



Nigritella widderi (Orchidaceae) from central Apennines (Italy): evidence of plastome differentiation events

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Abstract

The present study focuses on the taxonomic and systematic classification of the tetraploid species *Nigritella widderi*, a rare orchid distributed in the eastern Alps (i.e., Austria, Germany, Italy, Slovenia) and in central Apennines (Italy). The research was fueled by the hypothesis that geographic isolation—or alternatively, long-distance seed dispersal—is driving the genetic differentiation of the Apennine population from its original Alpine lineage. Thus, a representative series of Apennine samples were subjected to genetic analyses, that is, characterization of 5 plastid Variable Number Tandem Repeats (VNTRs) (specifically loci 1, 2, 10b, 11b, and 19) and a nuclear barcode region (i.e., Internal Transcribed Spacer of rDNA, ITS). The plastid markers proved to be the key, providing clear elements of distinction at loci 10b and 11b between the Apennine and Alpine series. Interestingly, a potential novel Single Nucleotide Polymorphism (SNP) at locus 10b able to discriminate specifically *N. widderi* from the Apennines was identified. Conversely, the ITS barcode region showed insufficient discriminatory power for inter- and intra-specific distinction, perhaps due to its low mutation rate or the limited comparative dataset in the literature. In conclusion, this contribution represents the first molecular evidence that the Apennine population of *N. widderi* is genetically distinct, at least at the plastid level, from its Alpine counterpart. These findings are consistent with active microevolutionary processes shaped by unique environmental pressures, opening new questions about the biogeographical history of this taxon.

Keywords Genetic differentiation · Glacial relict · ITS barcode region · Orchid evolution · Plastid microsatellites · Population isolation

Introduction

The systematic position of the genus *Nigritella* Rich. (subtribe Orchidinae Dressler & Dodson, tribe Orchideae Dressler & Dodson, family Orchidaceae Juss.) has been extensively debated over the past decades, with some

botanists treating it as a distinct genus (Hedré et al. 2018a,b; Brandrud et al. 2019) and others as a subgenus within the large genus *Gymnadenia* R.Br. (i.e., Bateman et al. 2018; Bateman 2021). The question is clearly still open (Bateman 2021) but, considering the high morphological divergence of the *Gymnadenia* subgenus *Nigritella* (Rich.) O.Gerbaud & P.Quentin from the subgenus *Gymnadenia*, we prefer to provisionally consider *Nigritella* as a distinct genus.

The genus *Nigritella* is endemic to Europe and restricted to mountainous habitats (Hedré et al. 2018b). Within this genus, diploid and polyploid species were comprehensively analyzed by Hedré and colleagues (2018) to characterize nuclear and plastid DNA polymorphisms, providing the most up-to-date phylogenetic framework available. As reported in earlier works, diploid taxa reproduce sexually and predominantly outcross, leading to high genetic variation within and among populations, whereas polyploid ones reproduce asexually through apomixis and exhibit lower levels of gene diversity (Teppner 1996, 2004; Hedré et al. 2000, 2018b).

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Specifically, the present study focuses on the tetraploid ($2n=4x=80$) *N. widderi* Teppner & E.Klein, a rare orchid species with a predominantly Alpine distribution, occurring along the Austrian side of the Alps, in Slovenia, Germany, and Italy (Veneto and Trentino-Alto Adige) (Rossi et al. 1987; Perazza 2005; Dakschobler et al. 2012; Bartolucci et al. 2024; POWO 2025). Its occurrence has also been reported in the central Apennines (Italy), specifically in Abruzzo, Lazio, Marche, and Molise administrative regions (Rossi et al. 1987; Bartolucci et al. 2024). The mountains of central Italy mark its southern distribution limit, where this sub-endemism is considered a glacial relict. Here, this plant typically grows on calcareous substrates at elevations of approximately 1300–2450 m a.s.l. in alpine and subalpine grasslands (Delforge 1995; Rossi et al. 1987; Rossi 2002; Bartolucci et al. 2012; Hedrén et al. 2018b; Biagioli and De Simoni 2024).

Like all orchid species, *N. widderi* is protected under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2025). In addition, it has been specifically assessed as “EN—endangered” by the International Union for Conservation of Nature (Rankou 2011). It is known that glacial relict habitats are increasingly threatened by climate warming; thus, the plant biodiversity in these *cold-spots* of exceptional ecological and conservation importance is considered at risk (Dullinger et al. 2012; Fragnière et al. 2020). In such types of contexts, comprehensive genetic and morphological analyses are essential to delineate evolutionarily significant units and guide conservation actions appropriately.

