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Biosystematic investigation on *Hieracium symphytifolium* (*Asteraceae*)

Abstract

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The results of karyological and isoenzymatic analyses carried out to verify the hybrid status of *Hieracium symphytifolium* proposed by Zahn and to evaluate the within population genetic variability of this taxon are reported.
The purposed parents of *H. symphytifolium* i.e. *H. lucidum* and *H. crinitum* and, in addition, *H. pignattianum* were also examined. The karyological analyses showed that *H. symphytifolium* is tetraploid (2*n*=36) while the *H. crinitum* is triploid (2*n*=27).
Concerning the isoenzymatic analysis, 13 loci from 8 systems (ADH, IDH, LAP, MDH, 6PGD, PGI, PGM and SKD) were examined. A total of 23 alleles were recognised, 20 of them in *H. symphytifolium* one of them exclusive. *H. symphytifolium* shows the largest genetic distance from *H. lucidum*. Considering these results, the geographical distribution relevant and some remarkable morphological characters with respect to the assumed parents, the hybrid origin of *H. symphytifolium* from *H. lucidum* and *H. crinitum* is not confirmed.

Introduction

Hieracium L. is a large genus of perennial herbaceous plants, very critical from the taxonomical point of view. Hybridization together with polyploidy and apomixis are very frequent so that variation is notably affected.

In Italy, 131 intermediate species (*species intermediae collectivae*, "Zwischenarten" *sensu* Zahn 1921-1923) are reported by Pignatti (1982). However a large number of them have not originated by of hybridization, but because of environmental factors. In fact many of them occur at a large distance from one or both of the supposed parents (Fiori 1928).

The subgenera of *Hieracium* occurring in Europe are: *Hieracium*, *Orthoteca* Froel. and *Pilosella* Tausch. They include more than 9000 entities; most of them are hybrids with reduced sexual reproduction because of apomixis and poliploidy (Pignatti 1982).

In Sicily, *Hieracium* comprises both widely distributed species (*H. crinitum* Sibth. & Sm., *H. atrovirens* Froel. and *H. pallidum* Biv.) and endemics more or less widespread (*H. macranthum* Zahn), or with very restricted range as *H. cophanense* Lojac., *H. lucidum* Guss., *H. pignattianum* Raimondo & Di Gristina and *H. symphytifolium* Froel.

This last taxon, firstly described by Froelich (1838) as *H. symphytifolium,* and, also known as *H. siculum* Guss. (Gussone 1844) in Sicily and Italy, it has variously been considered from the taxonomical point of view firstly generally accepted as a good species (Fries 1862, Strobl 1878, Lojacono 1903, Belli 1904), in the last century it has been considered as a nothotaxon (Zahn 1922, Fiori 1928, Sell & West 1976, Pignatti 1982).

The hybrid origins were suggested by Zahn, who purposed *H. lucidum* and *H. crinitum* as the parents. This Zahn's treatment was generally followed probably without any field observation. Indeed, the range of *H. symphytifolium,* in the Madonie Mountains (N-Sicily), is quite distinct from that of *H. lucidum*, which is endemic to the Monte Gallo near Palermo (NW-Sicily). For these reasons the hybrid origin of *H. symphytifolium* appeared uncertain.

In the present contribution, the results of a caryological and isoenzymatic study has been carried out in order to verify biochemically the purposed hybridity of *H. symphytifolium* and to evaluate its within-population genetic variability.

The taxa taken into account in this study were: *H. symphytifolium, H. lucidum, H. crinitum* (two distinct populations from the Madonie and the Nebrodi mountains, respectively), and *H. pignattianum,* a taxon close to *H. crinitum* recently described by Raimondo & Di Gristina (2004), soon after the "VI Conference on Plant Taxonomy" held in Alghero.

Eco-morphological features

Hieracium symphytifolium (Fig. 1) is a chasmophyte exclusive to calcareous-dolomitic rocks located on the Madonie Mountains (NC-Sicily). The whole population consists of very few individuals, growing scattered on the rocky slopes N-NW facing from 1000 to 1600 m a.s.l.

