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Chloroplast *trn*^{LEU} intron sequences reveal a rapid radiation in the Italian species of *Cistus* L. (Cistaceae)

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Riassunto. *Sequenze plastidiali dell'introne trnLEU rivelano una rapida radiazione nelle specie italiane di* Cistus *L. (Cistaceae)*
Le relazioni tra le specie italiane di *Cistus* sono state valutate attraverso l'analisi delle sequenze plastidiali dell'introne del *trn*^{LEU}. Tutte le specie esaminate, alcune ad ampia distribuzione, altre con areali ristretti, hanno evidenziato una bassa variabilità delle sequenze esaminate, dovuta esclusivamente alla presenza di transizioni/transversioni. Probabilmente le differenze nei caratteri morfologici (colore e dimensione del fiore), così come la presenza di differenti composti secondari, sono il risultato di molteplici eventi adattativi alle condizioni microambientali. In questo contesto, la bassa variabilità molecolare osservata tra le specie esaminate suggerisce che il genere *Cistus* possa essere il risultato di eventi di rapida e recente speciazione.

Key words: *Cistus*, cpDNA, *trn*^{LEU} intron

INTRODUCTION

Genus *Cistus* L. (rock-rose), includes sixteen species of termophilous perennial shrubs, which are typical members of the Mediterranean evergreen maquis and garigue, although in inland areas they may be found either in the understory of *Pinus* and *Quercus* woodlands or in arid and degraded slopes (DANSERAU, 1939; RIZZOTTO, 1979). Previous studies pointed out that *Cistus* is phenotypically plesiomorphic in the family and is chromosomically stable (2n=18) (RIZZOTTO, 1979). Since chromosomal evolution seems to be indicative of the necessity of adapting to habitats different from those of origin (STEBBINS, 1974), it has been hypothesised that the genus originated in the same regions

were it is still living, more precisely it diffused from western to eastern Mediterranean area (RIZZOTTO, 1979).

The flower of *Cistus* species is white or pink to red-purplish in colour, radially symmetrical and pentamerous, sometimes with three sepals, with numerous stamens and a plurilocular ovary. DANSERAU (1939) regards both the white flowers and the reduced calyces as derived characters. The different species have been classically discriminated and taxonomically arranged on the basis of the aforesaid floral characters as well as on their leaf morphology (WARBURG, 1968; PIGNATTI, 1982), which offers a set of features allowing, for the Italian species, the proposal of a leaf analytical key (PIGNATTI, 1982).

Eight wild species occur in the flora of Italy, some of them with a wide distribution and some others restricted to one or few disjunct areas. The widespread species are *C. salvifolius* L., *C. monspeliensis* L. and *C. incanus* L., the latter including three subspecies, ssp. *incanus*, ssp. *creticus* and ssp. *corsicus*. The other species are *C. albidus* L., distributed in the Liguria region as well as in Sardinia and Corsica, *C. laurifolius* L., known for Tuscany, *C. clusii* Dunal and *C. crispus* L., reported for Sicily, and *C. parviflorus* Lam., presently surviving only in the island of Lampedusa (RIZZOTTO, 1979; PIGNATTI, 1982).

Although molecular biology nowadays supplies effective analytical tools to asses species relatedness, genus *Cistus* has not been hitherto examined under this respect, and molecular techniques have been scantily applied also in the rest of family Cistaceae (JUDD *et al.*, 1999).

This study aims at investigating the relationships among the Italian species of *Cistus* by means of sequence analysis of chloroplast DNA trn^{LEU} (UAA) intron, since this region proved to be informative to evaluate phylogenetic relationships at low taxonomic levels (VAN HAM *et al.*, 1994; BOHLE *et al.*, 1994; GIELLY & TABERLET, 1996). For a more effective evaluation of the data, the same sequences were analysed also in two species of genus *Heliantemun* Miller, another genus of the family, strictly related to *Cistus*.

MATERIAL AND METHODS

Plant materials of Italian species of *Cistus* and *Heliantemum apenninum* (L.) Miller and *H. canum* (L.) Baumg. were field collected by the authors (Tab. 1).

Tab. 1 - Investigated specimens, with origin and acronyms.

