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Phylogenetic relationships among annual and perennial species of the genus *Cicer* as inferred from ITS sequences of nuclear ribosomal DNA

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Abstract

The cladistic analysis of the DNA sequences of the internal transcribed spacers of ribosomal cistrons (ITS1 and ITS2) for 20 species of *Cicer* L. (among which all the annuals), shows that various sections of the genus are not monophyletic. Annual species do not form a clade: *C. arietinum*, in fact, is closely related to both *C. echinospermum* and *C. reticulatum*, whereas *C. bijugum*, *C. judaicum*, and *C. pinnatifidum* form a separate clade. The annual *C. cuneatum* is sister group to the perennial *C. canariense* and both are archaic species within the genus. *C. yamashitae* is, on the contrary, the only annual species belonging to a group of perennials, within which close relationships are evident between *C. graecum* and *C. montbretii* as well as among a group of mainly Asian species.

Additional key words: DNA sequences, ribosomal cistrons.

Introduction

Genus *Cicer* L. (*Fabaceae*) consists of 42 wild species, including 9 annuals and 33 perennials, categorized into four sections based on life-cycle, morphological and geographical criteria: section *Monocicer* contains eight annual species (*C. reticulatum*, *C. echinospermum*, *C. pinnatifidum*, *C. bijugum*, *C. judaicum*, *C. yamashitae*, *C. cuneatum* and *C. arietinum*); sect. *Chamaecicer* includes two species only, of which one annual (*C. chorassanicum*); sects. *Polycicer* and *Acanthocicer* comprise 25 and 7 perennial species, respectively (Van der Maesen 1987).

The only cultivated taxon, *Cicer arietinum* L. (chickpea), is an economically important crop but is susceptible to a number of diseases and its world average yield is much below its potential (Singh *et al.* 1994). On the contrary, wild species of *Cicer* possess both disease resistance genes and in some cases agronomically interesting characters for plant breeding (Van der Maesen and Pundir 1984, Singh and Ocampo 1997).

The annual species of the genus *Cicer* form four crossability groups (Ladizinsky *et al.* 1988 and references therein). The first crossability group contains the cultigen, *C. arietinum*, its presumable ancestor *C. reticulatum* and *C. echinospermum*. The second group comprises *C. bijugum*, *C. pinnatifidum*, *C. judaicum* and *C. yamashitae*. The remaining two annual species, *C. chorassanicum* and *C. cuneatum* form the third and fourth group, respectively.

In the recent years, the relationships among annual species have been investigated by plant morphology (Robertson *et al.* 1997), karyotype analysis (Ocampo *et al.* 1992, Tayyar *et al.* 1994, Galasso *et al.* 1996), seed storage protein fractionation (Ahmad and Slinkard 1992), isozyme analysis (Kazan and Muehlbauer 1991, Ahmad *et al.* 1992, Tayyar and Waines 1996) and by using different molecular markers (Ahmad 1999, Staginnus *et al.* 1999, Choumane *et al.* 2000, Iruela *et al.* 2002, Rajesh *et al.* 2002, Sudupak 2004, Sudupak *et al.* 2002, 2004).

Perennial species have been studied and compared with annual species for the first time using Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers (Iruela *et al.* 2002) and later by ISSR polymorphism (Rajesh *et al.* 2002) and by

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Amplification Fragment Length Polymorphism (AFLP) fingerprinting (Sudupak *et al.* 2004), whereas only *C. songaricum* and/or *C. anatolicum* were compared with annual species using isozymes (Kazan and Muehlbauer 1991, Tayyar and Waines 1996) and Sequence-Tagged Microsatellite Sites (STMS) markers (Choumane *et al.* 2000).

To shed better light on the relationships among *Cicer* species, we have determined the nucleotide sequences of the internal transcribed spacers (ITS1 and ITS2) of the ribosomal cistrons in all the annual species of *Cicer* and in 11 perennial species, in this way sampling from all the sections of the genus.

Materials and methods

Plants: The names, accession numbers, section, source, and type of cycle of the analysed species are listed in Table 1.

Amplification of ITS region and DNA sequencing: Nuclear DNAs were extracted and purified as described in Maggini *et al.* (1978) from root tips or from leaves of herbarium specimens. Either the entire region including ITS1, 5.8S and ITS2 rDNA was amplified by the standard Polymerase Chain Reaction (PCR) or ITS1 and ITS2 were amplified separately. Amplifications were carried out using the following parameters: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min and finally 72 °C for 5 min.