H. lucidum is an endemic sexed species (Brullo & Pavone 1978) confined to the N facing cliffs of the Monte Gallo, a carbonate promontory West of the Palermo Gulf, at 200-300 m a.s.l. This population consists of few individuals too.

H. crinitum, is an European-Caucasian species, occurring in several areas within the Sicilian floristic District. It is differentiated in several populations morphologically distinct occurring in open woods in the highest mountains of Central and North-Eastern Sicily and in the Eolian Islands (Fiori 1928). As far as the edaphic features are concerned, in the Madonie mountains *H. crinitum* grows on dolomitic substrata between 1400 and 1500 m, but it generally occurs on acid soils within *Castanea sativa* woods, from 400 to 1000 m a.s.l..

H. pignattianum is a calcicolous endemic to the Madonie mountains, frequent at the borders of *Fagus sylvatica* woods, between 1300 and 1700 m a.s.l. (Raimondo & Di Gristina 2004).

The flowering period of *H. symphytifolium* is in the summer between June and July, sometimes until the first decade of August. In the other three taxa the flowering falls from late summer to autumn.

Concerning morphology, *H. symphytifolium* characteristic features are the involucral bracts with both simple eglandular hairs and glandular hairs dark-yellowish (Fig. 2a, 2b), while the involucral bracts in the presumed parents have only glandular yellowish hairs (Fig. 2c, 2d). Furthermore *H. symphytifolium* has stigma and achenes yellow and brown-

Fig. 1. Individual of *Hieracium symphytifolium.*

blackish respectively, while *H. lucidum* and *H. crinitum* have stigma always yellow-blackish and the fruits are light yellow (Fig. 3).

Material and methods

The karyological analyses were carried out on *H. symphytifolium* and *H. crinitum*. The chromosomes were counted in at least fifteen metaphase plates obtained by root tips pretreated with 0.3% colchicine for 3 hours, and fixed in a misture 3:1 absolute ethyl alcohol-glacial acetic acid for at least one hour and finally stained by Feulgen method after hydrolysis with HCl 1 N for seven minutes at 60°C (Darlington & La Cour 1960).

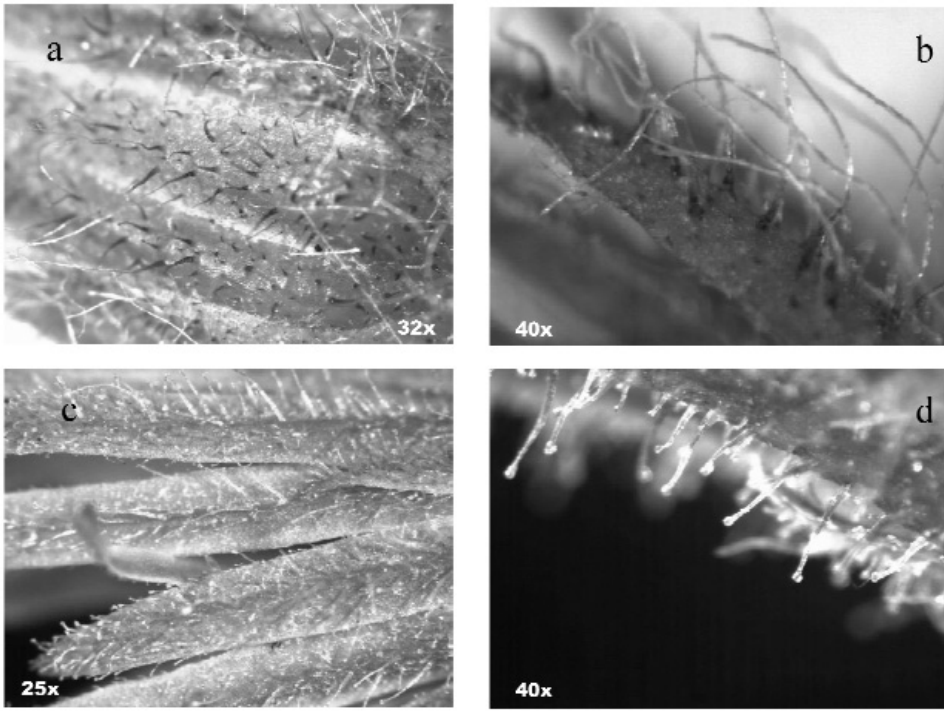

Fig. 2. Involucral bracts of *Hieracium symphytifolium* (a, b) and *H. crinitum* from the Nebrodi mountains (c, d).