Given the increased potential for allopatric speciation in fragmented or geographically remote populations (Hoelzer et al. 2008), the current work tries to assess whether the isolation of *N. widderi* population from the central Apennines is driving phenomena of genetic differentiation, with respect to their Alpine counterpart, and to evaluate if targeted conservation measures are required. This research is also supported by preliminary evidence documenting slight phenotypic differences between Apennines and Alpine populations of *N. widderi* (Rossi et al. 1987). Thus, following the approach of Hedrén et al. (2018b), five loci of plastid variable number tandem repeats (VNTRs) and the region coding for the nuclear internal transcribed spacer of rDNA (ITS) were selected as molecular markers and used to profile *N. widderi* accessions from central Apennines.

Materials and methods

Since *N. widderi* is protected under international conventions and assessed as *endangered* by the IUCN, field sampling was strictly conducted in the least invasive manner possible to prioritize the conservation of this glacial relict.

Consequently, a targeted and ethically sourced series of twelve specimens was analyzed; although numerically constrained (due to above-mentioned preservation principle), this collection comprehensively covers the entire known distribution of the species in the Central Apennines, ensuring the findings are geographically representative. In detail, samples were collected in early summer 2024 at the Gran Sasso massif (Table 1) by picking up only small portions of leaf tissue, which were subsequently dried in silica gel and stored at room temperature (Chase and Hills 1991). This material was supplemented by historical specimens from other Apennine areas (Table 1 and Fig. 1) sourced from the *Herbarium Apenninicum* (APP, located at the Apennine Floristic Research Center, Gran Sasso e Monti della Laga National Park—University of Camerino) (Conti et al. 2024; code follows Thiers 2025).

Prior to extraction, leaf surfaces were sterilized with 2.5% sodium hypochlorite solution and rinsed with sterile water. Approximately, 10 mg of leaf tissue per sample were ground in liquid nitrogen using mortar and pestle, and total DNA was isolated by the NucleoSpin Plant II kit (Macherey–Nagel), according to the manufacturer’s guidelines. Extracted DNA samples were quantified and evaluated in purity by a Nanodrop spectrophotometer (Thermo Fisher Scientific), reading the absorbance of the samples at 260 nm and 280 nm, and then stored at -80°C .

PCR amplifications of five plastid variable number tandem repeats (VNTRs) and the nuclear internal transcribed spacer of rDNA (ITS) were performed with a Bio-Rad IQ5 thermocycler. Every reaction was carried out in a volume of 50 μL containing 50 ng of template DNA, 0.2 mM of each dNTP, 1.5 units of Taq DNA polymerase (GeneDireX), 5 μL Taq buffer (GeneDireX), 20 μM of each primer (Table 2), and sterile water to reach the final volume. Amplification of the VNTR markers was performed under the following conditions: (a) initial denaturation at 94°C for 3 min; (b) 36 cycles of denaturation at 94°C for 30 s, annealing at the primer-specific temperature for 30 s (Table 2), and extension at 72°C for 45 s; (c) final extension at 72°C for 10 min. The ITS region, instead, was amplified as follows: (a) initial denaturation at 95°C for 5 min; (b) 60 cycles of denaturation at 95°C for 30 s, annealing at 58.5°C for 30 s, and extension at 72°C for 50 s; (c) final extension at 72°C for 10 min.

PCR products were verified for expected length (Table 2) by electrophoresis on 1.5% agarose gels containing 10 mg/mL ethidium bromide in TAE buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid, pH 8.5). Sequencing was performed as reported in Gismondi et al. (2012). Briefly, PCR products were treated with ExoSAP-IT (Thermo Fisher Scientific) and subjected to BigDye labeling procedure (Applied Biosystems). Then, DNA fragments were precipitated, by adding 95% ethanol and 3 M Sodium Acetate pH 5.2, washed in 70% ethanol, resuspended in 100% formamide, and analyzed using ABI

Table 1 *Nigritella widderi* specimens investigated in this study with related information

Code of the sampling site	Sample type	Locality	Coordinates (WGS84)	Collectors (collection date)	Voucher codes (and number) of the specimens
1	Fresh leaf portion	Italy, Abruzzo, Pietracamela (Teramo), Gran Sasso, between La Madonnina and Rifugio Franchetti	42.479835°N, 13.566188°E	<i>F. Bartolucci, F. Conti, A. Marziali, G. Zangari</i> (01/07/2024)	APP* no. 73593—(7)
2	Dry leaf portion	Italy, Abruzzo, Isola del Gran Sasso (Teramo), Gran Sasso, Vallone San Pietro	42.453790°N, 13.633771°E	<i>F. Conti</i> (05/08/2002)	APP no. 1837—(1)
3	Dry leaf portion	Italy, Molise, Pizzone (Isernia), Mainarde, between La Metuccia and Monte a Mare	41.677766°N, 13.960163°E	<i>F. Conti</i> (08/07/1992)	APP no. 23408—(1)
4	Dry leaf portion	Italy, Marche, Acquasanta Terme (Ascoli Piceno), Monti della Laga, between Passo il Chino and Macera della Morte	42.711349°N, 13.346840°E	<i>F. Bartolucci, F. Conti</i> (23/06/2011)	APP no. 50645 – (1)
5	Dry leaf portion	Italy, Abruzzo, Opi (L'Aquila), Monte Marsicano	41.802872°N, 13.869713°E	<i>F. Bartolucci, F. Conti</i> (03/07/1997)	APP no. 49695 – (1)
6	Dry leaf portion	Italy, Abruzzo, Barrea (L'Aquila), Monte Greco—La Capriola	41.775028°N, 13.993948°E	<i>F. Bartolucci, F. Conti</i> (02/08/2001)	APP no. 48657 – (1)

