Relationships among the Italian species of genus *Antirrhinum* L. (Scrophulariaceae) based on chloroplast DNA restriction fragment length polymorphisms

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Riassunto

E' stato intrapreso lo studio dei polimorfismi di restrizione del DNA plastidiale delle quattro specie italiane del genere *Antirrhinum* L. (Scrophulariaceae). La necessità di tale studio deriva dalle discordanze presenti nel trattamento sistematico proposto da vari autori per tali entità, dagli scarsi e variabili caratteri morfologici che le discriminano e dalla frequente formazione di ibridi. Sono stati individuati 90 frammenti di restrizione diversi. I dati sono stati analizzati impiegando sia il metodo di FITCH-MARGOLIASH sia la cluster analysis (UPGMA), dopo trasformazione in una matrice di distanze di NEI e LI. I fenogrammi ottenuti indicano che *A. siculum* è la specie più isolata; *A. majus* e *A. tortuosum sono* le due specie con distanza minore; *A. latifolium* si colloca ad una distanza intermedia.

INTRODUCTION

The genus *Antirrhinum* L. (Scrophulariaceae) is composed of about 30 species, distributed in the Western Mediterranean area (sect. *Antirrhinum*) and in Northern California (sect. *Saeorrhinum* Rothm.) (ROTHMALER, 1956; HONG, 1983). The genus is present in Italy with four species (PIGNATTI, 1982): *A. latifolium* Miller, *A. majus* L., *A. siculum* Miller and *A. tortuosum* Bosc. The status of these species is still unclear, as the major floristic works and monographies treat them in a very different fashion. FIORI (1923-29) recognizes only two species in *Antirrhinum* sect. *Antirrhinastrum* Chav.: *A. latifolium* and *A. majus*; the latter species contains three subspecies corresponding to *A. majus sensu stricto*, to *A. siculum* and to *A. tortuosum*. ROTHMALER (1956) indicates only *A. siculum* and *A. majus* for Italy, regarding the other taxa as subspecies of the

latter. ZANGHERI (1976) recognizes three species, considering *A. tortuosum* as a subspecies of *A. majus*. WEBB (1971, 1976) recognizes only the same three species as ZANGHERI.

The characters used to discriminate among the taxa, however, are few and of uncertain value (leaf length/width ratio, pubescence of the inflorescence, length and color of the corolla). The taxonomic treatment is further complicated by a common tendency in various species within the genus to form hybrids (WEBB, 1971); among our species, especially *A. majus* is prone to hybridization (WEBB, 1971, 1976).

This paper has the aim of verifying the distance among the Italian taxa of *Antirrhinum* and their possible relationships by using the Restriction Fragment Length Polymorphisms (RFLPs) of the chloroplast DNA (cpDNA).

MATERIAL AND METHODS

All plants were collected in the field and some of them transferred in cultivation at the Botanical garden of Naples. As some taxa may easily form hybrids, the specimens have been identified by each author separately and care has been taken to choose only plants the identification of which was unequivocal. Voucher specimens for all taxa examined are deposited at NAP.

Total DNA was extracted by following the method of CAPUTO *et al.* (1991). DNAs were then digested with *Bam* HI, *Bcl* I, *Bgl* II, *Cla* I, *Eco* RI, *Eco* RV, *Hind* III, *Xba* I restriction endonucleases, electrophoresed (0.5 μg/lane, 0.8% agarose gel, 16 h, 25 V), denatured, neutralized, and transferred overnight to nylon filters, as reported in SAMBROOK *et al.* (1989). Filters were prehybridized (24 h) and hybridized at 37 °C (50% formamide).

Hybridization probes were isolated from *Bam*HI clones of a *Nicotiana tabacum* cpDNA library (SUGIURA *et al.*, 1986). The hybridization probes were labelled with α-^{32}P-dATP, using the random primed DNA labelling method (FEINBERG and VOGELSTEIN, 1984). Unbound probe was removed by washing filters for 40 min at room temperature in 2 x SSC (20 x SSC = 3 M NaCl, 3.3 M sodium citrate, pH 6.8) plus 0.1% SDS, and then for 30 min at 55 °C in 0.1 x SSC, 0.1% SDS. Filters were exposed at -70 °C to X-ray sensitive film.