The isoenzymatic analysis were carried out in a sample of at least 20 individuals of *H. symphytifolium*, *H. lucidum*, *H. crinitum* and *H. pignattianum*.

These individuals, germinated from seeds collected in the natural sites, were cultivated under surveyed conditions.

The following enzyme systems were examined: alcohol dehydrogenase (ADH, E.C. 1.1.1.1), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), leucine aminopeptidase (LAP, E.C. 3.4.11.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), 6-phosphogluconato dehydrogenase (6-PGD, E.C. 1.1.1.44), phosphoglucoisomerase (PGI, E.C. 5.3.1.9), phosphoglucomutase (PGM, E.C. 2.7.5.1.) and sikimate dehydrogenase (SKD, E.C. 1.1.1.25).

Horizontal electrophoresis was performed on 11% starch gel (Sigma, St. Louis, MO. USA) according to Kephart (1990). Two buffer systems were used: Tris-citrate, pH 7.0 for ADH, LAP, PGI, PGM and SKD and Morpholine-citrate, pH 6.1 for IDH, MDH and 6-PGD (Geraci & al. 2004). Fresh young leaves were crushed in 100 ml buffer containing Tris-HCl pH 7.5, and KCl 10 mM, EDTA 1 mM, 0.1% 2-mercaptoethanol and $MgCl_2 \times 6\ H_20$ 10 mM. Crude extracts for each sample were absorbed on paper wicks and inserted into a slit made across the gel. The enzymes migrated towards the anode. 40 mA were applied in pH 6.1 gels and 175 V in pH 7 gels. After migration, the gel slices were incu-

bated at 30°C in the dark, in a staining solution specific for each enzyme following Wendel & Stuber (1984). The loci and alleles were counted and numbered from the anode to the cathode.

For the data analysis BIOSYS –1 software (Swofford & Selander 1989) has also been used and the following parametrs were calculated: the allozyme frequencies, the mean number of alleles per locus (A), the mean percentage of polymorphic loci (P), observed (Ho) and expected (He) heterozygosity (according to the Hardy-Weinberg law). The Wright (1951) fixation (F) index was calculated as F=1-Ho/He. The Chi-square test was used to evaluate the significance of the deviation from the Hardy-Weinberg law.

Gene diversity, H, as described by Nei (1973), was calculated for each locus in each population.

Genetic relationships between populations were calculated by computing the standard genetic distance according to Nei (1972) as D = -logI where I is genetic identity $I = I_{ab}/\sqrt{I_a I_{b,}}$ (Iab, Ia and Ib are the mean for the whole loci, $\sum a_i b_i$, $\sum a_i^2$, $\sum b_i^2$ respectively).

Hierarchical Cluster analysis was performed by the SPSS 10.0 software using the Nearest Neighbour Method measuring the distance by Squared Euclidean Distance.

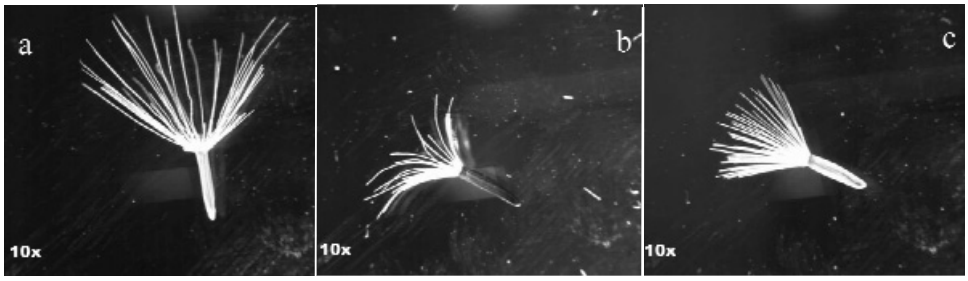

Fig. 3. Achenes of *Hieracium crinitum* from the Madonie mountains (a), *H. symphytifolium* (b) and *H. lucidum.*