* APP: *Herbarium Apenninicum* (Conti et al. 2024)

SeqStudio Flex 8 (Thermo Fisher Scientific). To guarantee the highest scientific rigor and confirm the reproducibility of the data, all molecular analyses were performed in triplicate across two independent laboratories. Indeed, in parallel, sequencing was also outsourced to an external provider (Microsynth, Switzerland), obtaining identical results. Resulting sequences were visualized and aligned using BioEdit v.7.7.1.

Nucleotide sequences were compared with those registered in the GenBank database (Nucleotide-NCBI) using the Nucleotide Basic Local Alignment Search Tool (BLAST) algorithm, specifically the MEGABLAST (blastn) function, which is optimized for identifying highly similar sequences. The ITS sequence obtained in this study was deposited in GenBank under accession number PV800901.

A geographical map was produced by ©Mapbox software in a ©OpenStreetMap format, while an evolutionary net was generated using Network software (Copyright 2004–2006 Fluxus Technology Ltd., version 10.2.0.0), following the median-joining approach described by Bandelt et al. (1999).

Results and discussion

From a morphological perspective, previous studies have reported minimal differences between Alpine and Apennine populations of *N. widderi* (Del Prete 1982; Rossi and Bassani 1982; Rossi et al. 1987). Specifically, Rossi and colleagues (1987) have documented a slight variation in the width of the bellied basal part of the labellum, ranging from 2.5 to 2.7 mm in Apennine specimens to 2.7–3.2 mm in Alpine ones. However, since some Alpine flowers also showed values below 2.7 mm, this overlap made the distinction doubtful. Given this evidence and considering the geographical isolation of the Apennine populations, we hypothesized that some degree of genetic differentiation might have occurred in Central Italy. Therefore, the present research aimed to investigate this possibility, analyzing a set of Apennine *N. widderi* samples (Fig. 1, Fig. 2 and Table 1) from a molecular standpoint. Both Alpine

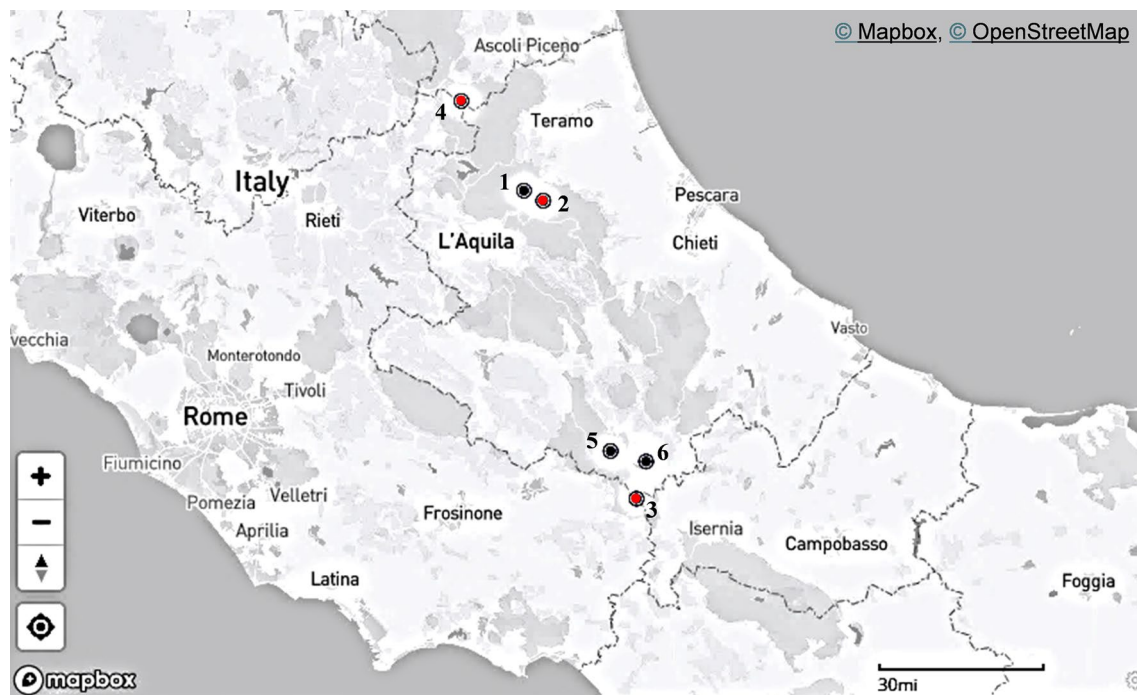


Fig. 1 Geographical map. Map (produced by ©Mapbox software in a ©OpenStreetMap format) of the sampling sites (from 1 to 6) described in Table 1; red spots indicate the areas where Apennine haplotype 1 was detected, while blue dots represent sites showing only haplotype 2

