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Extinct or Not? Confirming the "Extinct" Status of *Hieracium tolstoii* (Asteraceae) with Integrated Taxonomic Investigation

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Abstract: *Hieracium* is one of the largest flowering plant genera that has recently experienced a rapid evolutionary radiation. Due to the massive morphological variation among species, frequent hybridization, and polyploidization events, combined with apomictic reproduction in polyploids, the concept of species in *Hieracium* is widely debated, and species identification is incredibly challenging. Taxonomic investigation can lead to changes in conservation status or, in some cases, to de-extinctions of species. Taxa with doubtful classification are particularly sensitive to this kind of problem. *Hieracium tolstoii* was a narrow Italian endemic species that is nowadays considered extinct. Described as a morphological intermediate form between two other species, no further investigations were conducted after its first description. To clarify *H. tolstoii*'s origin and ensure the validity of its current conservation status, we conducted a taxonomic investigation on herbaria specimens of the early XX century. Specimens were studied using a morphometric analysis that compared *H. tolstoii* with other closely related taxa. Moreover, we performed phylogenetic investigations using three plastid intergenic spacers to evaluate the relationship between species. Plastid markers revealed the presence of indels and SNPs in *H. tolstoii* sequences that differ from sequences of the supposed parental species. Morphometry revealed differences among species that led us to confirm the validity of *H. tolstoii* as an independent apomictic species and, therefore, unfortunately, its extinction.

Keywords: Asteraceae; herbaria; *Hieracium*; extinct species; morphometry; plastid markers

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1. Introduction

Recent studies have highlighted significant conservation concerns for an increasing number of plant species [1] and have clearly shown that the ongoing rate of plant extinctions is up to 500 times the pre-Anthropocene background extinction rate for plants [2]. Although extinction is an irreversible process (although see IUCN 2016: International Union for Conservation of Nature Annual Report 2016 [3]; Seddon & King [4] for a technical and ethical discussion on de-extinctions), sometimes, rediscovery and reclassification can contribute to "resurrect" taxa from extinction [5,6]. This kind of "resurrection" is particularly common for species belonging to critical taxa, characterized by unclear taxonomy and difficult identification.

Hieracium L. (Asteraceae) represents a typical case of a taxonomically critical genus. Species in this genus show incredibly high morphological variability, which, in combination with apomictic reproduction in polyploid lineages [7], has led authors to describe more than 10,000 name combinations [8]. The most complete taxonomic treatment of the genus *Hieracium* was published by Karl Hermann Zahn, in the 1920s of the 20th century [9]. Zahn distinguished two distinct kinds of species: (i) the "basic" species or "*Hauptarten*" having a unique set of morphological characters; and (ii) the "intermediate" species or "*Zwischenarten*", which combines the morphological characters of two or more "basic"

species [10] making their identification challenging. Thus, *Hieracium* species lend themselves to misleading identification and are particularly subject to changes in their taxonomic treatment. However, the origin of both principal and intermediate species remained largely unexplained. Recent genetic studies on this genus revealed intricate phylogenetic history, suggesting multiple independent hybridization events occurred in the past [11]. However, only in a few cases the hybrid origin and genetic introgression were demonstrated for some polyploid taxa [12].

There are still contentious issues in *Hieracium* taxonomy, but as frequently occurs in modern treatments of species-rich and apomictic genera, taxonomic units are organized in informal aggregates (sections) according to their morphology [13]. Moreover, because of the apomictic reproduction, some authors consider every morphologically distinct taxon as a unique taxonomic unit defined as a microspecies. This kind of treatment is commonly used outside Central Europe [10]. On the other hand, the current taxonomic treatment in Italy [14,15] is still strongly based on Zahn's scheme and adopts a broader definition of species. The latest checklist of the native Italian flora [14] reports 278 species and 1080 subspecies (1198 microspecies if we adopt the narrower species concept) belonging to *Hieracium*, with 173 endemic species and 336 endemic subspecies. However, the number of taxa described for Italy constantly increases as new taxa are described every year [16–18].

Due to the high morphological variability, the alternative reproduction system related to the ploidy, and the supposed hybrid origin of most of the taxa, their distribution and number of individuals are almost unknown. Consequently, most of the *Hieracium* taxa in Italy are assessed as DD in the National Red List, including endemic ones [19].

Among the Italian endemic *Hieracium* taxa, the species *Hieracium tolstoii* Fen. & Zahn is currently assessed as extinct (EX) at a global level [20,21]. This species was described in 1927 by Fenaroli and Zahn [22] on specimens collected by Fenaroli on the walls of Sforza Castle in Milan (Italy) and distributed through the Flora Italica Exsiccata series [23]. About a century earlier, in 1848, the species was supposedly collected (but no specimens exist and only a generic reference to a glaucous form occurring in the area is given) on the walls of Milan by the Italian botanist Giuseppe De Notaris, together with another species described under the name *Hieracium australe* Fr. by the Swedish mycologist and botanist Elias Magnus Fries [24]. Only later, Fenaroli and Zahn considered these two plants as two different species, describing the new species as *H. tolstoii*. *H. australe* was considered by these two authors as an intermediate species between *H. racemosum* Waldst. & Kit. ex Willd. and *H. laevigatum* Willd., and *H. tolstoii* as an intermediate (supposed of hybrid origin) between a species of sect. *Italica* (Fr.) Arv.-Touv. (like *H. australe* or *H. racemosum*) and *H. pospichalii* Zahn (sect. *Dragicola* Gottschl.) or *H. porrifolium* L. (sect. *Drepanoidea* Monnier) [15,22]. *H. tolstoii* differs from *H. australe* in a series of characters, among which it is possible to quote the narrow bluish-green leaves with numerous sharp teeth and the involucral bracts with dense stellate hairs. The most recent treatment places *H. tolstoii* in the recently defined sect. *Dragicola* Gottschl., together with other morphologically similar species like *H. pospichalii* and *H. dragicola* (Nägeli & Peter) Zahn, which was formerly placed in sect. *Drepanoidea*. *H. australe* is placed in sect. *Italica* (Fr.) Arv-Touv. [15].