Hybridizing restriction fragments were scored on a presence/absence basis for each terminal taxon, paying attention not to score any fragment twice (CAPUTO *et al.*, 1991; MORETTI *et al.*, 1993); the resulting binary data matrix (Tab. I)

was analyzed using distance methods. The binary matrix was transformed in a square matrix by calculating the coefficient of NEI and LI (1979). Each value in the matrix was then transformed in its complement to 1 (NEI and LI's distance is in fact really a similarity index). On this matrix (Tab. II) both the FITCH-MARGOLIASH method (FITCH and MARGOLIASH, 1967) and an algorithm of cluster analysis (UPGMA) were then used, as respectively implemented in the FITCH and NEIGHBOR programs of the PHYLIP 3.5 software (FELSENSTEIN, 1993). For the FITCH software, the G and J (random seed 12345, 1000 replicates) options were invoked.

RESULTS

Ninety different restriction fragments were observed, of which 32 unique to single terminals. In particular, 10 fragments were unique to *A. latifolium*, 5 to *A. majus*, 16 to *A. siculum* and one to *A. tortuosum*.

The phenogram resulted from the FITCH-MARGOLIASH analysis (Fig. 1), which required the examination and rejection of over 7000 worse-fitting trees, has a sum of squares of 0.0014 and an average percent standard deviation of 1.1960 (Tab. III). In the phenogram, *A. siculum* is the most isolated terminal and *A. majus* and *A. tortuosum* are the closest to each other (Tab. III). *A. latifolium*, which is also fairly isolated, is closer to the pair *A. majus*-*A. tortuosum* than to *A. siculum*, with which the maximum distance in the phenogram is recorded (Tab. III).

Also the phenogram obtained from the UPGMA analysis (Fig. 2) indicates that *A. siculum* is the most isolated terminal (Tab. IV); this species is equidistant from the other three. *A. latifolium*, very far from the first terminal, is more close to *A. majus* and *A. tortuosum*, which are in turn the most closely related terminals (Tab. IV).

DISCUSSION AND CONCLUSIONS

Among the crucial issues in any molecular study are the choice of the molecule, the technique used to obtain characters and the method used to analyze them. Our choice of cpDNA depends upon the fact that cpDNA restriction fragments and/or mapping have the appropriate conservativity (and have been

Tab. I - Binary matrix (90 characters) for the taxa in study.

A.latifolium	001010000111101111111110001010111001100011001000111101111000000000000000000000000011111111110
A.majus	111101011011110111111110111010111111100111001101110111111111111110000000000000000000000000000000
A.siculum	11010111110101000000000101010110000011010101010101100011000000000011111111111111100000000000
A.tortuosum	111110111100001100000001110101111111010101010101110110011111000000000000000000000000000000001

Tab. II - Complemented NEI and LI's (1979) matrix for the taxa in study.

A.latifolium	0.000	0.532	0.814	0.556
A.majus	0.532	0.000	0.587	0.356
A.siculum	0.814	0.587	0.000	0.671
A.tortuosum	0.556	0.356	0.671	0.000

widely used) for infrageneric comparisons among angiosperms (COATES and CULLIS, 1987; MORETTI *et al.*, 1993; PALMER *et al.*, 1985; PALMER and ZAMIR, 1982; PERL-TREVES and GALUN, 1985).
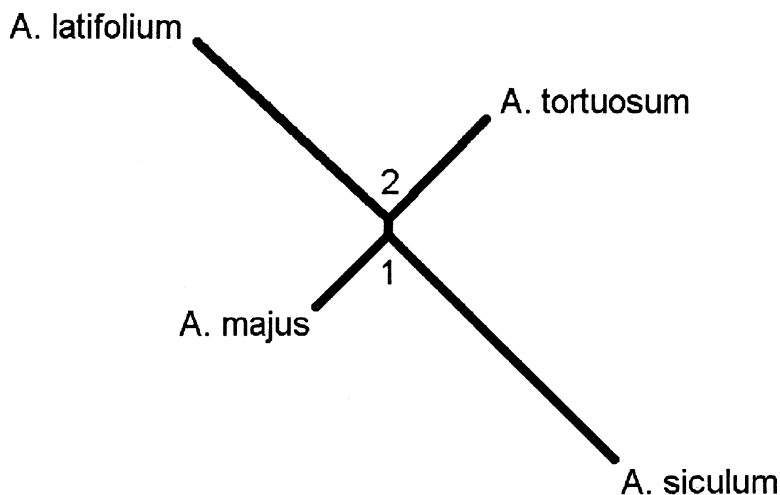

Fig. 1 - Phenogram calculated by FITCH-MARGOLIASH method in the four taxa in study. Numbers 1 and 2 refer to the nodes, the distance between which is indicated in Tab. III.