Results

The chromosome number of *H. symphytifolium*, obtained on more than 30 metaphasic plates resulted 2*n*=36=4*x* (Fig. 4).

The studied *H. crinitum* populations have 2*n*=27=3*x*. The same number is reported for *H. pignattianum* (Raimondo & Di Gristina 2004).

H. lucidum, according to Merxmüller (1975) is diploid with 2*n*=18.

As regards the isoenzymatic analysis in the 8 examined systems, 13 loci were detected, three of them (ADH, 6PGD and SKD) are monomorphic for all populations.

The IDH, MDH, PGI and PGM systems showed two loci each one, the LAP system, in contrast, have only one locus *Lap-1* (Tab. 1). In these systems, *Idh-2*, *Mdh-2*, *Pgi-1* and *Pgm-1* loci, were monomorphic in the tested plants, while *Idh-1*, *Mdh-1*, *Lap-1*, *Pgi-2* and *Pgm-2* loci were polymorphic having more than one allele with different distribution and frequency in the populations studied (Tab. 1).

Table 1. Allelic frequences detected in the 5 *Hieracium* population examined.

System	Locus	Allele	*H. symphytifolium*	*H. crinitum* (Madonie)	*H.crinitum* (Nebrodi)	*H. lucidum*	*H. pignattianum*
ADH	*Adh-1*	a	1.000	1.000	1.000	1.000	1.000
IDH	*Idh-1*	a	0.107	0.000	0.000	0.000	0.000
		b	0.464	0.300	0.545	0.167	0.074
		c	0.357	0.540	0.364	0.000	0.870
		d	0.072	0.160	0.091	0.833	0.056
	Idh-2	a	1.000	1.000	1.000	1.000	1.000
LAP	*Lap-1*	a	0.000	0.000	0.850	0.000	0.000
		b	0.571	0.670	0.116	0.223	0.157
		c	0.250	0.330	0.034	0.750	0.771
		d	0.179	0.000	0.000	0.027	0.072
MDH	*Mdh-1*	a	0.857	0.583	0.606	0.833	0.444
		b	0.143	0.417	0.394	0.167	0.444
		c	0.000	0.000	0.000	0.000	0.112
	Mdh-2	a	1.000	1.000	1.000	1.000	1.000
6PGD	*6Pgd-1*	a	1.000	1.000	1.000	1.000	1.000
	6Pgd-2	a	1.000	1.000	1.000	1.000	1.000
PGI	*Pgi-1*	a	1.000	1.000	1.000	1.000	1.000
	Pgi-2	a	1.000	1.000	1.000	1.000	0.921
		b	0.000	0.000	0.000	0.000	0.079
PGM	*Pgm-1*	a	1.000	1.000	1.000	1.000	1.000
	Pgm-2	a	0.678	0.000	0.045	0.000	0.079
		b	0.322	1.000	0.955	1.000	0.921
SKD	*Skd-1*	a	1.000	1.000	1.000	1.000	1.000

Table 2. Genetic variability at 13 loci in all populations.