Today, a small population of *H. australe* still survives on the ancient walls of Sforza castle in Milan [25]. On the contrary, all the attempts that we carried out in the last years to find individuals of *H. tolstoii* have failed, and this taxon is known only from a few herbarium specimens.

In this work, in order to disentangle the taxonomic uncertainty in *H. tolstoii* and clarify its conservation status, we compared herbarium specimens of *H. tolstoii* with *H. australe* and other *Hieracium* species by morphometric and molecular analysis, also aiming to better understand the position of *H. tolstoii* among other supposedly closely related species.

2. Materials and Methods

2.1. Morphometric Analysis

The aim of these analyses is mainly to test the morphological distinctiveness of the species in question. Therefore, a broad concept of species has been applied, and subspecies are not considered in the present analysis; instead, they are merged together, considering only *sensu lato* species.

Measurements were taken on herbarium specimens (FI, PAV) or on scanned specimens, notably for *H. tolstoii* (G, TR, HAL Herbaria, acronyms follow Thiers 2024 [26]). Only complete, well preserved, and typical (not evidently altered by pathogens or extreme climatic conditions) plants were selected. Microscopic characters were observed with an assembled Wild Heerbrugg stereomicroscope. The scanned material was measured with ImageJ software (version 1.54i). Microscopic characters, such as the indumentum hair that could not be observed from the scanned material, were generalized by extension from other seen specimens belonging to the same taxon.

Following previous studies on other *Hieracium* sections [27–29], forty-nine characters (Table 1) of presumably taxonomic importance were selected based on the literature [9,15] and coded for the 77 specimens included in the analysis (see Supplementary Materials, Table S1). A wide set of characters was employed to test their importance and correlations, considering that no previous morphometric studies on late flowering species of *Hieracium* were published. The selection of characters was carried out to get a complete overview of the different parts of the plant: 23 refer to the leaves, 6 to the stem, and 20 to synflorescence and floral parts; 19 regard the shape of leaves and phyllaries, 20 the indumentum, and 10 the general habit and growth form (Table 1).

Table 1. List of characters selected for morphometric analysis. QD = quantitative discrete, QC = quantitative continuous, BI = binary, CO = ordinal.

Description of the Character	Character Name	Type
Number of cauline leaves	L_n	QD
Leaves distribution along stem	L_Dis	CO
Stem indumentum low simple	St_Low_Si	CO
Stem inumentum mid stellate	St_Mid_St	CO
Stem indumentum mid glandular	St_Mid	CO
Base of leaves	L_B	CO
Leaf colour	L_C	CO
Leaf color (upper/lower side)	L_Cc	BI
Shape of lowermost cauline leaves	L_Sh	CO
Leaf indumentum lower side	L_In_Low	CO
Upper leaf surface	L_In_Up	CO
Leaves 1/3 up	L_1/3_Up	CO
Leaf margins upper leaves	L_In_Mar_Up	CO
Glandular hairs on leaf margins	G_L	CO
Hairs on leaves/lower stem	H_L	CO
Leaf length (lowermost well-developed)	L_L	QC
Leaf width (lowermost well-developed)	L_W	QC
Leaf length/Leaf width	L/W	QC
Position of the largest teeth	L_Th	CO
Shape/direction of teeth	Th_Sh	CO
Largest teeth (cm)	Th	QC
Width of leaf (at largest teeth, excluding teeth)	Th_L_W	QC
Width of leaf/largest teeth	L_W/Th	QC
Pattern of leaf dentation	Th_Pa	CO
Dentation along stem leaves	Th_St	CO
Internode length (mean of lowermost three nodes)	In_L	QC
Dentation on petiole (pseudopetiole)	Pe_Th	CO
Leaf margin	L_M	CO
Phyllaries disposition	Phy_Dis	CO

Table 1. *Cont.*

Description of the Character	Character Name	Type
Phyllaries margin	Phy_Ma	CO
Involucre length (from the base of the secondary capitulum to the apex of the longest)	Inv_L	QC
Phyllaries width max	Phy_W	QC
Apex of phyllaries	Phy_Ap	CO
Simple hairs on phyllaries	S_H	CO
Simple hair length	S_H_L	CO
Glandular hairs on phyllaries	G_H	CO
Glandular hairs length	G_H_L	CO
Stellate hairs on phyllaries and distribution	St_H_D	CO
Abundance of stellate hairs	St_H	CO
Peduncle indumentum	Ped_In	CO
Bracts below capitulum	B_Cap	CO
Bracts on peduncle	B_Ped	CO
Style pigmentation (in exsiccata)	Sty_Pig	CO
Orders of branching	Ord_Bra	CO
Number of branches	N_Bra	QD
Number of well-developed capitula	N_Cap	QD
Acladium length	Acl	QC
Presence of aborted capitula	Cap_Ab	CO

Taxa included in the analysis were chosen for their morphological similarity to members of sect. *Dragicola* (particularly to *H. tolstoii*), for being characterized by an aphyllopodous habit with the presence of more than 10–15 cauline leaves, and for their distributional range, including lowlands habitats in Northern Italy and adjacent regions. Considering the amount of available material differs significantly among species, and this study is focused on *H. tolstoii*, the number of available specimens of this taxon determined how many specimens of the other taxa were included (to have a more uniform dataset).