Species	Origin	Acronyms
Cistus albidus L.	Sardinia	ALBI
Cistus clusii Dunal	Vittoria (RG) Sicily	CLUS
Cistus crispus L.	Messina Sicily	CRIS
Cistus incanus L. subsp. *incanus*	Orto Botanico UNICAL	INCA
Cistus incanus L. subsp. *corsicus*	Corsica	CORS
Cistus incanus L. subsp. *creticus*	Sibari (CS) Calabria	CRET
Cistus monspeliensis L.	Castrovillari (CS) Calabria	MONS
Cistus salvifolius L.	Orto Botanico UNICAL	SALV
Heliantemum apenninum (L.) Miller	Castrovillari (CS) Calabria	HAPE
Heliantemum canum (L.) Baumg.	Castrovillari (CS) Calabria	HCAN

Total DNA was extracted from 0.5 g of silica gel dried leaves of individual plants according to PELLEGRINO *et al.* (2001).

Chloroplast trn^{LEU} intron DNA was amplified by polymerase chain reaction (PCR) using specific primers (5'-GGG GATAGAGG-GACTTGAAC-3' forward primer and 5'-CGAAATCGGTA-GACGCTACG-3' reverse primer), as described in TABERLET *et al.* (1991).

All PCR reactions, with 10 ng of DNA as template (100 µl final volume), were conducted in a thermal cycler (Perkin Elmer 2600) for 30 cycles. Initial conditions were as follows: 30 sec denaturation at 94 °C, 1 min annealing at 55 °C, 45 sec extension at 72 °C; extension time was increased of 3 sec/cycle, and extension was further prolon-

ged for 5 min at the end of the last cycle. Amplified fragments were purified using Microcon 100 microconcentrators (Amicon MWCO 100,000). PCR fragments were then double-strand sequenced in both directions by using a modification of the Sanger dideoxy method (SANGER *et al.*, 1977) as implemented in a double strand DNA cycle sequencing system with fluorescent dyes. All sequence reactions were loaded into a 373A Automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.). The electropherograms of the investigated specimen were aligned by using the Sequence Navigator software (Perkin Elmer, USA) and compared by using the Clustal W (THOMPSON *et al.*, 1994) with which a dendrogram was also produced.

RESULTS

The *trn*^{LEU} introns of all sequenced specimens of *Cistus* were 319 bp long, with G+C contents of 28% and a total identity of approx. 97%. The *trn*^{LEU} introns of *Heliantemum apenninum* and *H. canum* were 323 bp and 320 bp long, respectively, with a total identity of approx. 95% (between each other) and 92-94% with *Cistus* sequences. The alignment of *Cistus* sequences showed no insertion or deletion and eight nucleotide substitutions (Fig. 1). The dendrogram resulting from the alignment (Fig. 2) showed three different species groups with dissimilar degrees of identity. In detail, a group, with 100% sequence identity, includes all the subspecies of *C. incanus* (ssp. *incanus*, ssp. *corsicus* and ssp. *creticus*) and *C. albidus*. A second group includes *C. clusii* and *C. monspeliensis*, and shows only one nucleotide substitution as compared to the first group. Finally, the sequences of *C. crispus* and *C. salvifolius* display several (5) nucleotide substitutions different from members of other groups.

	1	2	3	4	5	6	7	8	9	10	11
ALBI	G	T	C	G	T	C	A	T	C	G	T
CORS	.	.	.	.	.	.	.	.	.	.	.
CRET	.	.	.	.	.	.	.	.	.	.	.
INCA	.	.	.	.	.	.	.	.	.	.	.
CLUS	.	.	.	.	.	.	.	.	.	.	.
MONS	.	.	.	.	.	.	.	.	.	.	.
CRIS	T	.	.	.	.	.	.	.	.	.	.
SALV	.	C	.	.	.	.	.	.	.	.	A
HCAN	**.**	**.**	**.**	**A**	**C**	**T**	**C**	**G**	**A**	**T**	**.**
HAPE	.	.	T	.	C	.	C	G	A	.	.