The primers, designed by using the conserved coding regions of the 18S and 26S ribosomal genes, were "18Sdir" 5'-CGTAACAAGGTTTCCGTAGG-3' and "26Scom" 5'-AGCGGGTAGTCCCGCCTGA-3' in the case of single amplifications (Venora *et al.* 2000), or those reported in Aceto *et al.* (1999) for the separate ITS1 and ITS2 amplifications.

The PCR products were either sequenced directly or ligated in pUC18 vectors by using the *Sure Clone Ligation Kit* (*Amersham Pharmacia Biotech*, UK) and then sequenced for both strands. Sequences were obtained by using the automated sequencer *ABI Prism 310* (*Applied Biosystems*, USA)

Outgroups were selected by using the ITS1, 5.8S and ITS2 of *C. arietinum* for a FASTA query (Pearson and Lipman 1988) in the EMBL database. Among the sequences most similar to that of *C. arietinum* but not belonging to genus C*icer*, four were selected, belonging to three genera: *Medicago lupulina* (Z99216), *Trifolium ciliatum* (AF053152), *T. lupinaster* (AF053163), and *Vicia montbretii* (AF228075). *Cicer* sequences, together with those of the outgroups, after removal of the 5.8S, were aligned by using Clustal W version 1.8 (Thompson *et al.* 1994) with default parameters (except for the transition/transversion ratio, which was set to 1). Jukes-Cantor (Jukes and Cantor 1969) distances were

Table 1. Origins, sections, types of cycle and EMBL accession numbers of ITS1 and ITS2 sequences for the investigated *Cicer* species. ICARDA - International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria; USDA - United States Department of Agriculture, Pullman, USA; RNG - Herbarium, the University of Reading, Reading, UK

Species	Origin	Section	Cycle	EMBL accession number
C. arietinum L.	ICARDA FLIP82-150C	*Monocicer*	A	AJ237698
C. bijugum Rech.	ICARDA ILWC 65	*Monocicer*	A	AJ237701
C. chorassanicum (Bge) Pop.	ICARDA ILWC 90	*Chamaecicer*	A	AJ250886
C. cuneatum Rich.	ICARDA ILWC 37	*Monocicer*	A	AJ238480
C. echinospermum Dav.	ICARDA ILWC 35	*Monocicer*	A	AJ237699
C. judaicum Boiss.	ICARDA ILWC 20	*Monocicer*	A	AJ238479
C. pinnatifidum J.& S.	ICARDA ILWC 29	*Monocicer*	A	AJ238478
C. reticulatum Lad.	ICARDA ILWC36	*Monocicer*	A	AJ237700
C. yamashitae Kit.	ICARDA ILWC 3	*Monocicer*	A	AJ238477
C. anatolicum Alef.	RNG Nesbitt 736	*Polycicer*	P	AJ639946, AJ639947
C. canariense San. Guer.& Lew	USDA PI 557453	*Polycicer*	P	AJ639941
C. flexuosum Lips.	RNG Maxted *et al.* 8062	*Polycicer*	P	AJ639948, AJ639949
C. graecum Orph.	RNG Herb. Goulandrium 12317	*Polycicer*	P	AJ639950, AJ639951
C. macracanthum M. Pop	USDA PI 599080	*Acanthocicer*	P	AJ639942
C. microphyllum Benth.	USDA PI 599083	*Polycicer*	P	AJ639943
C. montbretii Jaub. Sp.	RNG Maxted *et al*. 4699	*Polycicer*	P	AJ639952, AJ639953
C. multijugum van der Maes.	USDA PI 599085	*Polycicer*	P	AJ639944
C. oxyodon Boiss.& Hoh.	USDA PI 561084	*Polycicer*	P	AJ639945
C. pungens Boiss.	RNG Maxted *et al*. 8015	*Acanthocicer*	P	AJ639954, AJ639955
C. songaricum Steph. ex *DC.*	RNG Maxted *et al.* 8201	*Polycicer*	P	AJ639956, AJ639957

calculated by using the *DNADIST* software, and the corresponding phenogram was obtained by using neighbor-joining method as implemented in the *NEIGHBOR* software, both programs of the *PHYLIP* package (Felsenstein 1993). The alignment was also subject to a parsimony analysis employing the cladistic software *Winclada*, running *Nona* as a daughter process, with the following parameters: hold 10000; hold/100; mult*50; max. Gaps were coded as missing data and all characters were treated as unordered. *Winclada* was also used to calculate bootstrap percentages, out of 1000 replicates (Felsenstein 1985).