The selected taxa include both "basic" species (or "Hauptarten") and "intermediate" species (or "Zwischenarten") *sensu* Zahn. These belong to sect. *Dragicola*. (*H. dragicola*, *H. leiocephalum* Bartl. ex Griseb., *H. pospichalii*, and *H. tolstoii*), sect. *Italica* (*H. australe*), sect. *Tridentata* (Fr.) Arv.-Touv. (*H. laevigatum* Willd.), sect. *Sabauda* (Fr.) Arv.-Touv. (*H. sabaudum* L.) and sect. *Hieracioides* Dumort. (*H. vasconicum* Jord. ex Martrin-Donos, *H. brevifolium* Tausch).

Despite the fact that it was supposed to contribute to the origin of *H. tolstoii* [15,22], *H. racemosum s.l* was not included in the morphometric analysis as it is generally readily recognizable by the pseudophyllopodous habit, the green leaf color (never glaucous), the scarcely imbricate involucre with the inner series of phyllaries considerably longer than the outer ones usually with the floccose hairs not restricted to (or denser on) margins. The same applies to *H. umbellatum* L., possibly the most distinctive and easily recognized hawkweed, rather unique in the genus for its clearly umbellate synflorescence and the reflexed tips of phyllaries which are mostly devoid of any indumentum or almost so. Plants in the sect. *Drepanoidea* were excluded due to their phyllopodous habit and the presence of lanceolate or lanceolate-linear leaves.

2.2. Statistical Analysis

Before proceeding with any multivariate statistical analysis, the normality of distribution for characters was tested with the Shapiro–Wilk test at the taxon level. Significantly correlated characters (>0.95) were then individuated via Spearman's correlation coefficient and excluded from the dataset to ensure that no distortion would affect the results of the analyses. A principal coordinates analysis (PCoA) performed on a distance matrix calculated with Gower 1971 [30] was preferred for exploring the morphology of the different taxa, due to the presence of both quantitative and qualitative characters. We then tested the hypothesis via discriminant analyses [31] based on the results of the exploratory analy-

sis. A canonical discriminant analysis (CDA) was chosen to visualize the morphological variation within groups, and a jackknifed linear discriminant analysis (LDA) was used to test how robust the groupings are. The aforementioned analyses were all computed using R Studio version 4.2.764 [32]. All functions were performed using the package Morpho-Tools2 [33], which conveniently wraps different functions scattered in different packages for the analysis of morphological data.

2.3. DNA Extraction

Since, for some specimens, it was not possible to extract DNA (i.e., plants consulted virtually), or we faced difficulties in obtaining good quality DNA from old herbaria specimens, in a few cases, recent herbaria specimens or fresh material were used for DNA extraction. A total of 50 specimens belonging to 16 species (using a narrow species concept) were selected for molecular investigations (Supplementary Materials Table S2). We also included species of sect. *Drepanoidea* (*H. willdenowii* Monnier, *H. porrifolium*), and other species of sect. *Sabauda* (*H. scaboisum* Sudre, and *H. sabaudiforme* (Zahn) Prain), sect. Italica (*H. racemosum*, *H. virgaurea* Coss., *H. spinidentatum* [=*Hieracium racemosum* subsp. *spinidentatum* Gottschl. & M.Villa]), and sect. *Dragicola* (*H. racemosiforme* Zahn). *Pilosella officinarum* F.W.Schultz & Sch.Bip. and *Pilosella piloselloides* (Vill.) Soják were used as an outgroup.

DNA was extracted using a modified CTAB protocol [34]. For each specimen, ca. 20 mg of plant tissue was ground in a sterile 1.5 mL reaction tube using a pestle and liquid nitrogen, then, 1 mL of CTAB extraction buffer (CTAB buffer (Promega) and 0.3% v/v β-mercaptoethanol) was added and incubated for an hour at 65 °C. After cooling them down at room temperature, they were centrifuged at 10,000× g for 2 min, and the aqueous phase was transferred in a sterile 2 mL tube, then 1 mL chloroform was added and mixed thoroughly. Samples with chloroform were centrifuged at 15,000× g for 2 min. The upper phase was transferred in a new 2 mL tube, and it was mixed with 400 µL of 2-Propanol. Samples were centrifuged again at 15,000× g for another 5 min, and the supernatant was removed. The pellet was washed with 500 µL of 70% ethanol. After a final centrifuge at 15,000× g for 2 min, it was dried at room temperature. Finally, it was eluted in 15–30 µL of distilled sterile water.

2.4. Markers Amplification

Three different plastid intergenic spacers were selected for molecular studies: trnH-psbA, trnL-trnT, and trnV-ndhC. Details about primers and annealing temperatures used for amplification are reported in Table 2. Due to the length of trnV-ndhC (800–1200 bp) and the difficulties in amplifying it due to ancient DNA degradation, we designed new primers to amplify portions of this region. A similar approach was conducted for the trnL-trnT region when it was not possible to obtain an entire sequence. In that case, we amplified using the primers a+ and b+ developed by Fehrer et al. (2007) [35].

