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A fine restriction map of the ITS1 in some Dipsacaceae and allied taxa

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Riassunto

E' stata effettuata una mappatura di restrizione fine dello spaziatore intragenico I (ITS1) del DNA ribosomiale nucleare nei seguenti taxa delle Dipsacaceae e Valerianaceae: *Centranthus ruber* (L.) DC., *Dipsacus fullonum* L., *Lomelosia crenata* (Cyr.) Greuter et Burdet subsp. *crenata* (Cyr.) Greuter et Burdet, *Pterocephalidium diandrum* (Lag.) G. López, *Sixalix atropurpurea* (L.) Greuter et Burdet subsp. *maritima* (L.) Greuter et Burdet. L'ITS1 è risultato lungo 230 paia di basi (bp) in tutti i taxa presi in considerazione tranne che in *P. diandrum*, in cui è presente un'inserzione di circa 15 bp.

I siti di restrizione individuati sono stati impiegati per un'analisi cladistica con *C. ruber* come outgroup. Il singolo cladogramma ottenuto è completamente congruente con le ipotesi filogenetiche proposte per le Dipsacaceae su base morfologica. L'ITS1 è da considerarsi pertanto una molecola appropriata per effettuare ipotesi filogenetiche a livello intergenerico nell'ambito delle Dipsacaceae.

INTRODUCTION

Among the regions of nuclear DNA which have been closely scrutinized by plant systematists, nuclear ribosomal DNA (n-rDNA), i.e., the DNA which codes for ribosomal RNA, is one of the most promising. Its widespread use depends upon both its abundance and different rates of conservation, which allow systematists to use some of its regions for high rank comparisons and some others for studies of narrow taxonomic scope. A n-rDNA repeat consists of three highly conserved coding units, 18S, 5.8S and 26S, which have been used to provide data at the highest level of plant phylogeny (MISHLER *et al.*, 1994 and references therein). These regions are separated by two quite variable Internal Transcribed Spacers (ITS 1 and 2), normally employed in infrageneric comparison (SUH *et al.*, 1994; KIM and JANSEN, 1994 and references therein; BALDWIN *et al.*, 1995); each transcription unit is in turn separated by a highly variable intergenic spacer (IGS).

Key words: Dipsacaceae, restriction mapping, ribosomal DNA.

The object of this paper is testing the feasibility of a ITS1 fine restriction mapping approach for inferring phylogenetic relationships in Dipsacaceae. Five species belonging to different genera of Dipsacaceae Juss. and to one genus of Valerianaceae Batsch. were chosen.

MATERIALS AND METHODS

Plants of *Centranthus ruber* (L.) DC. (Valerianaceae), *Dipsacus fullonum* L., *Pterocephalidium diandrum* (Lag.) G. López, *Lomelosia crenata* (Cyr.) Greuter et Burdet subsp. *crenata* (Cyr.) Greuter et Burdet, *Sixalix atropurpurea* (L.) Greuter et Burdet subsp. *maritima* (L.) Greuter et Burdet (Dipsacaceae) were grown from seeds at the Botanical Garden of Naples (Italy). Vouchers of all the examined plants are deposited at NAP.

Leaves (1 g per sample) were ground on liquid nitrogen and total DNA was extracted following the procedure described in CAPUTO *et al.* (1991).

The sequence of interest was obtained via polymerase chain reaction (PCR). Amplification of ITS1 was carried out by using two primers which anneal in the 3' region of the 18S (5'-GGAGAAGTCGTAACAAGGTTTCCG-3') and in the 5' region of the 5.8S (5'-ATCCTGCAATTCACACCAAGTATCG-3'), respectively. PCR reactions were conducted in a thermal cycler (Perkin Elmer Cetus 9600) for 30 cycles. Initial conditions were as follows: 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 45 sec extension at 72 °C. Samples were denatured for 5 min at 94 °C before the beginning of the first cycle; extension time was increased of 3 sec/cycle; extension was further prolonged for 7 min at the end of the last cycle. All PCR fragments were tested for homology via hybridization against a clone containing a complete sunflower ribosomal repeat obtained through the courtesy of M. Arnold (University of Gainsville, Florida), acknowledged here.

In order to avoid length estimation errors due to mistermination of PCR products, PCR fragments were all cut with *Bin* I and *Eco*R V prior to any other digestion experiment. From our observation of the 18S and 5S sequences available in the literature, in fact, these are among the restriction endonucleases which have sites in the 3' region of the 18S (downstream to our 5' primer) and in the 5' region of the 5.8S (upstream to our 3' primer).