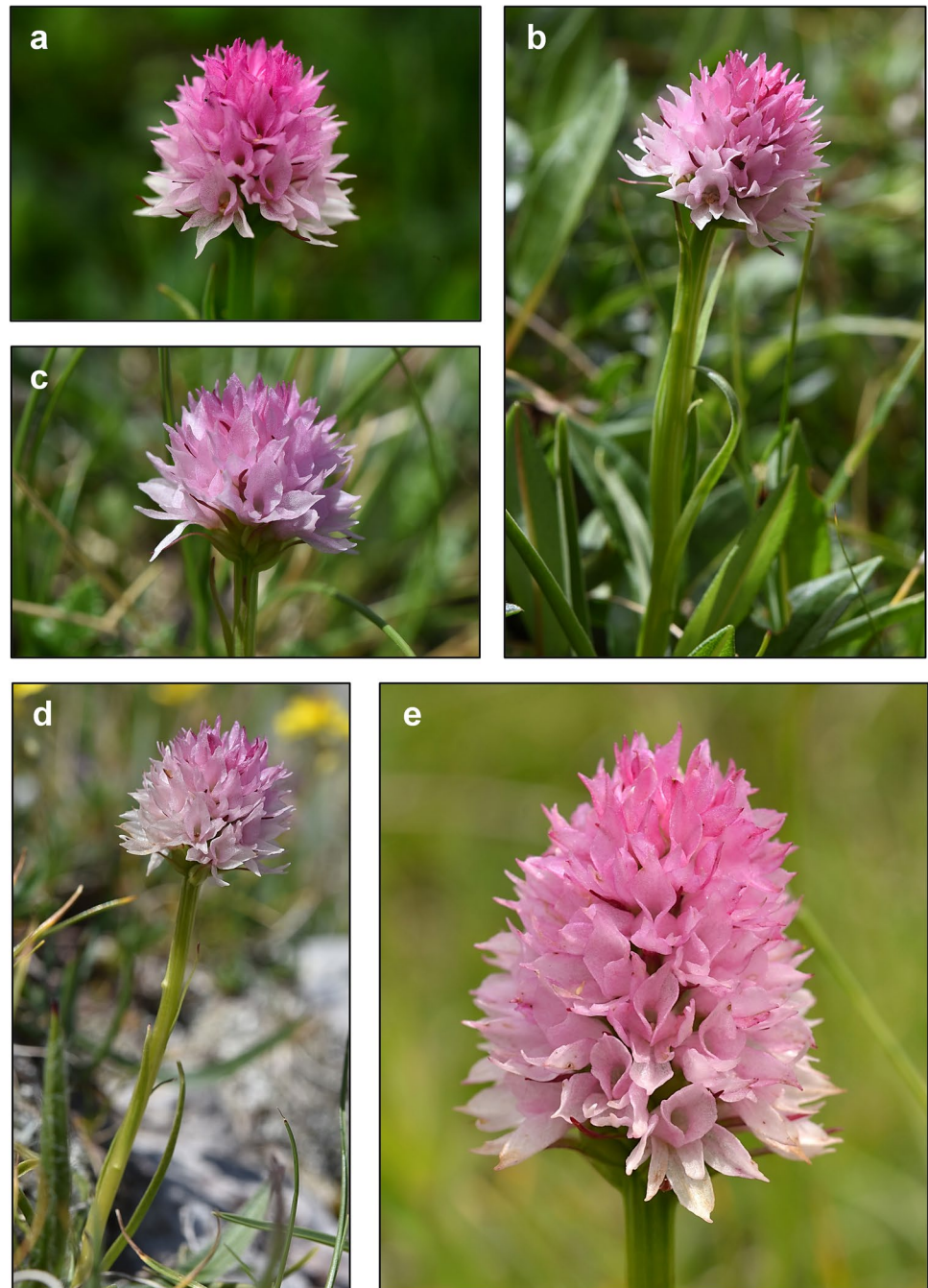
Table 2 Identity and PCR details of chloroplast (cp) and nuclear markers used in the present study are reported

