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tide distances converge in supporting the distinctiveness of *V. triloba*, whereas ITS and *trn*L–*trn*F reveal limited resolution and minor cytonuclear discordance, indicative of historical reticulate evolution. This integrated approach highlights the importance of combining morphological traits with multiple molecular markers for accurate species delimitation in recently diverged or reticulate lineages (Webb et al., 1972; Fischer, 1981; Albach et al., 2004).

Our findings confirm earlier molecular phylogenies in Plantaginaceae (Albach et al., 2004; Meudt & Albach, 2005b) and align with recent genomic-scale studies that highlight the widespread role of reticulation and polyploidy in related plant groups (Kates et al., 2017; Johnson et al., 2019). Within *Veronica*, similar patterns are evident: rapid radiation identified through target enrichment sequencing (Thomas et al., 2021), and repeated polyploidization and hybridization events, both ancient and recent, documented (Meudt et al., 2015). These processes are consistent with the cytonuclear discordance observed in the *V. hederifolia* complex, particularly the incongruence between the plastid (*trn*L–*trn*F) and nuclear (ITS, CYC2) datasets, as shown in (Figures 2-4) .

The differing phylogenetic signals strongly suggest plastome capture and hybridisation, further supported by the relatively high nucleotide distances among taxa presented in (Tables 4-6) .

Conclusion

This study provided new insights into the taxonomy and evolutionary history of the *V. hederifolia* complex (*V. hederifolia*, *V. cymbalaria*, *V. panormitana*, and *V. triloba*). Clear morphological differentiation was observed in leaf shape, calyx and pedicel orientation, corolla size and color, sepal morphology, and seed characters. By combining morphological and molecular evidence, we demonstrated that low-copy nuclear markers, particularly CYC2, are essential for resolving relationships within recently isolated or reticulate species, whereas plastid and ribosomal markers have clear limitations. These findings emphasize the importance of marker selection in phylogenetic inference and highlight the value of integrating nuclear and morphological data for accurate species determination. Beyond the genus *Veronica*, our results contribute to broader discussions on the role of networking and polyploidy in plant evolution and emphasize the methodological importance of low-copy nuclear genes in phylogenetic studies.

To further refine species boundaries and elucidate evolutionary histories within the complex, we recommend: Expanded geographic sampling, incorporating multiple populations per species .Inclusion of additional low-copy nuclear markers and complete plastome sequencing to better resolve cytonuclear incongruences. Application of genomic approaches such as target enrichment or genome skimming to comprehensively investigate hybridization and polyploidization events.

Authors' contributions

Hamideh Ahmadian Yazdi: Thesis student, responsible for sample preparation, conducting experiments, data collection, phylogenetic analyses, and interpreting the results, as well as drafting the article.

Azarnoosh Jafari and Jamil Vaezi: Thesis supervisors, research design, overseeing research stages, reviewing and controlling results, and editing and finalising the article.

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Ehsan Karimi: Thesis advisor, participating in research design, supervising the research, and reviewing and editing the article.

All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data available on request from the authors.

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Ethical considerations

The authors avoided data fabrication, falsification, plagiarism, and misconduct.

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Conflict of interest

The authors declare no conflict of interest.

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