Table 2. Regions and primers used in this study. T_a is referred to as annealing temperature.

Region	5′ Sequences 3′	T_a (°C)	Source
trnH-psbA	pbAF: GTTATGCATGAACGTAATGCTC trnHR: CGCGCATGGTGGATTCACAAATC	56	Sang et al., 1997 [36]
trnT-trnL	a: ATTACAAATGCGATGCTCT b: TCTACCGATTTCGCCATATC	46	Taberlet et al., 1991 [37]
trnT-trnL portion1	a: ATTACAAATGCGATGCTCT b+: TATACATCTGTCTCTCTTCC	46	Taberlet et al., 1991 [37] Fehrer et al., 2007 [35]
trnT-trnL portion2	a+: AAGAGAGACAGATGTATAGC b: TCTACCGATTTCGCCATATC	46	Fehrer et al., 2007 [35] Taberlet et al., 1991 [37]
trnV-ndhC	trnV-a: GAAGGTCTACGGTTCGAGTC ndhC-a: AGAAATGCCCAAAAAATATCATATTC	52	Krak et al., 2013 [11]

Table 2. *Cont.*

Region	5′ Sequences 3′	T_a (°C)	Source
trnV-ndhC portion 1	trnV-a: GAAGGTCTACGGTTCGAGTC V.C-R1: CCTCCATCGGGATTGGATTC	57	Krak et al., 2013 [11] New design
trnV-ndhC portion 2	V.C-F2: CGCAGGAAAATTTATATGGA V.C-R2: CCAAATTCTCTTGTTTTCAT	50	New design
trnV-ndhC portion 3	V.C-F3: CTCGGTAAGATTGAGATGAAAACA ndhC-a: AGAAATGCCCAAAAAATATCATATTC	55	New design Krak et al., 2013 [11]

PCRs were performed in 25 mL reactions containing 12.5 µL of VWR ® Red Taq DNA polimerasi 2X MasterMix 1.5 mM $MgCl_2$ (VWR International, Haasrode, Belgium), 1 µL of each primer, 8.5 µL of water and 2 µL of genomic DNA. After the first denaturation step at 95 °C for 5 min, 40 cycles of denaturation (95 °C for 30 s), annealing (annealing temperature Ta for 30 s), extension (72 °C for 1 min), and a final extension at 72 °C for 7 min. PCR products were purified using the Monarch® PCR & DNA Cleanup Kit (5 µg) (New England Biolab, Ipswich, MA, USA). Amplicons were finally analyzed with Sanger sequencing by the Ez-Seq service at Macrogen Europe (Amsterdam, The Netherlands) using the same primer for amplification.

2.5. Phylogenetic Analysis

Only specimens from which we were able to obtain all the sequences were included in the phylogenetic analysis. Trimming, alignment, and editing were performed with Geneious Prime® software (Dotmatics, build 2024.0.5). Sequences were trimmed, removing regions with more than 0,1% of error per base. Partial sequences of trnV-ndhC were concatenated manually to reconstruct the entire sequence and then combined with sequences of other markers. Missing data were annotated as "N". Ambiguous bases were represented following the IUPAC ambiguity code. Alignment was performed with Clustal W and then corrected manually. The combined dataset was used for phylogenetic analysis. As described in previous works [11,35], indels were considered to be single characters and the length variation in poly-A regions in trnV-ndhC and in trnH-psbA were omitted. Alignments are presented in the Supplementary Materials (Files S4 and S5).

Phylogenetic analyses were conducted with maximum parsimony (MP) using PAUP 4.0a (build 169) [38] and Bayesian inference using MrBayes [39]. Parameters were set following the same method reported in Krak et al. (2013) [11]. We performed MP analysis by heuristic searches with 10 random sequence addition replicates; for branch swapping the TBR method was used, saving no more than 100 trees with length ≥ 1. The same settings were used for calculating bootstrap values on 1000 replicates. Nodes with a percentage < 50% were retained. For Bayesian analysis, the most fitting model of molecular evolution was determined with MrModeltest V2.4 [40] for each marker using hierarchical Likelihood Ratio Tests. For TrnH-psbA and trnT-trnL, the best-fitting model was the F81 model. For the trnV-ndhC, it was GTR + I. Four Markov chains were run simultaneously for 5 million generations, using the default prior settings (one substitution rate and gamma distribution) and sampling every 1000th tree. The final average standard deviation of split frequencies reached values <0.01 (0.003244). The first 1250 trees per run were discarded as burn-in, and the remaining 7502 trees were summarized.

3. Results

3.1. Morphometric Analysis

The normality of distribution has been tested via Shapiro–Wilk for each quantitative character at the taxon level, with most characters normally distributed for most taxa. An attempt at correcting the few exceptions to the normality of distribution could be made, but since the analyses we chose have been shown to be quite robust to violation of this assumption [41], we decided to use the data without transforming them. To further support this choice, it is to be noted that, to better fit a normal distribution, all the values had

to be transformed independently from the taxon, leading to an attenuation of the actual variability between taxa. Spearman's correlation coefficient was also calculated for the characters. The only significantly correlated characters (=0.972) were the leaf width (L_W) and the leaf width at the largest teeth (Th_L_W). We decided to remove Th_L_W from the analysis due to it being a more elaborated character compared to L_W and being almost the measurement of the same feature.