Marker name (genome type)—reference	Repeat motif	Forward and reverse primer sequences* (5'—3')	Expected product dimension (bp)	Annealing temperature (°C)
1 (cpDNA)—Solina and Widmer 1999	poly(A)	F: CGA AAT CGG TAG ACG CTA CGC R: CGT TAG AAC AGC TTC CAT TG	187	57.2
2 (cpDNA)—Bateman 2001	5'-ATAGTATA-3'	F: CAT AGC AAA TGA TTC ATC GC R: TAG GGG GTT TCT ATA GAA CC	150–164	55
10b (cpDNA)—Hedrén et al. 2008	poly(A)-poly(TA)-poly(T)	F: CCG ACT AGT TCC GGG TTC GA R: GAA AGG CTT GTT ATT TCA CAG	138–149	56.3
11b (cpDNA)—Hedrén et al. 2008	poly(A)	F: GCT ATG CTT AGT GTG TGA CTC GTT G R: AGT TTA TAG TGG GGT CAG CC	87–91	56
19 (cpDNA)—Hedrén et al. 2008	poly(T)	F: GAG TAA TAG TGT TCT AAT AAG AG R: CAG ACG CAG TCA AGA TAG CA	146–155	58
ITS (nuclear) – Kress et al. 2005	—	F: TCC TCC GCT TAT TGA TAT GC R: CCT TAT CAT TTA GAG GAA GGA G	630	58.5

and Apennine plants have been found to have the same chromosome number and similar embryological features (Rossi et al. 1987); therefore, this study was based on the molecular framework established by Hedrén et al. (2018b),

who have identified five plastid VNTR loci (i.e., 1, 2, 10b, 11b, and 19) and the nuclear ITS barcode region as the most informative markers for *Nigritella* species, whereas nuclear SSR loci have been excluded due to their limited

Fig. 2 *Nigritella widderi* from central Apennines. Images of *N. widderi* specimens captured in situ: **a, b** Abruzzo, between La Madonnina and Rifugio Franchetti, photos by F. Bartolucci and F. Conti; **c, d** Lazio, Monte Terminillo, photos by F. Bartolucci; **e** Abruzzo, close to Rifugio del Monte, photo by F. Bartolucci



discriminatory power. In fact, the use of these standard nuclear markers is quite discouraged due to the scarcity of validated loci (i.e., Gc51 and Gc77), their highly conserved nature at intra-specific level, and the inherent interpretation complexities arising from polyploidy and apomixis in *Nigritella* (Hedrén et al. 2018a, b).

DNA extracted from all *N. widderi* specimens showed high quality and concentration, allowing both plastid microsatellite (VNTR) and barcoding analyses to be successfully performed (Fig. 3, panel a and b).

Plastid markers proved crucial, revealing clear differences in the genetic profiles of the Apennine (haplotypes 1 and 2) and Alpine (haplotypes NF and NG) samples (Table 3). It is important to specify that Alpine haplotypes were not genotyped de novo in this work but retrieved directly from the dataset published by Hedrén et al. (2018b), while the haplotypes 1 and 2 are those detected and sequenced in the present study. In particular, the number of repeats at loci 1, 2, and 19 (respectively, 13, 0, and 12 base pairs [bp], as shown in Fig. 3 panels c, d, and e) were uniform across all

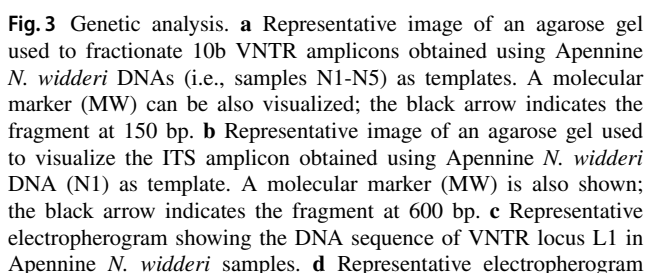
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Table 3 Comparison of repeat-region lengths (in bp) in 5 plastid VNTR loci of *Nigritella widderi* from Alpine and Apennine accessions. Haplotypes NF and NG, corresponding to Alpine accessions, were not genotyped in this work but retrieved directly from the dataset published by Hedrén et al. (2018b), while the haplotypes 1 and 2 are those detected and sequenced in the present study from Apennine samples

	Estimated length of repeat region (bp) per locus				
	1	2	10b	11b	19
Alpine haplotype NF	13	0	32	12	12
Alpine haplotype NG	13	0	33	12	12
Apennine haplotype 1	13	0	29	13	12
Apennine haplotype 2	13	0	30	13	12

Apennine samples and corresponded exactly to the schemes characterized for the Alpine *N. widderi*. By contrast, loci 10b and 11b displayed polymorphisms that were unique to Apennine populations.