Population	**N° alleles**	**A**	**P99**	**Ho**	**He**	**F**
H. symphytifolium	20	1.538	0.308	0.209	0.236	0.145
H. crinitum (Madonie)	17	1.308	0.231	0.110	0.169	0.350
H. crinitum (Nebrodi)	19	1.461	0.308	0.144	0.155	0.071
H. lucidum	17	1.308	0.231	0.077	0.072	-0.069
H. pignattianum	21	1.615	0.384	0.135	0.164	0.762

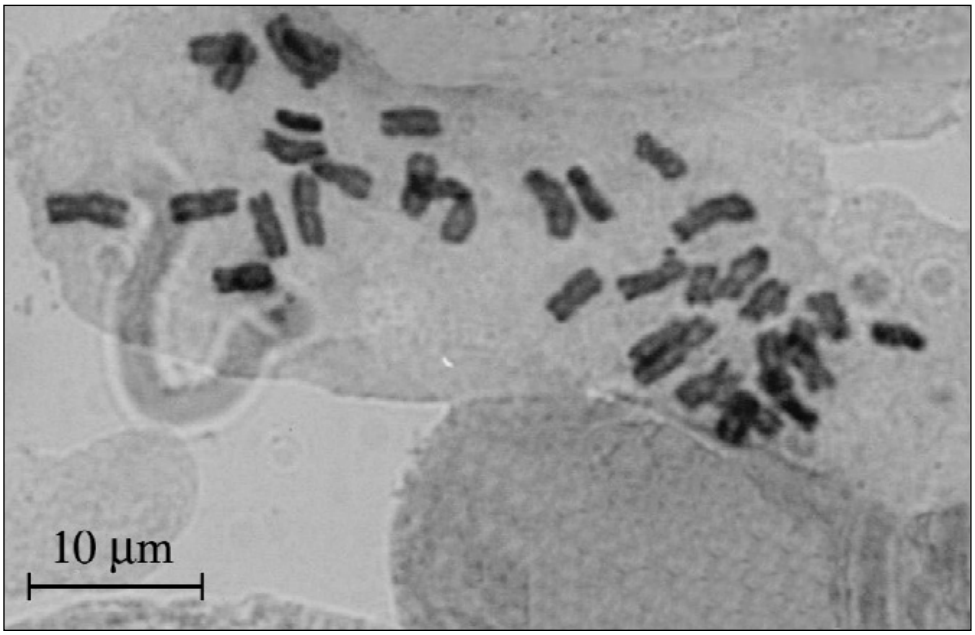

Fig. 4. Metaphasic plate (2*n*=4*x*=36) of *Hieracium symphytifolium.*

A total of 23 alleles were recognised, 20 of them in *H. symphytifolium* that in the polymorphic loci revealed four alleles at *Idh-1* locus, one of which exclusive (allele "a") with respect to *H. crinitum* and *H. lucidum*; three alleles at *Lap-1* locus, two alleles at *Mdh-1 e Pgm-2* loci respectively.

As regards *H. crinitum*, the population from Nebrodi mountains showed a specific allele at *Lap-1* locus (allele "a") with high frequency (0.850), while the population from Madonie mountains revealed on the whole less alleles (17).

In *H. lucidum* only 17 allelic forms were found. Allele "d" at *Idh-1* locus showed a frequency (0.833) generally higher in comparison with the other populations.

The genetic variability parameters as the mean number of alleles per locus (A), the mean proportion of polymorphism (P99), the observed (Ho) and expected (He) heterozygosity, and the fixation index (F) calculated at 13 loci for each population are shown in Table 2.

The mean number of alleles per locus is 1.538 in *H. symphytifolium* while in the other taxa ranges from 1.308 in *H. lucidum* and *H. crinitum* from Madonie Mts. to 1.615 in *H. pignattianum*.

The polymorphism rate was 30.8% in *H. symphytifolium* and in *H. crinitum* from the Nebrodi mountains. The lowest values were observed in *H. lucidum* and in the *H. crinitum* population from the Madonie mountains (23.1%).

The observed and expected heterozygosity values are higher in *H. symphytifolium* (0.209 and 0.236), very low for *H. lucidum* (0.077 and 0.072), in which the observed heterozygosity is weakly higher than expected, as showed by negative values of F (-0.069).

Nei's gene diversity index (H) was calculated for each polymorphic locus and for each population (Tab. 3); this value is a measure of the intra-populational genetic diversity. It

Table 3. Nei (1973) diversity (H) for each population at each locus.