The main two axes of the PCoA account for 42.89% of the variance (Figure 1a). Specimens result separated into three morphologically distinct groups: *H. tolstoii*, H1 (comprehensive of *H. australe*, *H. brevifolium*, *H. laevigatum*, and *H. sabaudum*) and H2 (consisting of *H. dragicola*, *H. leiocephalum*, and *H. pospichalii*). The CDA fully supports the three groups individuated in the exploratory PCoA (Figure 1b), with the most significant characters distinguishing *H. tolstoii* from other taxa being the number of cauline leaves (L_n), the dentation on the petiole (Pe_Th), the type of leaf margin (L_M) and the color of phyllaries margin (Phy_Ma) (Figure 1c). LDA performed maintaining these three morphological groups and confirms the hypothesis that *H. tolstoii* is a morphologically well-defined taxon from the other *Hieracium* considered in this study (Figure 1b), with a 100% correct classification for *H. tolstoii* and an overall 93.3% correct classification for the groups (Table 3). It is to be noted that only a subset of 18 characters was used for the CDA and LDA, compared to the 49 of the PCoA, as it is a fundamental requirement of these analyses that no character is invariant in any group.

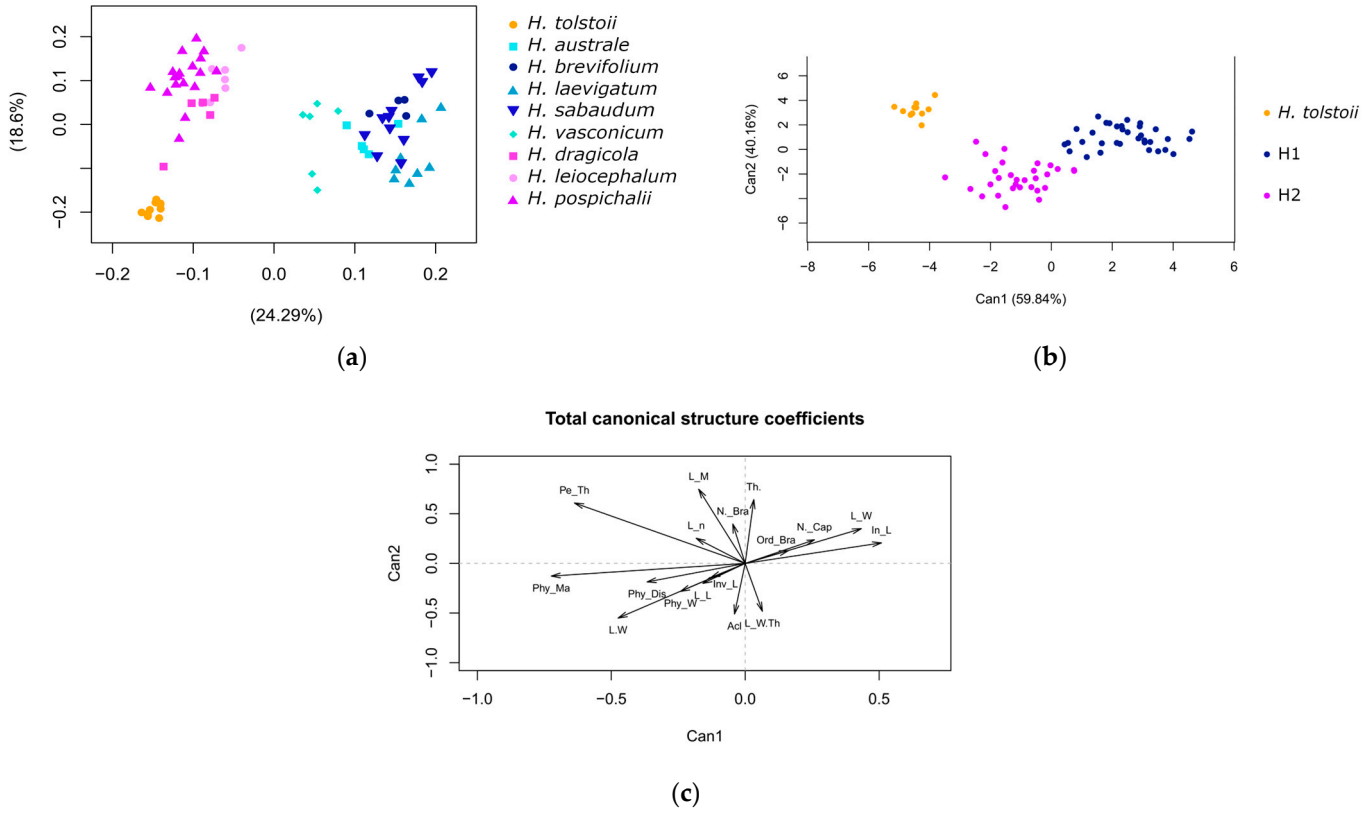

Figure 1. Results of morphometric investigation that show three main groups *H. tolstoii* (in orange); H1 (in blue) and H2 (in purple). Both plots obtained from PCoA (**a**) and from CDA (**b**) *H. tolstoii* create a distinct group that is isolated from other taxa included in the analysis. (**c**) Plot obtained by the LDA presenting the most discriminant traits.