Locus 10b, described as a complex polyA-polyTA-polyT repeat (Hedrén et al. 2018b), exhibited two variant patterns within the Apennine series: the first, characterized by a shorter polyA stretch (9 bp) and found in three samples, presented a total length of 29 bp (Fig. 3, panel f), while the second one, showing a 10 bp polyA stretch and detected in the remaining specimens, revealed a dimension of 30 bp (Fig. 3, panel g). This variability put in evidence how strong evolutionary pressures exist even on *N. widderi* specimens living in areas sharing similar ecological and pedoclimatic features (central Italy, Adriatic side). Anyway, it is interesting to notice that a comparable phenomenon occurred in Alpine *N. widderi* as well. Indeed, Hedrén et al. (2018b) have distinguished two haplotypes of Alpine *N. widderi* according to the genetic variability of the same VNTR locus (i.e., haplotype NF with a total length of 32 bp and NG with 33 bp). All these data would suggest that 10b microsatellite site is highly prone to mutations, confirming its suitability as discriminatory molecular marker for *Nigritella* species. This can be affirmed also considering the notable feature registered for this locus in all Apennine samples but absent in the Alpine haplotypes: the insertion of a TC dinucleotide interrupting the otherwise regular and expected TA repeats (Fig. 3, panels f and g). The change of an adenine (A) to cytosine (C) would represent an unusual event because generally transversions are produced at a lower frequency than transitions, due to complexity of their molecular mechanisms and the possibility they determine significant aminoacidic substitutions implying phenotypic alterations (Quicke 1993; Morton 1997). Therefore, this punctiform mutation, never registered before for *Nigritella* species (Hedrén et al. 2018b), could be considered a novel single nucleotide polymorphism

(SNP) useful to discriminate specifically the population of *N. widderi* from the Apennines.

In parallel, locus 11b, a simple mononucleotide repeat (polyA), also showed differences between Alpine and Apennine haplotypes. In the Apennine samples, the length of the microsatellite was equal to 13 bp (Fig. 3, panel h)—matching the haplotypes ND (from *N. stiriaca* (Rech.) Teppner & E.Klein), NJ (from *N. stiriaca* and *N. miniata* (Crantz) Janch.), NK (from *N. stiriaca* and *N. miniata*), and NL (from *N. miniata*, *N. corneliana* (Beauverd) Gözl & H.R.Reinhard, *N. lithopolitana* Ravník and *N. rhellicani* Teppner & E.Klein) described by Hedrén et al. (2018b)—whereas the Alpine NF and NG profiles contained a motif consisting of 12 repeats (Table 3). This evidence is noteworthy, considering that Alpine specimens should be most closely related to the Apennine ones. However, the haplotypes mentioned above are not exclusive to single species—for instance, haplotype NL has been independently documented across four different *Nigritella* species (Hedrén et al. 2018b)—reflecting the size homoplasmy phenomenon known to affect fast-evolving plastid microsatellites, whereby identical repeat lengths can arise independently in unrelated lineages without necessarily indicating close phylogenetic relatedness. For this reason, single-locus comparisons alone should be interpreted with caution, whereas the multilocus analysis carried out in this work provides a more robust representation of the overall genetic relationships among samples. In fact, the data from VNTR analyses demonstrated that the plastid genomes (i.e., haplotypes NF and NG) dominating *N. widderi* populations located in Austria (Styria), Slovenia (Gorenjska and Primorska), and Germany (Bavaria) share some traits with those characterizing the Apennine specimens (i.e., haplotypes 1 and 2) but also exhibit clear distinctive elements (i.e., loci 10b and 11b). While this reflects the existence of independent evolutionary events or localized mutational processes within the Apennine lineage, a finer resolution of these dynamics was achieved by reconstructing a median-joining haplotype network (Fig. 4). This network provides key insights into the discrete evolutionary steps of the investigated specimens, clearly demonstrating that the Apennine orchids uniquely and unequivocally derive from the Alpine ancestral pool, specifically rooted in the haplotype NF. Indeed, from this pivotal Alpine genotype, evolution appears to have proceeded along two distinct parallel branches. On one side, haplotype NF transitioned into NG via a single mutational event at the microsatellite locus 10b, characterized by a size increase from 32 to 33 bp. In parallel, along a separate evolutionary trajectory, haplotype NF gave rise to the Apennine haplotype 2 through three sequential mutations: an expansion at locus 11b (from 12 to 13 bp), followed by two successive contractions at locus 10b (first from 32 to 31 bp, and subsequently from 31 to 30 bp). Finally, the Apennine haplotype 1 developed as the

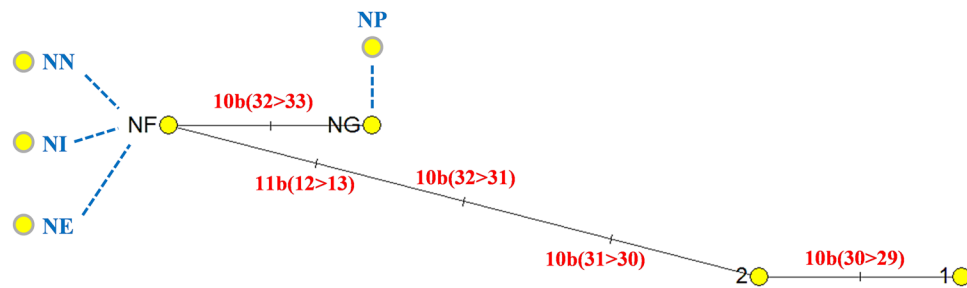


Fig. 4 Evolutionary network. Network focusing on the four haplotypes detected in *N. widderi*: the Alpine haplotypes NF and NG (described by Hédren et al. 2018) and the Apennine haplotypes 1 and 2 (identified in the present study). Only the Alpine haplotypes NF and NG are shown, as these represent the closest relatives and direct ancestral nodes to the Apennine haplotypes identified in this study; the remaining ones described by Hedré et al. (2018b) for other *Nigritella* species were omitted as they fall outside the specific evolutionary trajectory addressed here. Red labels indicate specific