Tab. III - Length of the branches of the phenogram in Fig.1, calculated by FITCH-MARGOLIASH method in the four taxa in study. Numbers 1 and 2 refer to the corresponding nodes in the phenogram.

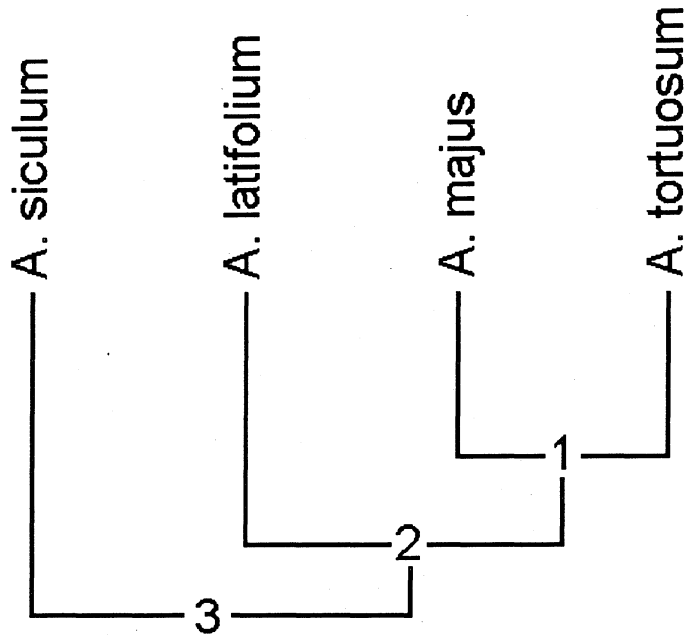| Distance	Length
1-*A.majus*	0.14225
1-2	0.02205
2-*A.latifolium*	0.36156
2-*A.tortuosum*	0.19444
1-*A.siculum*	0.44475

Fig. 2 - Phenogram calculated by UPGMA method in the four taxa in study. Numbers 1, 2 and 3 refer to the nodes, the distance between which is indicated in Tab. IV.

Tab. IV - Length of the branches of the phenogram in Fig. 2, calculated by UPGMA method in the four taxa in study. Numbers 1, 2 and 3 refer to the corresponding nodes in the phenogram.

Distance	Length
3-2	0.07333
2-*A.latifolium*	0.27200
2-1	0.09400
1-*A.majus*	0.17800
1-*A.tortuosum*	0.17800
3-*A.siculum*	0.34533

Our choice of RFLPs, instead of restriction mapping, depends on the fact that, in the narrow evolutionary span involved among *Antirrhinum* species, RFLPs do not show a significant increase in the information/noise ratio, which is one of the most relevant disadvantages of RFLPs indicated by PALMER (1987). Furthermore, precautions have been taken not to score the same fragment twice and to exclude deletions/insertions from our character matrix, by using a sequential hybridization approach described in MORETTI *et al.* (1993). Finally, the danger of including nonhomologous fragments in a RFLP analysis is grossly overestimated, as shown by BREMER (1991).

Another relevant issue in any study of relationships is the technique employed to analyze characters. We regarded cladistics, possibly the most widely used method at present, as not suitable in our system, as gene flow among our terminals cannot be ruled out, and this would violate one of the basic principles of cladistics. Thus, we preferred to use methods which are less burdened with strict prerequisites to application, i. e., phenetic methods. FITCH-MARGOLIASH method produces an unrooted tree with branch lengths unconstrained (FITCH and MARGOLIASH, 1967; FELSENSTEIN, 1993) and, from this point of view, it is a very generalistic method (i.e., with very few assumptions). However, assumptions of distance additivity, independent errors, as well as the fact that relative (percentage) error is more constant than absolute error, are a requirement for the usage of such method (FELSENSTEIN, 1993). Therefore, we decided to use also cluster analysis, which is perhaps the broadest method available to infer propinquity, albeit possibly the least accurate. The only difficulty with such methods is that the expected amount of evolution in any lineage (i.e., the length of the branches of the rooted tree obtained) should be proportional to elapsed time (FELSENSTEIN, 1993), and this has not yet been demonstrated with cpDNA (but see ALBERT *et al.*, 1994).