Table 3. A confusion matrix based on the LDA was performed on the subset of 18 characters, using the morphological groups found in the exploratory PCoA as priori groups. Each row shows the a priori group, the number of samples analyzed for that group, and how they have been classified according to the LDA model.

Group	N. Samples	As *H. tolstoii*	As H1	As H2	Correct	Correct (%)
H. tolstoii	11	11	0	0	11	100
H1	34	0	29	5	29	85.29
H2	30	1	1	28	28	93.33
Total	75	12	30	33	68	90.67

3.2. Molecular Analysis

We successfully obtained all three marker sequences for a total of 21 *Hieracium* individuals belonging to 11 different species, 10 of them for the first time in literature (See Supplementary Materials, Table S3). The most informative regions were trnL-trnT and trnV-ndhC sequences. Within *Hieracium*, the length of amplified sequences varied from 643–657 bp for trnL-trnT and 827–1212 bp for trnV-ndhC region due to the presence of multiple gaps as observed also in previous studies [11,35,42]. The trnH-psbA region was the smaller (366), and only one SNP was observed. Considering indels as single traits, our combined dataset had 1737 characters: 69 of them were variable and 53 parsimony-informative. Both MP and Bayesian inference produced trees topologically similar (Figure 2).

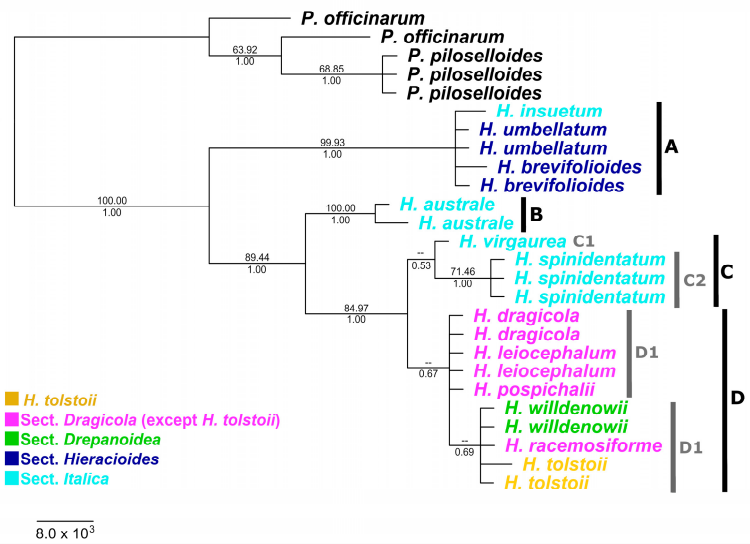

Figure 2. Phylogenetic tree of combined plastid markers (trnH-psbA, trnLtrnT, trnV-ndhC). MP and Bayesian analyses present similar topology. Bootstrap values on the MP analysis are presented above the branches. Posterior probability values from Bayesian analysis are presented. Six different haplotypes are presented. Both analyses did not resolve with a high statistical support relationship among clade C and clade D, where *H. tolstoii* is inserted.

The phylogenetic tree revealed *Hieracium* as a monophyletic clade. Four main clades were observed in the tree (Figure 2). One well-supported clade (Clade A) occurred in a basal position and is characterized by species belonging to the sect. *Hieracioides* (*H. umbellatum*, *H. brevifolioides* Zahn) and *H. insuetum* Boreau. (sect. *Italica*)

Species belonging to sect. *Italica* occurred in different clades. *H. insuetum* is inserted in the same clade of species of the sect. *Hieracioides* (Clade A). *H. australe* was the only species that presented the smaller variant of trnV-ndhC because of a large deletion of 355 bp, and clustered in a distinct clade with strong statistical support (Clade B). Other two main haplogroups (C and D) were observed. However, our tree was not able to definitively resolve relationships between them (Figure 2). In clade C, *H. virgaurea* and *H. spinidentatum*

are included (sect. *Italica*). Plants of sect. *Dragicola* and *H. willdenowii* (sect. *Drepanoidea*) cluster in the same monophyletic clade (clade D) because they present a peculiar insertion 14 bp long in trnT-trnL region. Two haplotypes were observed in Clade D (D1 and D2), which differed for only one SNP in trnV-ndhC. *H. pospichalii* presents the same haplotype of *H. dragicola* and *H. leiocephalum*. *H. tolstoii* has the same haplotype of *H. racemosiforme* and *H. willdenowii*.

4. Discussion

Molecular studies on *Hieracium* phylogeny are extremely rare [7,11,12,42], and in only one case, morphological and molecular data were combined to verify hybridization patterns between apomictic species [12]. This is the first time that morphological and molecular approaches were conducted on an extinct *Hieracium* species to assess its taxonomic treatment. Due to the absence of fresh material of *H. tolstoii*, it was not possible to apply ploidy estimation methods (chromosome counts, genome size estimation) commonly used for these plants [7,12,43]. Despite this, we can assume that *H. tolstoii* could be a triploid taxon, as resulting for many other apomictic *Hieracium* microspecies in Northern Italy, like *H. australe* and *H. spinidentatum*.

Herbaria are an invaluable source of biodiversity data and results that are particularly useful in conservation actions [44].