	12	13	14	15	16	17	18	19	20	21	22
ALBI	C	G	G	-	T	A	A	A	A	A	C
CORS	.	.	.	-	.	.	.	.	.	.	.
CRET	.	.	.	-	.	.	.	.	.	.	.
INCA	.	.	.	-	.	.	.	.	.	.	.
CLUS	.	.	.	-	G	.	.	.	.	.	.
MONS	.	.	.	-	.	.	.	.	.	.	.
CRIS	.	.	.	-	.	.	.	.	.	.	.
SALV	.	.	.	-	G	.	.	.	.	.	.
HCAN	**G**	**C**	**C**	**C**	**G**	**G**	**-**	**G**	**T**	**G**	**-**
HAPE	G	C	C	-	G	G	-	G	T	G	.

	23	24	25	26	27	28	29	30	31	32	33
ALBI	A	C	G	A	A	A	G	T	T	-	-
CORS	.	.	.	.	.	.	.	.	.	-	-
CRET	.	.	.	.	.	.	.	.	.	-	-
INCA	.	.	.	.	.	.	.	.	.	-	-
CLUS	.	.	.	.	.	.	.	.	.	-	-
MONS	.	.	.	.	.	.	.	.	.	-	-
CRIS	.	.	.	.	.	.	.	.	A	-	-
SALV	.	.	.	.	.	.	.	.	.	-	-
HCAN	**C**	**T**	**T**	**-**	**-**	**-**	**A**	**A**	**A**	**G**	**A**
HAPE	C	T	T	T	T	G	A	A	A	G	A

	34	35	36	37	38	39	40	41
ALBI	T	C	G	A	-	-	T	G
CORS	.	.	.	.	-	-	.	.
CRET	.	.	.	.	-	-	.	.
INCA	.	.	.	.	-	-	.	.
CLUS	.	A	.	.	-	-	.	.
MONS	.	.	.	.	-	-	.	.
CRIS	.	.	.	.	-	-	.	.
SALV	.	A	.	.	-	-	.	.
HCAN	**C**	**.**	**T**	**T**	**A**	**C**	**A**	**.**
HAPE	C	.	T	T	A	C	C	A

Fig. 1 - All variable nucleotide sites among *trn*^{LEU} intron sequence of the Italian species of *Cistus*, *Heliantemum apenninum* and *H. canum*. Dots indicate sequence matches to the first taxon. Site numbers 1-41 correspond to the actual sites: 38, 56, 61, 86, 94, 101, 119, 135, 136, 142, 162, 171, 175, 182, 187, 196, 207, 232, 240, 241, 251, 255, 262, 270, 273, 274, 275, 276, 279, 280, 282, 286, 287, 289, 290, 294, 298, 300, 301, 312, 313.

The comparison of *trn*^{LEU} intron sequences of *Heliantemum apenninum* and *H. canum* shows five insertion/deletions and five nucleotide substitutions, while there are various other substitutions (14-16) and insertions/deletions (7-10) between sequences of *Heliantemum* and *Cistus* (Fig. 1).

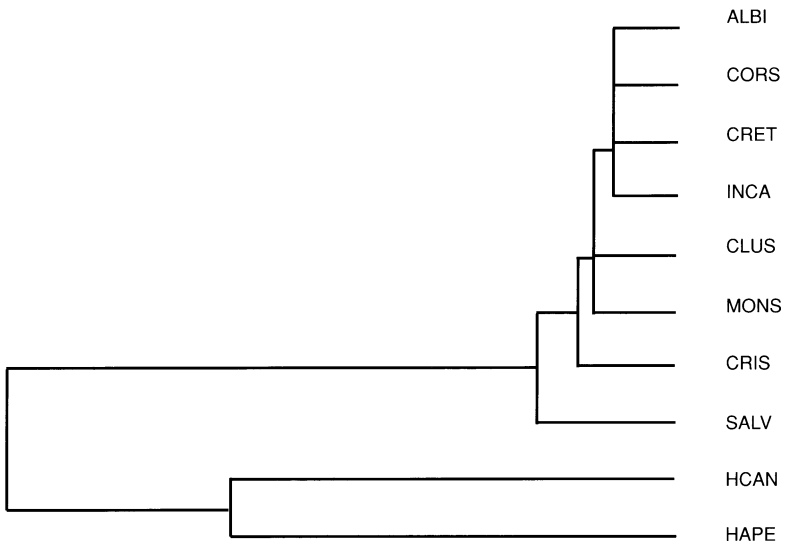

Fig. 2 - Dendrogram based on the *trn*^{LEU} intron sequences of the Italian species of *Cistus*, *Heliantemum apenninum* and *H. canum*.