The four taxa examined here all belong to the European sect. *Antirrhinum*, in the range of which Italy is the Easternmost boundary. The section has its present center of variation in the Iberian peninsula, where all 17 species reported for the section are present (WEBB, 1976). Among the four taxa in study here, *A. majus* is very likely a naturalized escape from cultivation, at least in continental Italy (PIGNATTI, 1982; WEBB, 1976). For Sicily there is some doubt, as shown by the contradictions between the two just mentioned authors. A similar situation

characterizes *A. tortuosum*, which is regarded as originally present only in Southern Spain and Sicily, and naturalized elsewhere (WEBB, 1976; PIGNATTI, 1982). *A. latifolium*, on the contrary, is a narrow North-West Mediterranean endemic, distributed from Central Italy to North-Eastern Spain. Also *A. siculum* has a rather narrow range, being distributed mainly in Sicily and Malta, and probably naturalized in continental Italy and Spain (Webb, 1976). This would be the only species of sect. *Antirrhinum* whose present range is not centered in the Iberian peninsula.

The latter two species appear very removed from each other in our FITCH-MARGOLIASH and UPGMA phenograms (Figs. 1 and 2), as they are divided by the highest distance possible (Tabs. III and IV). As far as their relationships with *A. majus* and *A. tortuosum* are concerned, *A. latifolium* is the closest, regardless of the method used (Figs. 1 and 2; Tabs. III and IV). However, when observing the FITCH-MARGOLIASH calculated distances (Tab. III), it is possible to note that *A. siculum* and *A. latifolium* are closer to *A. majus* than to *A. tortuosum* (see also Fig. 2).

On what said above, and keeping in mind that the length of the branches in the UPGMA analysis are proportional to the amounts of evolution (FELSENSTEIN, 1993), that is, to the time elapsed from the origin of the taxa, we may hypothesize the following: *A. siculum* separated as first from the original stock of the ancestors of the modern *Antirrhinum* species examined here, remaining isolated on large islands (Sicily and Malta); this is also asseverated by the high number of unique characters (Tab. I). A later yet still remote event caused the origin of *A. latifolium* (which also shows a high number of unique characters). The isolation of *A. tortuosum* from *A. majus* is comparatively recent (the former has only a unique restriction fragment). During all these events, *A. majus* accumulated little diversity as compared to the ancestral stock (it is the closest one to the center of the phenogram in Fig. 1, and has only 5 unique characters). As a consequence, we may infer that *A. latifolium*, *A. siculum* and *A. tortuosum* are geographical isolates from a continuous range which had to characterize ancestral *Antirrhinum* species in the past in Europe. This hypothesis has been already drawn by other authors on phytogeographical evidence (WEBB, 1971). Finally, no one of the species in consideration derives from a recent hybridization event, given the fact that all cpDNA contain unique characters.

Further study is doubtlessly needed to asseverate this, which is at the moment only a working hypothesis. In particular, extraliminal species should be added to our analysis and a quantitative evaluation of the amounts of hybridization and introgression among taxa should be obtained in order to understand the phylogeny of the European section of the genus, to identify the boundaries between species and to better define the taxonomic circumscription of several entities.

Abstract
A chloroplast DNA restriction fragment length polimorphism study was undertaken on the four Italian species of the genus *Antirrhinum* L. (Scrophulariaceae). The need for this study depends upon the discrepancies in the taxonomic treatment of these species by various authors, upon the few and variable diagnostic characters and upon the frequent production of natural hybrids. Ninety different restriction fragments were observed. Data were analysed by both FITCH-MARGOLIASH and cluster analysis (UPGMA) methods, after transformation into a NEI and LI's distance matrix. The resulting phenograms indicate that *A. siculum* and *A. latifolium* are rather isolated; *A. majus* and *A. tortuosum* are the two most closely related species.

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