Unfortunately, several limitations prevent the inclusion of a larger number of species in this study. Since *Hieracium* is a quite neglected genus, a reduced number of herbarium specimens were available. Most of them were collected at the beginning of the 20th century, and some taxa were no longer recorded in Italian flora [14] since their first description. Some species are known only for a few or single specimens or from a few or single localities [45]. *Hieracium* specimens, like other Asterales, are also frequently attacked by insects that eat flowers and leaves, causing severe damage or complete loss of specimens [46]. Having well-preserved material is crucial for morphometric analysis because microscopic details are used for species identification in *Hieracium*.

Herbarium specimens also provide DNA that finds important applications in phylogenetics and evolutionary studies [47,48]. Being associated with an accurately identified individual, sequences obtained from herbarium material can have a crucial role in modern taxonomy, allowing the creation of an accurate dataset for genetic references [49]. This could be particularly useful for taxonomically challenging taxa. Despite their importance, molecular techniques are destructive methods that necessarily cause damage to specimens [48,50]. Not all the specimens are suitable for this kind of study due to insufficient material for DNA extraction and herbaria policies [44].

Moreover, DNA is known to undergo a degradation process over time. The more a specimen is old, the less are the chances to obtain good sequences [47,48,50]. Also, the probability of obtaining markers is strictly related to their length, so longer sequences are more difficult to obtain from herbarium specimens.

To avoid a reduced number of individuals per taxa, a broad species concept was used for morphometric analysis. This permitted the inclusion of more individuals in the analysis, obtaining more statistically robust results. Particular attention was reserved to consider plants belonging to sect. *Dragicola*, the section including *H. tolstoii*. According to the most recent treatment of the Italian flora [15], the sect. *Dragicola* is composed of eight species and it has the center of diversity in the Central-Eastern Italian Alps, where almost all the species occur in rock crevices and calcareous screes. The only exception is *H. tolstoii*, that was reportedly growing on ancient brick walls. Unfortunately, some of the species included in this section had to be excluded because of the insufficient number of individuals suitable for solid morphometric analysis, in some cases due to their poor conditions (*H. racemosiforme*) or because the species was known only from a few specimens (e.g., *H. atrocalyx* Gottschl.; [51]). However, leaf fragments were used for DNA extraction (i.e., *H. racemosiforme*) when possible. Nevertheless, morphometric analysis allowed to distinguish three main clusters: H1, including members of the sect. *Dragicola* (*H. dragicola*,

H. leiocephalum, and *H. pospichalii*) except *H. tolstoii*, H2 including members of the other sections (*H. australe*, *H. brevifolium*, *H. laevigatum*, *H. sabaudum*, and *H. vasconicum*, despite the latter shows a slight separation from the other species) and an isolated cluster made by individuals of *H. tolstoii*. Although not all the species of the section are included, both PCoA and LDA clearly show that *H. tolstoii* differs from all the other taxa in this study.

Members of the sect. *Dragicola* clustered together (H1) due to several morphological traits like aphyllopodous habit, glaucous leaves, the number of cauline leaves, and the presence of abundant stellate hairs on the indumentum of the capitula (Figure 1c) [15]. These traits are also common in *H. tolstoii*, which however, presents also some unique traits like the decurrent dentation on petiole (pseudopetiole) and the revolute leaf margin that are somewhat typical of species belonging to sect. *Italica* or *Hieracioides* respectively (Figure 1b,c).

Phylogenetic studies on *Hieracium* are still at an early stage because molecular diversity in this genus was only recently investigated [11,12,42]. Because of this, most of *Hieracium* species and entire sections still lack molecular data. In this study, for the first time, sequences from an extinct *Hieracium* species (*H. tolstoii*) and from historical *Hieracium* accessions were obtained and used in a phylogenetic analysis, including species belonging to sect. *Dragicola* (*H. dragicola*, *H. leiocephalum*, *H. pospichalii*, *H. tolstoii*), sect. *Italica* (*H. australe*, *H. insuetum*, *H. virgaurea*, *H. spinidentatum*), sect. *Hieracioides* (*H. brevifolioides*), and sect. *Drepanoidea* (*H. willdenowii*) for which no molecular data exist. In fact, *Hieracium* sect. *Dragicola* taxa were never studied from a genetic point of view, and no sequences were available in online databases. Also sect. *Drepanoidea*, which includes species from the Central-Eastern Alps and has typical glaucous leaves, is underrepresented, and molecular data from only a few species (*H. bupleuroides* C.C. Gmel., *H. glaucum* All., and *H. porrifolium*) are available [42]. Moreover, *Hieracium* sequences stored in NCBI rarely originated from plants collected from Italian regions. Since the main goal of the analysis was to assess possible parental relationships with other Italian species, the few sequences stored online were not included in the analysis.

Plastid markers are often used to resolve phylogenetic relationships among plants and identify species due to their versatility [52,53]. Two regions (trnT-trnL and trnV-ndhC) were selected because they had already been used in previous studies [11,42]. The trnH-psbA marker was selected because it is a short region commonly used as a barcode. A first trial revealed the presence of an indel among *Hieracium* specimens. Due to its smaller dimension (around 360 bp), trnH-psbA had a higher probability of amplification, especially from ancient herbarium DNA samples. To improve the probability of amplification, trnT-trnL and trnV-ndhC markers were amplified and sequenced in parts and reconstructed successively. Unfortunately, the indel observed in the trnH-psbA region was in between the extremity of the sequence and a poli-A region, which affected the sequencing of the following part of the sequence. Because of that, this portion was always discarded after trimming, and the remaining part was almost identical to all *Hieracium* species. Nevertheless, the polymorphisms observed for trnT-trnL and trnV-ndhC revealed that plastid sequences of *H. tolstoii* are more closely related to species in the sect. *Dragicola* differs significantly from sequences obtained from *H. australe*, the other species that previously shared the same habitat and growing site with *H. tolstoii*.

