Investigation of *Helleborus* Genus (Ranunculaceae) Using RAPD Markers as an Aid to Taxonomic Discrimination

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Abstract

The RAPD technique was used to investigate the taxonomic position of some *Helleborus* species of Italian flora. The taxonomy of the genus is rather complicated due to the variability of the diacritic characters and to the presence of transitional forms. At present, some discrepancies exist among the authors about the systematic position of species of *Helleborus* characterised by green flowers. Because of this taxonomical uncertainty, it appears of interest to explore the potential of some molecular markers as a diagnostic feature of these entities. In this study, we used RAPD markers to characterise eight populations of *Helleborus* growing in central and northern Italy, which belong to the species *H. bocconei, H. viridis,* and *H. odorus* (characterised by green flowers) and *H. niger* (white flowers). The RAPD and cluster analyses enabled the taxonomic position of the critical entities to be clarified in favour of the classification made by Pignatti: the populations from northeastern Italy (attributed to *H. odorus*) are in fact clearly differentiated from those collected in Piemont and Lombardy and attributed to *H. viridis*.

INTRODUCTION

Some *Helleborus* species are an important source of cardiac glycosides of the bufanolide type and have been widely studied because of their pharmacological properties. The taxonomy of the genus is rather complicated due to the variability of the diacritic characters and to the presence of transitional forms; at present, some discrepancies exist among the authors about the systematic position of *Helleborus* species of the Italian Flora. While no problems appear in the determination of *Helleborus niger* L. characterized by white flowers, over-wintering basal leaves and anthesis during winter, Pignatti (1982), following the classification of Merxmuller and Podlech (1961) for Italy, mentions the difficulties encountered in classifying the hellebores with green flowers, basal leaves not over-wintering and anthesis during spring. These entities are to be considered, according to Pignatti (1982) and Tutin et al. (1993), as belonging to *H. viridis* L. (growing in northwest Italy), *H. odorus* W.&K. (northeast and central Italy) and *H. bocconei* Ten. (central and southern Italy). Other authors (Zanotto and Cristofolini, 1994) hypothesize the possibility that all populations from Northern Italy belong to *H. viridis* with the exception of populations growing in the Maritime Alps (northwest Italy) attributed to *H. bocconei*.

The main morphological characters generally considered for the identification of the various taxa are the blade division, serration and pubescence of the leaves, the diameter of the flowers, the width of the tepals and the style/follicle ratio; however these characters are highly variable, even within the same population (Servettaz et al., 1988); the opinion of these authors is that the species present in Lombardy, commonly called *H. viridis* in reality is to be considered as *H. odorus*. As a matter of fact the taxonomy of hellebores is still a debated question: it appears therefore of interest to explore the potentiality of some molecular markers as a diagnostic feature. The use of RAPD (Random Amplified Polymorphic DNA) has resulted in a potentially useful tool for species discrimination (Williams et al., 1990; Demeke et al., 1992).

In this work we have characterised, using RAPD markers, some populations of *Helleborus* growing in northern and central Italy, belonging to the critical entities *H. odorus*, *H. viridis* and *H. bocconei* in order to clarify the taxonomic position of such species; furthermore, for the sake of comparison, the neatly different species *H. niger* has also been analysed.

MATERIALS AND METHODS

Plant Material

Eight populations of *Helleborus* belonging to the species *H. niger*, *H. odorus*, *H. viridis* and *H. bocconei* were collected during the spring of 1999 and 2000 and determined according to Flora d'Italia (Pignatti, 1982) and Flora Europea (Tutin et al., 1993). The voucher specimens are deposited in the Dipartimento di Biologia, Università di Milano (MI). Table 1 shows the localities and identification number of samples.

RAPD Analysis

The RAPD analysis was performed on about 90 individuals: 8 to 17 for each population. Samples (100 mg of fresh leaves) were frozen in liquid N_2 and ground in a mortar to give a fine powder from which DNA was extracted using QIAGEN DNeasy Plant Kit (Dellaporta, 1983; Doyle and Doyle, 1987).