Samples were then digested and double digested with *Alu* I, *Ava* II, *Hae* III, *Hha* I, *Mse* I, *Nar* I, *Nla* III, *Rsa* I restriction endonucleases according to the manufacturer's specifications for temperature and buffers.

Digested samples were electrophoresed (1-3 μg/lane, 2.0 to 4.0% agarose gel - according to desired resolution - 6 h, 25 V). The length of the fragments was estimated by using both a 50 and a 10 base pair (bp) DNA ladders (Gibco BRL) as markers.

Restriction maps were prepared by an inferential procedure using the data from single and double digestions.

The cladistic analysis was performed on a restriction site matrix, by using the exhaustive option in the HENNIG86 software (Farris, 1988) and delayed transformation character optimization.

RESULTS

Raw PCR products were 320 bp in length for CEN, DIP, LOM and SIX and 340 bp for PTE (see Tab. I for acronyms). After the two successive *Eco* RV and *Bin* I digestions, the single fragments obtained for CEN, DIP and SIX were 250 bp long. *Bin* I digestions of both LOM and PTE yielded two fragments, which added up to 245 and 260 bp, respectively. Of the chosen enzymes, only *Alu* I did not cleave any of the PCR fragments.

The complete restriction map for all the enzymes and taxa is reported in Fig. 1, and the estimated length of the fragments is reported in Tab. I. Notably, the endonucleases whose recognition sites are GC rich cleave sequences more often than those with AT rich recognition sequences (Tab. I).

The estimated length of the sequences obtained adding up all restriction fragments (Tab. I) was as follows: 245-250 (CEN, DIP, LOM, SIX); 260-270 (PTE). Therefore, PTE shows a small insertion ranging between the 10 and 20 bp. This insertion is located between the *Rsa* I and the *Nla* III sites (the upstream *Rsa* I fragment and the downstream *Nla* III fragment are of the same length as in DIP), and therefore between approx. position 40 and 90 of the PTE fragment. A more accurate evaluation, obtained comparing in both PTE and DIP the length of the *Rsa* I and *Nla* III fragments, would suggest that the insertion is approx. 15 bp long.

Tab. I - Length of the restriction fragment detected in ITS1 of the taxa in study. For each enzyme, the cleavage sequence is reported. Abbreviations are as follows: CEN = *Centranthus ruber*; DIP = *Dipsacus fullonum*; LOM = *Lomelosia crenata* ssp. *crenata*; PTE = *Pterocephalidium diandrum*; SIX = *Sixalix atropurpurea* ssp. *maritima*. A dashed line indicates absence of cleavage.

	Ava II GGWCC	*Bin* I GATCC	*Hae* III GGCC	*Hha* I GCGC	*Mse* I TTAA	*Nar* I GGCGCC	*Nla* III CATG	*Rsa* I GTAC
CEN	50	---	60	20	---	190	---	30
	200		45	15		60		220
			70	130				
			70	30				
				55				
DIP	50	---	90	130	---	---	75	40
	125		155	30			170	205
	70			90				
LOM	---	170	60	85	---	85	80	---
		75	190	35		160	65	
				10			100	
				30				
				85				
PTE	190	210	---	140	160	---	90	40
	70	50		10	105		170	220
				120				
SIX	175	---	60	85	---	85	145	---
	70		190	35		100	100	
				10		60		
				30				
				30				
				60				

A restriction site matrix was prepared (Tab. II) by scoring the 23 different restriction sites for presence and absence. Autapomorphic sites were scored to allow an easier comparison between Tabs. I and II, but were not used to compute length, consistency (c.i.) and retention (r.i.) indices. The cladistic analysis, for which CEN was used as an outgroup, yielded a single most parsimonious cladogram (length 23 steps, c.i. 0.73, r.i. 0.50) shown in Fig. 2.