Population	*Adh-1*	*Idh-1*	*Idh-2*	*Lap-1*	*Mdh-1*	*Mdh-2*	*6Pgd-1*
H. symphytifolium	0.000	0.937	0.000	0.889	0.460	0.000	0.000
H. crinitum Mad.	0.000	0.811	0.000	0.663	0.729	0.000	0.000
H. crinitum Nebr.	0.000	0.789	0.000	0.384	0.716	0.000	0.000
H. lucidum	0.000	0.278	0.000	0.387	0.278	0.000	0.000
H. pignattianum	0.000	0.341	0.000	0.537	0.823	0.000	0.000

Population	*6Pgd-2*	*Pgi-1*	*Pgi-2*	*Pgm-1*	*Pgm-2*	*Skd-1*	*H*
H. symphytifolium	0.000	0.000	0.000	0.000	0.778	0.000	0.236
H. crinitum Mad.	0.000	0.000	0.000	0.000	0.000	0.000	0.169
H. crinitum Nebr.	0.000	0.000	0.000	0.000	0.129	0.000	0.155
H. lucidum	0.000	0.000	0.000	0.000	0.000	0.000	0.072
H. pignattianum	0.000	0.000	0.218	0.000	0.218	0.000	0.164

ranges (excluding monomorphic loci in which it is zero) from 0.129 (*H. crinitum* Nebrodi Mts. population) to 0.937 in *Idh-1* locus in *H. symphytifolium* where four different alleles were detected. *H. symphytifolium* showed the highest values of intra-populational genetic diversity at loci *Idh-1*, *Lap-1* and *Pgm-2* (0.937, 0.889 and 0.778, respectively) in comparison with all other taxa investigated. In contrast the lowest values were found in the loci of *H. lucidum*. The mean value (H) of genetic diversity for each population in all loci ranges from 0.236 in *H. symphytifolium* to 0.072 in *H. lucidum*.

In order to evaluate the genetic affinity between *H. symphytifolium* and its assumed parents, the genetic distances (D) were calculated (Tab. 4).

H. symphytifolium shows a higher distance from *H. lucidum* (0.096) and from the population of *H. crinitum* from the Nebrodi mountains (0.089), while a lower value has been observed with *H. crinitum* from the Madonie mountains (0.054). This last population shows, in fact, lower genetic distance values and very similar with the other populations examined.

The dendrogram obtained using Nei's genetic distance (Fig. 5) shows four groups: the former includes *H. pignattianum* and the population of *H. crinitum* from the Madonie mountains.; the second, *H. lucidum*, the third *H. symphytifolium* and the fourth the *H. crinitum* population from the Nebrodi mountains.

Table 4. Matrix of Nei (1972) distances.

	H. symphytifolium	*H. crinitum* Madonie	*H. crinitum* Nebrodi	*H. lucidum*
H. symphytifolium	-			
H. crinitum (Madonie)	0.054	-		
H. crinitum (Nebrodi)	0.089	0.054	-	
H. lucidum	0.096	0.055	0.096	-
H. pignattianum	0.087	0.031	0.105	0.072

Discussion and conclusion

The above illustrated results allow to characterize *H. symphytifolium* from its assumed parents.

As regards morphology, the main differential characters of this taxon in comparison with the assumed parents are the involucral bracts indumentum, the coulor of the stigma and of the achenes. The constancy of these characters in the natural habitat and in cultivated plants after several generations confirms that they are genetically fixed.

Repeated karyological analyses confirm that *H. symphytifolium* is tetraploid (2*n*=4*x*=36) and agree with Sell & West (1976). For this taxon 2*n*=27 (Raimondo & al. 1983) had been detected in the Madonie mountains population (Monte Quacella).