The RAPD analysis was performed using the following mixture: genomic DNA (5 µg/ml) 5 µl, primer (5 pM) 1 µl, dNTPs (100 µM each) 2.5 µl, DNA-polymerase (2U/µl) 0.1 µl, 10 x buffer PCR 2.5 µl, H_2O millipore 13.9 µl, for a total of 25 µl reaction mixture. The DNA-polymerase (DyNAzyme II) and buffer were purchased from CELBIO (Milano).

Fifteen primers supplied by Operon Technologies Inc. (Alameda, CA), were used for the analysis. Amplification reactions were carried out on the DNA Thermal Cycler 960 (Perkin Elmer) and subjected to 45 cycles of PCR as follows: 94°C, 1 min; 36°C, 1 min; 72°C, 2 min.

Amplification products were analysed by electrophoresis on 1.4% of agarose containing 0.1 µg/ml of ethydium bromide. The DNA fingerprints were photographed with Polaroid print film type 667 (SIGMA).

The analysis was carried out on 33 RAPD polymorphic markers. The bands were recorded as present or absent. From RAPD data a binary matrix was obtained. The matrix was elaborated using the multivariate analysis program NTSYS-pc (Rohlf, 1993). The binary matrix was transformed into a similarity matrix using Jaccard coefficients, in which joint absences were excluded from consideration. From this matrix a phenetic dendrogram was obtained by cluster analysis (UPGMA method).

RESULTS AND DISCUSSION

The specimens were collected in triplicate at each collection site. After extraction, using the procedure proposed by QIAGEN, the analysis on agarose gel and the subsequent spectrophotometric measurements of the isolated DNA revealed a significant yield of high quality DNA in the samples.

A total of 97 DNA samples were analysed. Among the 15 random primers used for the initial screening, 9 gave optimum RAPD profiles with all the populations studied and were chosen for the analysis (Table 2). A total of 33 polymorphic markers were generated using these primers. The number of bands ranged from 18 to 27 per population and the amplified products varied between 320-2200 bp. RAPD data from the nine primers were used for cluster analysis.

Examination of the obtained dendrogram (Fig. 1) clearly shows the isolated position of the "outside" taxon *H. niger* (sample 1). *H. bocconei* (sample 2) appears to be separated from the other green flowered hellebores. It appears also that the utilized molecular markers well define the populations growing on each geographic site; most likely this is due to the fact that such plants are present in isolated areas and not

distributed all over northern Italy. Among the critical entities, the populations from northeastern Italy (samples 3,4) cluster together under the same branch and the ones from northwestern Italy (samples 5,6,7,8) under the other branch. The results obtained from the RAPD and subsequent cluster analyses enable clarification the taxonomic position of the studied populations: the populations attributed to *H. odorus* (samples 3,4) are well differentiated from those attributed to *H. viridis* (samples 5,6,7,8) and are therefore really to be considered two different species.

We can notice that the critical entities from Lombardy (samples 5,6) can be considered as belonging to *H. viridis*, thus confirming the classification made by Pignatti (1982) and Merxmuller and Podlech (1961), and not to *H. odorus* as hypothesized by others (Servettaz et al., 1988), even if the morphological characters would show a different conclusion. In addition the hypothesis (Zanotto et al., 1992) that populations from Maritime Alps (8) belong to *H. bocconei* is to be discarded as well.

We can conclude that difficulties in species recognition usually caused by the highly variable morphological characters could be tackled by using the molecular markers. The results obtained from the RAPD and cluster analyses elucidated more information about the taxonomic position of the critical entities growing in Lombardy and the presence in northern Italy of two different species of *Helleborus* genus, namely *H. odorus* and *H. viridis*.

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Tables

Table 1. *Helleborus* samples.

Samples (according to Flora d'Italia)	Locality	Herbarium No.
1 *H. niger* (N)	Monte Barro (LC)	Hn – 101
2 *H. bocconei* (BO)	Monte Catria (AN)	Hb – 101
3 *H. odorus* (X)	Monrupino (TS)	Ho – 101
4 *H. odorus* (B)	Buia (UD)	Ho – 102
5 *H. viridis** (C)	Colle Brianza (LC)	Hv – 101
6 *H. viridis** (V)	Alpe del Vicerè (CO)	Hv – 102
7 *H. viridis* (U)	Upega (CN)	Hv – 103
8 *H. viridis* (CT)	Col di Tenda (CN)	Hv – 104

Note: * = critical entities

Table 2. Primers used for the RAPD analysis.