mutation sites and related evolutionary steps between nodes (yellow circles). Blue haplotypes (NI, NN, NE and NP) are those detected by Hédren et al. (2018) in other *Nigritella* species and dashed lines indicate their association with NF and NG haplotypes, according to the same authors. The network was generated using Network software (Copyright 2004–2006 Fluxus Technology Ltd., version 10.2.0.0), following the median-joining approach described by Bandelt et al. (1999)

most recent evolutionary step, originating directly from haplotype 2 via a final one-step mutation at locus 10b, which further reduced its size from 30 to 29 bp. This reconstruction strongly supports a directional evolutionary radiation from the Alps to the Apennines, identifying haplotype 1 as the latest derived genetic profile within this lineage. Notably, all seven specimens sampled at site 1 (Table 1) shared the same plastid haplotype (haplotype 1), consistent with the hypothesis of a local haplotype fixation; however, the current sample size does not allow robust inference regarding seed dispersal dynamics between populations. From a phylogeographic perspective, it is worth noting that the sampling sites harboring haplotypes 1 and 2 are geographically intermingled across the Apennine range rather than forming spatially distinct clusters, precluding a clear-cut phylogeographic interpretation. Nevertheless, according to

the stepwise evolutionary model proposed here (Fig. 4), it is tentatively possible that sites carrying haplotype 2 correspond to earlier-established populations, while those carrying haplotype 1 reflect more recent within-Apennine colonization events. This hypothesis, however, remains speculative given the current sample size and would benefit from further investigation with expanded sampling.

To obtain further information about the Apennine *N. widderi* genome, the nuclear ITS region was successfully amplified and sequenced (Fig. 3, panel i). The sequences obtained were fully identical across all the individuals, highlighting the strong conservation of this DNA barcode within the taxon. Thus, it was registered in GenBank (ID: PV800901) and then compared with all the other accessions present in the same database by BLAST software. The analysis, carried out under stringent parameters, revealed that the

Table 4 Comparison between ITS barcode sequence from *Nigritella widderi* Apennine accessions (query) and GenBank nucleotide sequences (subjects) identified by BLAST software. Species name, % of query coverage, E-value (significance of the result, max value is

0.0), % of identity of the sequences, and GenBank accession number of the subjects are reported for each match. Only matches showing a query coverage percentage equal to 100% and an identity percentage > than 97% were reported

Species	Query cover	E-value	Identity	Accession
<i>Nigritella nigra</i> (<i>Gymnadenia nigra</i>)	100%	0.0	100.00	DQ022892.1
<i>Gymnadenia conopsea</i>	100%	0.0	98.46	MN221404.1
<i>Gymnadenia conopsea</i>	100%	0.0	98.15	DQ022888.1
<i>Gymnadenia conopsea</i>	100%	0.0	98.15	DQ022887.1
<i>Gymnadenia conopsea</i>	100%	0.0	98.00	KY512498.1
<i>Gymnadenia</i> sp. <i>BB Raskoti</i> 201,431	100%	0.0	97.69	MF944277.1
<i>Gymnadenia orchidis</i>	100%	0.0	97.38	MF944276.1
<i>Gymnadenia emeiensis</i>	100%	0.0	97.38	MF944275.1
<i>Gymnadenia crassinervis</i>	100%	0.0	97.38	DQ022890.1
<i>Coeloglossum viride</i> (<i>Dactylorhiza viridis</i>)	100%	0.0	97.38	MH711389.1
<i>Gymnadenia conopsea</i>	100%	0.0	97.23	DQ022891.1

