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A fine restriction map of the ITS1 in species of *Primula* L. (Primulaceae)

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Abstract. A fine restriction mapping of the intragenic spacer I (ITS1) of the nuclear ribosomal DNA was carried out in the following five species of genus *Primula* L. (Primulaceae): *P. auricula* L., *P. farinosa* L., *P. glaucescens* Moretti., *P. palinuri* Petagna and *P. vulgaris* Hudson. The ITS1 was approximately 250-255 bp long in all taxa but *P. vulgaris*, which is approx. 10 bp longer. Restriction sites were used for a cladistic analysis, in which *P. vulgaris* was used as an outgroup. The single fully resolved most parsimonious cladogram obtained is congruent with the classical taxonomy of the genus.

Riassunto. E' stata effettuata una mappatura di restrizione fine dello spaziatore intragenico I (ITS1) del DNA ribosomale nucleare nei seguenti taxa del genere *Primula* L. (Primulaceae): *P. auricula* L., *P. farinosa* L., *P. glaucescens* Moretti, *P. palinuri* Petagna e *P. vulgaris* Hudson. L'ITS1 è risultato lungo 250-255 paia di basi (bp) in tutti i taxa esaminati, tranne che in *P. vulgaris* in cui è risultato più lungo di circa 10 bp. I siti di restrizione individuati sono stati impiegati per un'analisi cladistica, con *P. vulgaris* come outgroup. Il singolo cladogramma ottenuto è completamente congruente con la tassonomia tradizionale del genere. L'ITS1 è da considerarsi pertanto una molecola appropriata per effettuare ipotesi filogenetiche a livello intragenerico in *Primula*.

Key words: *Primula*, ITS1, restriction mapping.

INTRODUCTION

Nuclear ribosomal DNA (n-rDNA), i. e., the DNA which codes for ribosomal RNA, is one of the regions of nuclear DNA most closely scrutinized by plant systematists. 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., 1993; KIM & JANSEN, 1994 and references therein; BALDWIN et al., 1995); each transcription unit is in turn separated by a highly variable intergenic spacer (IGS).

The object of this paper is testing the feasibility of a ITS1 fine restriction mapping approach for inferring phylogenetic relationships in genus *Primula*. Five Italian species belonging to three different subgenera were chosen.

MATERIALS AND METHODS

Specimens of *P. auricula* L. (Grigna Massif, Lombardia), *P. farinosa* L. (Passo dello Stelvio, Lombardia), *P. glaucescens* Moretti (Alben Massif, Lombardia), *P. palinuri* Petagna (Capo Palinuro, Campania), and *P. vulgaris* Hudson (Matese Massif, Campania) were collected in nature. Vouchers of all the examined plants are deposited at MI.

Leaves (0.2-0.5 g of silica gel dried leaves per sample) were ground on liquid nitrogen and total DNA was extracted following the procedure described in CAPUTO et al., 1991.

ITS1 was amplified 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'-ATCCTGCAATTCACACCAAG-TATCG-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; the extension was further prolonged for 7 min at the end of the last cycle. 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 upstream to ITS1) and in the 5' region of the 5.8S (upstream to our 3' primer and downstream to the end of ITS1).

Samples were then digested and double digested with *Aci* I, *Bst* E II, *Bsm* I, *Mae* I, *Mbo* II, *Mse* 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 carried out 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 approx. 380-390 bp in length for all the examined samples. After the *Eco* RV digestion, two fragments were obtained for all species, one of approx. 265-280 bp and the other of approx. 90-100 bp. *Bin* I digestions produced two fragments for *P. auricula* and *P. palinuri* (approx. 360 and 15-20 bp). *Bin* I digestion of *P. vulgaris* yielded four fragments, which added up to 390 bp, and digestions of *P. farinosa* and *P. glaucescens* with the same enzyme yielded three fragments which added up to 380 bp. Given the invariant position of the *Bin* I and *Eco* RV sites discussed in the previous section, ITS1 in the examined species is 250-260 bp long.