Primer	Sequence
OPA – 05	AGGGGTCTTG
OPA – 06	GGTCCCTGAC
OPA – 08	GTGACGTAGG
OPA – 10	GTGATCGCAG
OPA – 11	CAATCGCCGT
OPA – 14	TCTGTGCTGG
OPA – 17	GACCGCTTGT
OPA – 18	AGGTGACCGT
OPE – 14	TGCGGCTGAG

Figures

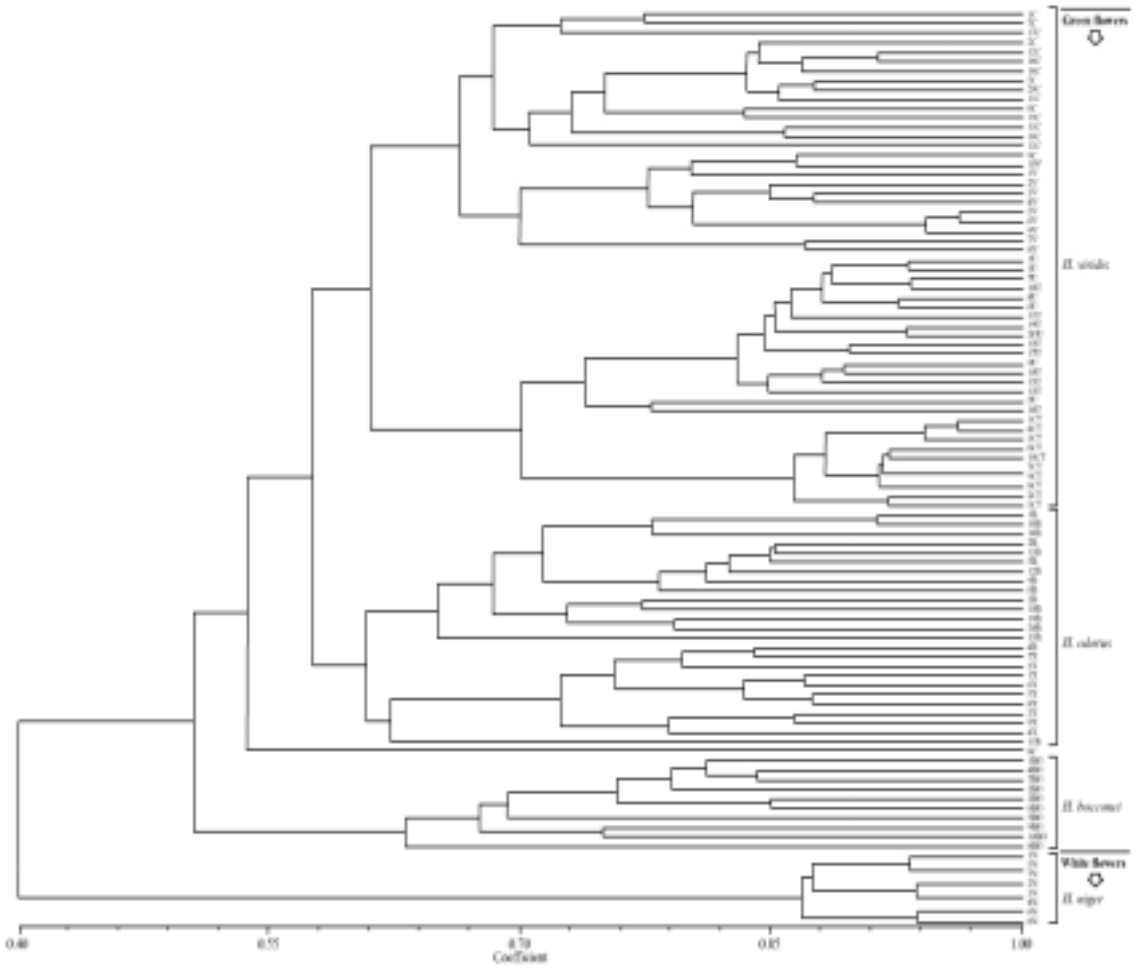

Fig. 1. Dendrogram of eight populations based on cluster analysis of genetic similarity from RAPD marker data.