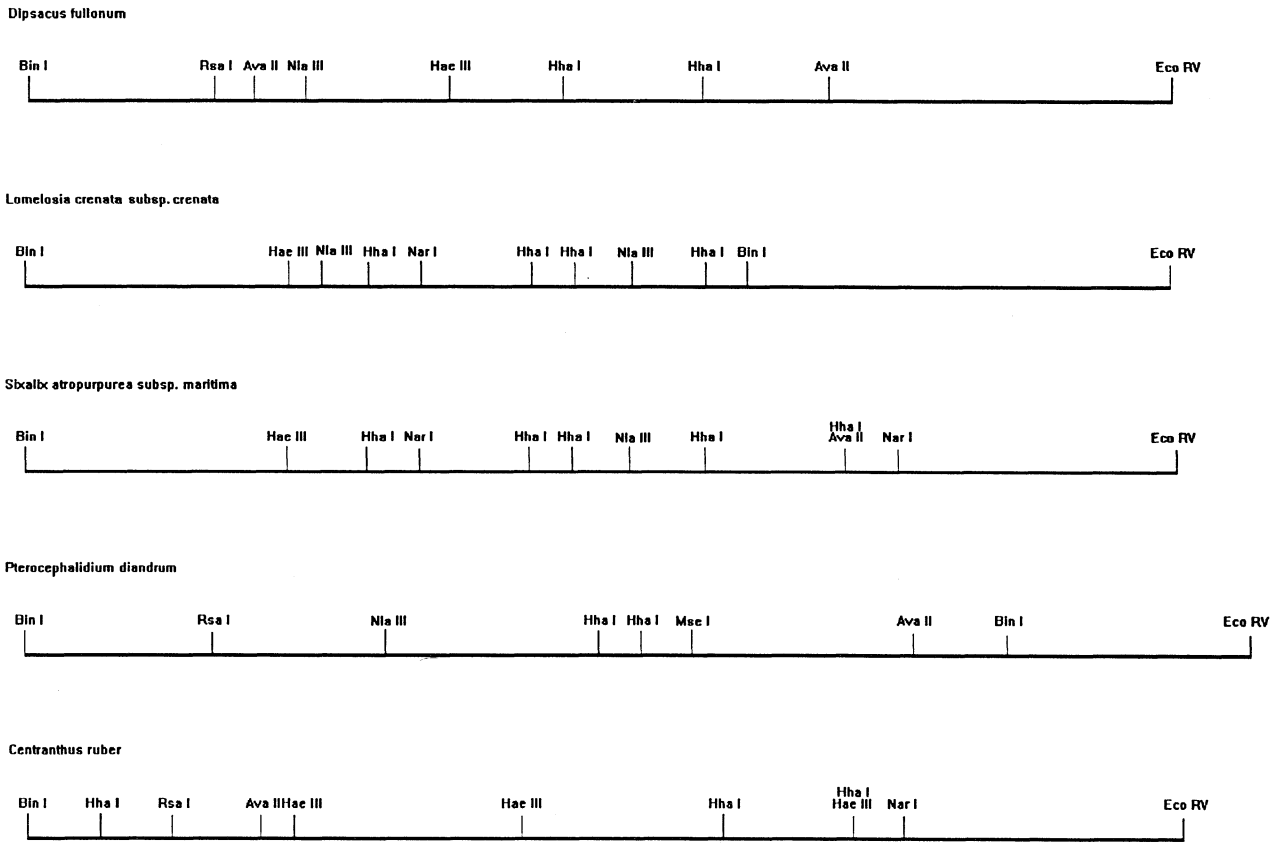

Fig. 1. - Restriction map of the *Bin* I-*Eco* RV fragments containing the complete ITS1 for the taxa in study.

Tab. II - Restriction site matrix for the taxa in study. 0 indicates absence of a site; 1 indicates its presence. Characters are as follows: 1-3: *Ava* II; 4-5: *Bin* I; 6-8: *Hae* III; 9-15: *Hha* I; 16: *Mse* I; 17-19: *Nar* I; 20-21: *Nla* III; 22-23: *Rsa* I. For acronyms see caption to Tab. I.

CEN	1 0 0 0 0 1 1 1 1 1 1 1 0 0 0 0 1 0 0 0 0 1 0
DIP	1 1 1 0 0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1 0 0 1
LOM	0 0 0 1 0 1 0 0 0 0 0 1 0 1 1 1 0 0 1 0 1 1 0 0
PTE	0 1 0 0 1 0 0 0 0 0 0 0 1 0 1 1 0 0 0 1 0 0 1
SIX	0 1 0 0 0 1 0 0 0 0 1 1 1 1 1 0 1 1 1 0 1 0 0