Interestingly, *H. tolstoii* has the same haplotype observed for *H. racemosiforme* and *H. willdenowii*, which were not included in morphometric analysis. Despite that now it is considered a distinct taxon [15], *H. racemosiforme* was originally classified as a subspecies of *H. pospichalii*, which tends more toward *H. porrifolium* (*H. porrifolium* $\leq$ *H. racemosiforme*) [9]. *H. willdenowii* is currently considered a subspecies of *H. glaucum* [8] and possesses phyllopodous habit, lanceolate, and lanceolate-linear leaves typical of plants of the sect. *Drepanoidea*. The high genetic similarity between species with different morphology is not particularly surprising since the sections are higher taxa defined by merely drawing arbitrary borderlines through a continuous space of variation [28], and in this specific case, all the species included in sect. *Dragicola* are somehow morphologically related to

plants of the sect. *Drepanoidea* [15] since both present glaucous leaves. All the species in this section presumably originated through past hybridization and polyploidization processes that involved *H. porrifolium*, the only known diploid species in this group [42]. *H. porrifolium* is restricted to the Southeastern Alps, a well-known glacial refuge area. It reaches its westernmost range limit in the Alps in Grigna massif, around 50 km from Milan. Moreover, species of the sect. *Dragicola* and *Drepanoidea* occur mostly in rock crevices and scree on limestone and have a very similar ecology.

Since its first description, *H. tolstoii* was described as an intermediate between *H. australe* and *H. porrifolium,* or *H. australe* and *H. pospichalii* (*H. porrifolium–H. racemosum*) [22]. Our data suggest that it is possible that some species of sect. *Italica* and especially sect. *Dragicola-Drepanoieda* are somehow involved in its origin, despite plastid sequences of *H. tolstoii, H. australe*, and *H. pospichalii.* having distinct maternal lineages. However, plastid sequences alone cannot take trace of hybridization events and are not efficient in resolving the reticulated phylogenetic history of such a complicated genus. In *Hieracium,* cpDNA is usually combined with data forming a nuclear marker like squalene synthase (sqs) and external transcribed spacers (ETS) [11,12,42]. Unfortunately, the probability of obtaining sequences of low-copy genes like sqs from ancient material is quite low, so they are not particularly suitable for this kind of study. ETS sequences, on the other hand, are already used for genetic studies of herbaria [54]. All attempts to obtain ETS sequences from our samples failed, and we decided to focus only on cpDNA due to the limited availability of plant material. However, future studies involving more performing technologies, like NGS sequencing, could facilitate obtaining nuclear DNA and provide a more complex view of the genetic relationships between the species.

About *H. insuetum*., this taxon is considered a subspecies of *H. australe* in the Italian flora [14,15]. However, our data highlighted that *H. insuetum*, treated as *H. australe* in the morphometric analysis, is morphologically and genetically different from other *H. australe* specimens. *H. insuetum* presents morphological traits more related to *H. sabaudum* and *H. brevifolium* than other conspecifics (Figure 1a) and the same haplotype of species ascribed to sect. *Hieracioides* (Figure 2). Moreover, *H. australe* individuals create a monophyletic clade since they all have a large deletion in trnV-ndhC region, which was observed for some species with phyllopod habit [11]. This could also be interpreted as evidence of hybrid origin for this species, but more investigations are required to confirm this hypothesis.

In this preliminary study, our results proved that, despite several limitations, genetic data can be obtained from ancient *Hieracium* specimens. These data can provide precious insights into this genus's complex taxonomy and phylogenetic history and should be considered in future *Hieracium* studies.

5. Conclusions

All our results concur in highlighting that the current taxonomic hypothesis that considers *H. tolstoii* an independent species seems to be supported, especially by morphometric analysis. Moreover, the idea that *H. tolstoii* represents a taxon somehow intermediate between a member of the sect. *Italica* and a species related to sect. *Drepanoidea* or sect. *Dragicola* is supported by molecular data. Nevertheless, it also should be noted that the plastid sequences alone do not allow to completely reconstruct *H. tolstoii* phylogeny and identify which species contributed to its genetics with absolute certainty. Nuclear or genomic data should be considered to provide a more complete picture of the phylogeny of these plants. The data collected so far allows us to confirm the validity of *H. tosltoii* as a valid independent species, and unfortunately, confirm its extinction since the last collection of this species is dated back to 1938 (specimen preserved in the HAL herbarium), and all attempts to find living plants in its only growing site were unsuccessful.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16090591/s1, Table S1: Dataset for morphometric analysis. Table S2: species included for molecular investigation, Table S3: Species included in phylogenetic

analysis. File S4: Alignment of sequences with indels. File S5: Alignment of sequences after indel substituted by single numeric variables to represent presence, absence, or variants.

Author Contributions: F.F., G.B. and S.O. conceived and designed the research; G.B. collected morphometric data; F.F. performed molecular experiments; M.P. and F.F. analyzed the data; F.F., G.B., M.P. and S.O. wrote the first draft of the manuscript. All authors provided edits and comments and participated in the revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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