Discussion and conclusions

Italian species of *Cistus* exhibit a low variability in their chloroplast DNA *trn*^{LEU} intron sequences, and this is due exclusively to transitions or transversions. An unexpected finding is the complete lack of insertions/deletions, which instead are been observed between *Heliantemum* species.

However, relationships among the Italian species of *Cistus* based on these molecular data are clearly different from those based on the classical morphological characters. In fact, species with white or red

coloured flowers show identical trn^{LEU} sequences (*C. incanus* and *C. monspeliensis*). In addition, *C. clusii*, the only species with three sepals, shows a close relationship with *C. monspeliensis*, which has five sepals. In the light of the present results, flower colour and number of sepals appear as characters with a less relevant evolutionary/ systematic value than previously thought.

However, discrepancies among morphological data and phytochemical (VOGT *et al.*, 1987; DEMETZOS *et al.*, 1994) and ultrastructural (GÜLZ *et al.*, 1996) analyses have been already observed. In fact, the species that group together on the basis of ultrastructural characters do not share the same flower colour or number of sepals. Similarly, a chemotaxonomic survey was carried out using phytochemical data from the literature and relative to both natural and cultivated taxa of *Cistus* (DEMETZOS & PERDETZOGLOU, 1999). This study showed that secondary compounds may be useful in revising the genus *Cistus*, but discrepancies were noted, once again, in comparison with classical, morphology-based taxonomy. Incongruence of different datasets may be indicative of various independent modification events as adaptive responses to microenviromental conditions experienced from time to time. This opinion is nowadays well founded on the acquired awareness that ecological and morphological divergences often proceed at a different speed as compared to genetic divergences (LEVIN, 1993).

Interestingly enough, some derived features of the rock-roses appear to be associated with primitive characters. For instance, *Cistus* species produce a great number of seeds, which are dispersed by gravity and can rapidly germinate (TALAVERA *et al.*, 1993), a character propitious in those Mediterranean habitats where anthropic disturbances are frequent. However, seeds quickly germinating are usually associated with a parietal placentation type, which is in turn correlated with a reduction of flower size (STEBBINS, 1974). Actually, *Cistus* exhibits this type of placentation, but flower size is still relatively large. According to STEBBINS (1974), *Cistus* may be regarded as an example of secondary increase in flower size and ovule number in response to selective pressure for greater fecundity in relatively favourable habitats.

Probably, the expansion of rock-roses was a consequence of the contemporary insurgence of similar reproductive strategies, as the development of a large, open flower, with large stigmata and numerous stamen, copious pollen and nectar production, and the acquisition of self-incompatibility, all of them observed in three co-occurring *Cistus* species (BOSCH, 1992).

In fact *Cistus* species have similar floral structures and flowering periods, so they may compete for the service of the same pollinators. Identity and abundance of pollinators (without the risk of extensive hybridisation and self-pollination), as well as an extremely limited seed dispersal render pollen a major component of the gene flow in this group and at the same time assure an high reproductive success to these species (TALAVERA *et al.*, 2001).

These features may have allowed a rapid radiation of *Cistus* in the Mediterranean area and the low molecular variability observed in this study may be explained in the framework of this scenario (HODGES & ARNOLD, 1994). However, further molecular analysis, employing nuclear markers, will contribute to the definition of a possible role of hybridisation in obscuring the patterns of relationships within the genus (SANG *et al.*, 1995), as well as to the understanding of its history.

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Abstract

Chloroplast DNA *trn*^{LEU} (UAA) intron sequences were used to investigate the relationships among the italian species of *Cistus*. All the examined species, some of them showing a wide distribution and some others restricted to few disjunct areas, exhibit low variability, due exclusively to transitions or transversions in their chloroplast sequences. Probably, the difference in morphological characters (as colour and size of flowers), as well as the different set of secondary compounds, are the results of many independent modification events deriving from adaptive responses to microenvironmental conditions. As a consequence, the low molecular variability observed in this study suggests that *Cistus* may have undergone a recent and rapid radiation event.

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