The two *H. crinitum* populations in the Madonie and Nebrodi mountains were triploid (2*n*=3*x*=27). This data which conforms with the report of Brullo & al. (1979) for a popu-

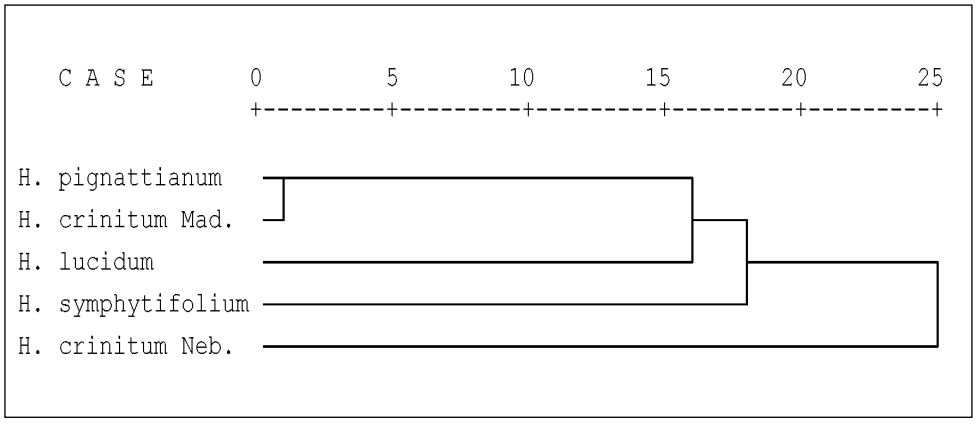

Fig. 5. Dendrogram of Nei's genetic distance using the Nearest Neighbour Method.

lation of Sella Croce in the Peloritani Mountains, but does not agree with the number reported for the same species (2*n*=36) by Sell & West (1976).

The genetic variability carried out using the isoenzymatic characterization could be useful for inferring hypothesis on the origin of *H. symphytifolium*.

Generally, hybrid taxa exibit heterozigous genotypes detecting different combinated alleles, resulting from their parents (Aparicio & al. 2000). The values of the genotypic and allelic frequencies are comparable with those of the same parents.

H. symphytifolium shows three alleles more than *H. crinitum* from the Madonie Mts. and *H. lucidum*. In this population, indeed, a specific allele (allele "a" at *Idh-1* locus) in any of the two presumed parents was not found and the frequency values of some alleles shared with *H. lucidum* and *H. crinitum* resulted rather different (allele "a" and allele "b" at *Pgm-2* locus).

The specific allele could be derived because of a sampling that not included the whole variability, although seeds were collected in several plants distant at least five meters each others and a great number of individuals have been tested (in some cases over 50 plants).

Moreover, the hypothesis of genic flow in *Hieracium* species is quite unlikely**,** because of the large apomictic reproductive cycles in this genus.

Likely it could be arised by mutation. In apomictic taxa, in fact, because of the lack of genetic recombination**,** mutations are fixed as clonal lines and quickly accumulated (Crawford 1990).

Among the taxa investigated *H. symphytifolium* shows the most genetic within population variabilty (0.236), probably because of its tetraploidic genome is wider than the *H. lucidum* and *H. crinitum* ones.

On the contrary, the small genetic variability detected in *H. lucidum*, could be considered the result of very frequent inbreeding within the populations of restricted endemic taxa (Hamrick & Godt 1990). Furthermore, the small size of this population and human impact (as fire, grazing, etc.) occurring in its habitat make *H. lucidum* a threatened species of the flora of Italy.

Concerning the genetic relationships among *H. symphytifolium*, *H. lucidum* and *H. crinitum*, the dendrogram shows *H. symphytifolium* in a separate cluster respect to *H. lucidum* and the populations of *H. crinitum* from the Nebrodi and the Madonie mountains. This last population is more similar to *H. pignattianum*; the two species share the same ecological habitat but differ significantly in the leaves indumentum in particular.

In conclusion, given the results of isoenzymatic analysis, the geographycal distribution of *H. symphytifolium* and some very important morphological characters respect to the assumed parents, the hybrid origin of this taxon is not confirmed. On the other hand, *H. lucidum* being one of the few sexual species of the whole genus (Pignatti 1979, 1982 and 1994) could be probably considered an ancestor for many European species of *Hieracium*, including *H. symphytifolium, H. crinitum* and *H. pignattianum*.

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