ITS sequence from the Apennine populations were identical to that of *N. nigra* (L.) Rchb.f. from Scandinavia, with 100% query coverage and identity (Table 4). Subsequent hits included other species of *Gymnadenia* and one *Coeloglossum viride* (L.) Hartm. accession (under the name *Dactylorhiza viridis* (L.) R.M.Bateman, Pridgeon & M.W.Chase) but with slightly lower identity values. Unfortunately, no *N. widderi* ITS sequence had previously been deposited in GenBank, underscoring how the present result provides novel data to the scientific knowledge and the genetic database. The lack of differentiation between the Apennine *N. widderi* and Scandinavian *N. nigra* highlights the highly conserved nature of the ITS region within the genus *Gymnadenia* sensu lato, where only two major nuclear ITS variants (ITS-Early and ITS-Late) have been recognized to date, with *Nigritella* species consistently fixed for the ITS-Late variant (Hedrén et al. 2018a, b). This indicates that despite the prolonged isolation of the Apennine populations, the ITS region lacks the resolution to detect recent evolutionary divergence, remaining strongly conserved even in specimens which are geographically and phylogenetically distant. This phenomenon could be expected, considering that partial mycoheterotrophy—a nutritional strategy combining photosynthesis with carbon uptake from mycorrhizal fungi—has been documented in the closely related species *Nigritella nigra*, which shows isotopic signatures distinguishable from fully autotrophic plants (Schiebold et al. 2018). Such a partially mixotrophic strategy, relatively common among green orchids growing in montane meadow habitats with high irradiance, could be associated with a reduced nuclear mutation rate compared to the plastid genome, as reported in other plant systems with altered photosynthetic metabolism (Schelkunov et al. 2025), thereby also justifying the observed polymorphisms at VNTR loci. However, direct evidence of this nutritional strategy in *N. widderi* itself is currently lacking and remains an interesting avenue for future investigation. Apart from the low mutation rate, the high degree of identity in ITS sequences from geographically distant *Nigritella* species might be also linked to other non-exclusive evolutionary phenomena. First, it could indicate a relatively recent population or species divergence, meaning that these lineages separated too recently to have accumulated diagnostic nucleotide substitutions in this nuclear marker. Alternatively, this pattern might be explained by incomplete lineage sorting, where ancestral ITS variants have been retained across different populations despite a prolonged history of geographical isolation. Furthermore, historical gene flow, such as past hybridization or introgression events, cannot be ruled out as a potential driver for the homogenization of ITS sequences. At the genomic level, the concerted evolution characteristic of ribosomal DNA regions, driven by unequal crossing-over and gene conversion, likely plays a major role in maintaining high similarity

among intra-genomic copies, thereby drastically reducing observed variations. Additionally, strong purifying selection might be acting on the ribosomal DNA region; although ITS is non-coding, its secondary structure is functionally linked to rRNA maturation, meaning that certain mutations could be evolutionarily disadvantageous and thus eliminated. Anyway, in this context, ITS barcode gene cannot represent an adequate discriminatory marker for *N. widderi*, possessing a limited resolution power at interspecific level, although widely used for taxonomic identifications (Letsiou et al. 2024). The findings reported in this contribution provide the first molecular evidence that the Alpine *N. widderi* populations are genetically distinct, at least at the plastid level, from their Apennine counterpart, consistently with the hypothesis that the latter represents an isolated population undergoing independent microevolutionary events. This geographic and genetic pattern could be explained by two alternative evolutionary scenarios: either the Apennine populations represent a historical glacial relict isolated after post-glacial warming or they originated from a more recent long-distance seed dispersal event from the Alps followed by a founder effect. In both cases, the observed plastid DNA variations need not necessarily be attributed to unique selective environmental pressures acting on the Apennine massifs; alternatively, they could simply reflect neutral genetic drift within a small, isolated founding population, with the two mechanisms not being mutually exclusive. In conclusion, taking into consideration the plastome divergence documented in the present research, the morphological variability already suggested by Rossi et al. (1987), and the rapid evolutionary rate of Orchidaceae (Cozzolino and Widmer 2005; Chase et al. 2015; Givnish et al. 2015), *N. widderi* living on the Apennines could represent an emerging evolutionary branch within this species (e.g., a specific ecotype), starting to exhibit genetic traits distinct from the Alpine ones. Conversely, the high conservation of the nuclear ITS region underscores the need for more discriminating nuclear markers to fully untangle these dynamics.

To complement the molecular analyses presented here, a morphometric study involving populations from the two geographical macroareas is currently in progress. Crucially, as a preliminary study, these initial genetic findings provide a vital scientific baseline. Indeed, this evidence will be instrumental in securing the necessary legal and conservation permits to collect a significant number of highly protected Alpine specimens in future. Ultimately, these encouraging results serve as a steppingstone to establish formal, long-term international collaborations, aiming to systematically resolve the evolutionary trajectory of this vulnerable orchid.

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Author contributions AG, FC, and FB conceptualized the research; SDR, ET, and GDM performed the experiments; FC and FB provided the plant material; AG and AC supervised the research; AG provided financial support; SDR, FB, FC, and AG wrote the manuscript; all the authors revised the final version of the manuscript.

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Data availability All data reported in this work are present in the text and registered in the scientific databases GenBank under accession number PV800901.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval The experimental research performed in this study complies with relevant institutional, national, and international guidelines and legislation; in particular, the collection of the plant material was carried out in respect of the IUCN Policy Statement. In addition, the current work promotes the conservation status of the investigated species.

Consent for publication All the authors provide their consent to publish the present article.

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