The inferred restriction map for all the enzymes and taxa is reported in Fig. 1, and the estimated length of the fragments is reported in Tab. 1.

A restriction site matrix was prepared (Tab. 2) by scoring the seven informative restriction sites for presence and absence. Autapomorphic sites were not scored. The cladistic analysis, for which *P. vulgaris* was used as an outgroup, yielded a single most parsimonious cladogram (length 8 steps, c.i. 0.87, r.i. 0.85) shown in Fig. 2. The ingroup is a ladderized sequence of *P. farinosa*, *P. glaucescens*, *P. auricula*, and *P. palinuri*..

Tab. 1 - Length of the restriction fragment detected in ITS1 of the species in study. For each enzyme, the cleavage sequence is reported. Abbreviations are as follows: PAUR = *P. auricula*; PFAR = *P. farinosa*; PGLA = *P. glaucescens*; PPAL = *P. palinuri*; PVUL = *P. vulgaris*.

	Aci I SCGS	*Bsm* I GAATGC	*Bst* E *II* GGTNACC	*Mae* I CTAG	*Mbo* II GAAGA	*Mse* I TTAA
PAUR	25 190 10 50	130 140	100 170	90 180	190 80	180 90
PFAR	130 90 55	---	---	---	---	---
PGLA	25 150 50 50	130 140	100 170	90 180	---	180 90
PPAL	25 190 10 50	130 140	100 170	90 180	190 80	180 90
PVUL	175 50 60	---	---	80 200	---	