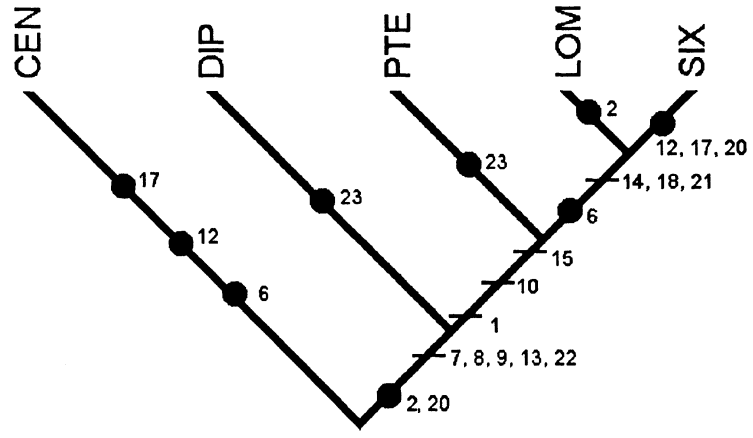

Fig. 2 - Cladogram obtained by the cladistic analysis of the restriction sites using the matrix in Tab. II. A dash indicates apomorphy; a circle indicates homoplasy. Acronyms and character numbers are indicated in the captions to Tabs. I and II, respectively.

The ingroup, for which 5 synapomorphies were recorded, consists of a ladderized sequence of DIP, PTE, LOM and SIX. The dichotomy between DIP and the rest of the taxa as well as the most internal one both show 3 synapomorphies. Homoplasies are present in the ingroup, and especially in the terminal taxa: DIP and PTE show one parallelism, LOM one reversion, and SIX two parallelisms and one reversion.

DISCUSSION AND CONCLUSIONS

In a restriction mapping study one of the most crucial issues is related to the minimum length of the fragments that the gel system used can discriminate. In order to prepare a fine map of a fragment which is about 250 bp long, we were compelled to use several different gel systems, containing from 2.0 to 4.0% agarose. The final gel often required several previous attempts at different concentrations. All the single digests were observed before the double digests, so to decide which pair of enzymes and which digestion order were less likely to produce fragments too short to be accurately sized. In one case (*Hha* I), we were compelled to use also a partial digestion approach to achieve accurate mapping of the fragment.

Sizing the ITS1 in Dipsacaceae is almost impossible in our system (in fact, the fragments we mapped also include the 3' end of the 18S and the 5' end of the 5.8 S). However, by comparing the angiosperm sequences available in public-access automated databases, it is possible to note that the *Bin* I site is invariably present exactly at the 3' terminus of the 18S, and that the *Eco* RV site is present at a location which is 20-22 bp downstream of the ITS1 3' terminus. On these grounds, the ITS1 of the taxa in study is 225-230 (CEN, DIP, LOM and SIX) to 240-250 bp long (PTE).

The cladogram obtained perfectly matches the previous evidence on the family (EHRENDORFER, 1964; VERLAQUE, 1985; CAPUTO and COZZOLINO, 1994, 1995), by showing PTE, LOM and SIX (all belong to the tribe Scabioseae) in a monophyletic unit which is in turn sister group to DIP (belonging to Dipsaceae). What is more, LOM and SIX, which are sister group to each other, were once belonging to genus *Scabiosa* L., and only recently have been segregated (GREUTER and RAUS, 1985) in the genera *Lomelosia* Raf. and *Sixalix* Raf.

The ITS1, therefore, appears to be a potentially appropriate tool to infer phylogenetic relationships at the intergeneric level in Dipsacaceae.

Abstract

A fine restriction mapping of the intragenic spacer I (ITS1) of the nuclear ribosomal DNA was carried out in four dipsacaceous and one valerianaceous taxa. The taxa in study were: *Centranthus ruber* (L.) DC. (Valerianaceae), *Dipsacus fullonum* L., *Lomelosia crenata* (Cyr.) Greuter et Burdet subsp. *crenata* (Cyr.) Greuter et Burdet, *Pterocephalidium diandrum* (Lag.) G. López, *Sixalix*

atropurpurea (L.) Greuter et Burdet subsp. *maritima* (L.) Greuter et Burdet (Dipsacaceae).

The ITS1 was approximately 230 bp long in all taxa but *P. diandrum*, in which a 15 insertion is present. Restriction sites were used for a cladistic analysis, in which *C. ruber* was used as an outgroup. The single fully resolved most parsimonious cladogram obtained is perfectly congruent with previous findings based on morphology. On these grounds, the ITS1 appears to be an ideal molecule to infer phylogeny in Dipsacaceae at the intergeneric level.

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