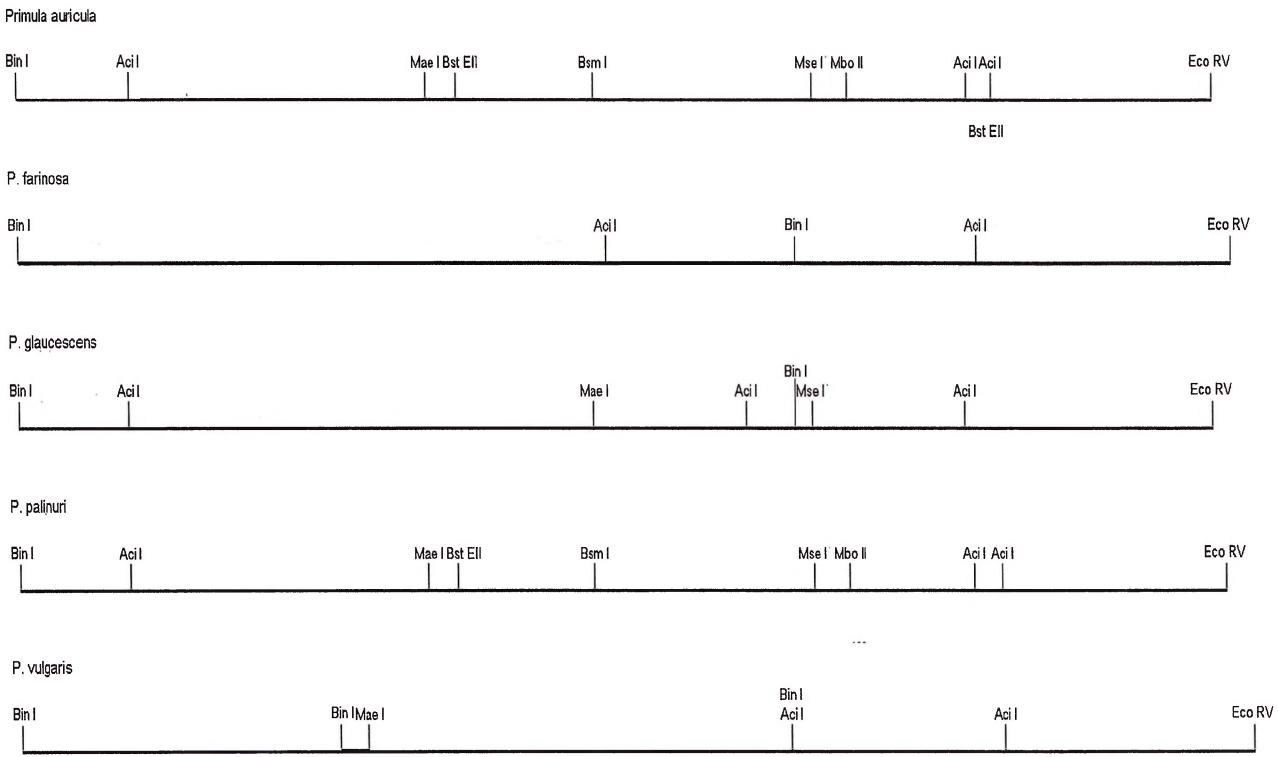

Fig. 1 - Restriction map of the *Bin* I-*Eco* RV fragments containing the complete ITS1 for the species in study.

Tab. 2 - Restriction site matrix for the species in study. 0 indicates absence of a site; 1 indicates its presence.

PAUR	1111111
PFAR	0100000
PGLA	1001111
PPAL	1111111
PVUL	0000000

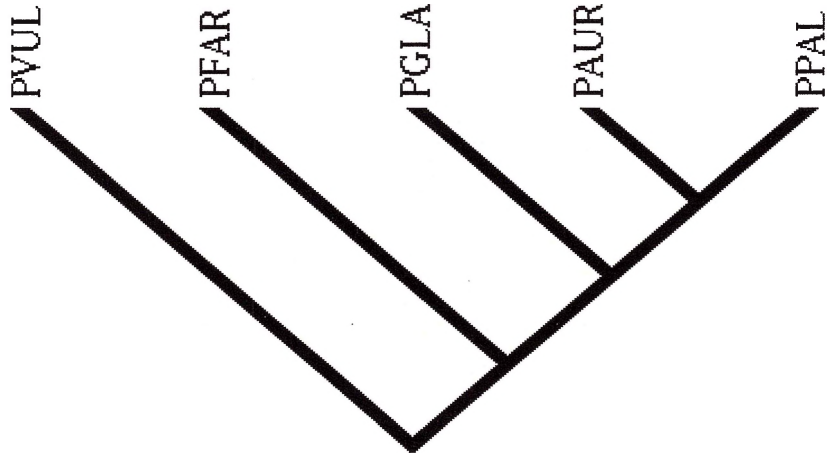

Fig. 2 - Cladogram obtained by the cladistic analysis of the restriction sites using the matrix in Tab. 2. Acronyms are indicated in the caption to Tab. 1.

DISCUSSION

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 1.8 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.

The size indicated for the ITS1 in our species is not necessarily entirely accurate (in fact, the fragments we mapped also included 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 2 bp upstream the 3' terminus of the 18S, and that the *Eco* RV site is present 20-22 bp downstream the ITS1 3' terminus. On these grounds, the ITS1 of the taxa in study is 250-255 bp long, with the exception of *P. vulgaris*, which appears to be approx. 10 bp longer.

The cladogram obtained completely corresponds to the classical infrageneric grouping in *Primula*. This genus, in fact, is customarily divided in three subgenera (VALENTINE & KRESS, 1972): *Primula* (of which *P. vulgaris* was chosen as a representative), *Aleuritia* (Duby) Wendelbo (with *P. farinosa* present in this study) and *Auriculastrum* Schott (with *P. auricula, P. glaucescens and P. palinuri* in our sample). The latter is not the only infrageneric treatment of *Primula* which finds support in our data. In fact, also the subgeneric divisions, proposed by SMITH et al., (1977) and reported by KÖHLEIN (1984), which partially go back to previous contributions (SMITH & FLETCHER, 1949; SMITH et al., 1948; SMITH & FLETCHER, 1948; SMITH & FORREST, 1928), are completely congruent with our results.

The infrageneric grouping in all cases is based mainly on differences in epicuticular wax deposition, leaf texture and leaf venation. These characters, within the limits of the present

study, seem to correctly depict the monophyletic pattern of descent in the investigated species.

However, the closer propinquity of *P. auricula* to *P. palinuri* than to *P. glaucescens*, albeit completely matching the majority of previous evidence (KÖHLEIN, 1984 and references therein), does not find entire correspondence in previous morphological and karyological investigations. CHIARUGI (1956), in fact, suggests the segregation of *P. auricula* in a separate section (sect. *Palaeoauricula* Chiarugi), on the basis of the chromosome number (2n=44), which is different from that of the other species of subgenus *Auriculastrum* (which mainly show 2n=62), placed in sect. *Neoauricula* Chiarugi. The relevance of this discrepancy may be verified only through further work on the species of subgenus *Auriculastrum*.

This study, although preliminary, showed that ITS1 in *Primula* species has a range of variation well suited to infer relationships across a representation of the wide morphological diversity in the genus.

On these grounds, the ITS1 appears to be an appropriate tool to infer interspecific relationships in *Primula*.

REFERENCES

BALDWIN B. G., SANDERSON M. J., PORTER M. J., WOJCIECHOWSKI M. F., CAMPBELL C. S. & DONOGHUE M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence in angiosperm phylogeny. Ann. Missouri Bot. Gard., 82: 247-277.

CAPUTO P., STEVENSON D. W. & WURTZEL E. T. 1991. A phylogenetic analysis of American cycads (Cycadales) using chloroplast DNA restriction fragment length polymorphisms. Brittonia, 43: 135-145.

CHIARUGI A. 1956. *Primula palinuri* Petagna - Posizione sistematica e significato fitogeografico attraverso l'indagine citogenetica. Webbia, 11: 861-888.

FARRIS J. S. 1988. Hennig 86. Reference manual. Published by the author, Port Jefferson Station, NY, USA.

KIM Y. D. & JANSEN R. K. 1994. Comparison of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*, Asteraceae): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. Pl. Syst. Evol., 190: 157-185.

KÖHLEIN F., 1984. Primeln. E. Ulmer, Stuttgart.

MISHLER B. D., LEWIS L. A., BUCHHEIM M. A., RENZAGLIA K. S., GARBARY D. J., DELWICHE C. F., ZECHMAN F. W., KANTZ T. S. & CHAPMAN R. L. 1994. Phylogenetic relationships of the "green algae" and "bryophytes ". Ann. Missouri Bot. Gard., 81: 451-483.

SMITH W. W. & FORREST G. 1928. The sections of the genus *Primula*. Notes Royal Bot. Gard. Edinburgh, 16: 1-50.

SMITH W. W. & FLETCHER H. R. 1948. The genus *Primula*: section *Vernales* Pax. Trans. Proc. Bot. Soc. Edinburgh, 34: 402-468.

SMITH W. W., FORREST G. & FLETCHER H. R. 1948. The genus *Primula*: sections *Cuneifolia*, *Floribundae*, *Parryi*, and *Auricula*. Reprinted in: SMITH W. W., FORREST G. & FLETCHER H. R., 1977 pp. 757-810.

SMITH W. W. & FLETCHER H. R. 1949. The genus *Primula*: sections *Cuneifolia*, *Floribundae*, *Parryi*, and *Auricula*. Trans. Royal. Soc. Edinburgh, 61: 631-686.

SMITH W. W., FORREST G. & FLETCHER H. R. 1977. The genus *Primula*. Plant Monograph Reprints Vol. 11. (Kramer Publish., Vaduz).

SUH Y., THIEN H. E. & ZIMMER E. A. 1993. Molecular evolution and phylogenetic implications of internal transcribed sequences of ribosomal DNA in Winteraceae. Amer. J. Bot., 80: 1042-1055.

VALENTINE D. H. & KRESS A. 1972. *Primula* L. In: Tutin T. G., Heywood V. H., Burgos N. A., Moore D. M., Valentine D. H., Walters S. M., Webb D. A. (Ed.). Flora Europaea 2: 15-20. Cambridge University Press.

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