Results

The lengths of ITS 1 for the taxa sequenced in this study ranged from 235 bp (*C. bijugum*, *C. canariense*, *C. cuneatum*, *C. judaicum*, *C. pinnatifidum*) to 239 bp (*C. arietinum*, *C. echinospermum*, *C. reticulatum*), with all the remaining species (Table 1) 238 bp long; the length of ITS2 was invariably 213 bp. Pairwise similarity within genus *Cicer* ranges from complete identity (between *C. echinospermum* and *C. reticulatum*, among *C. flexuosum*, *C. multijugum* and *C. songaricum*) to 93.65 % (between *C. cuneatum* and *C. graecum*). The phenogram was obtained from the distance matrix (Fig. 1). The matrix obtained from the alignment which was used as input for the cladistic analysis contained 24 taxa and 459 characters (ITS1 consensus length 240 characters, informative characters 19.6 %; ITS2 consensus length 219 characters, informative characters 15.9 %; overall consensus length 459 characters, informative characters 17.8 %, informative characters within the ingroup 7.0 %). The cladistic analysis yielded three equally parsimonious cladograms (length 195, C.I. 0.67, R.I. 0.89; after removal of uniformative characters length 123, C.I. 0.82, R.I. 0.89), the strict consensus of which is shown in Fig. 2. The ingroup is divided in two clades, one of which includes *C. canariense* and *C. cuneatum* and the other the rest of the taxa. This latter clade is poorly resolved, and includes an unresolved trifurcation with *C. arietinum*, *C. echinospermum*, *C. reticulatum*, a ladderized sequence of *C. judaicum*, *C. bijugum* and *C. pinnatifidum* and a clade with all the other species. This latter group shows a basal collapse of *C. anatolicum*, *C. chorassanicum*, *C. microphyllum*, *C. oxyodon* and *C. yamashitae*; a clade including *C. graecum* and *C. montbretii*; and a clade including *C. flexuosum*, *C. macracanthum*, *C. multijugum*, *C. pungens*, and *C. songaricum*. Bootstrap values (Fig. 2 shows bootstrap percentages > 50 %) indicate strong support for various clades; notably, all the major clades of the ingroup show a bootstrap support > 85 %. The terminal clades, however, show a weaker support (*e.g.*, the clade including *C. bijugum*, with 63 %, the clade of *C. flexuosum*, with 62 %).

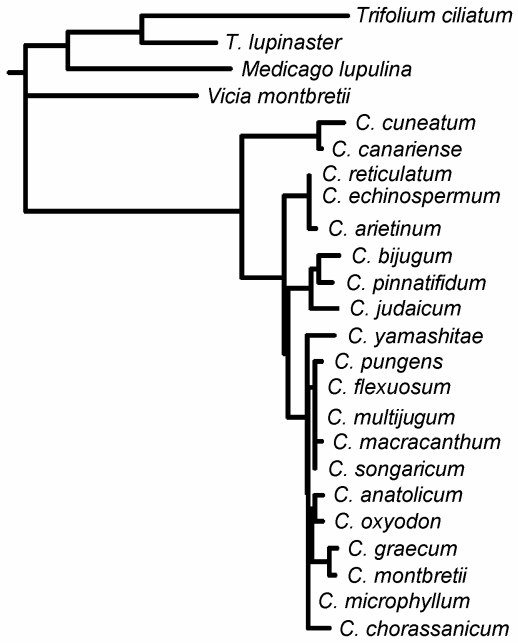

Fig. 1. Phenogram showing the overall affinity of the species in study.

Discussion

Our phylogenetic hypothesis (Fig. 2) does not concur in entirety with previous literature data. However, various our groups of closely related species match previous evidence. In particular, the results obtained here, as well as all the data present in the literature, suggest that *C. arietinum*, *C. echinospermum* and *C. reticulatum* form a closely knit unit. Our data, however, do not allow further comment on the origin of *C. arietinum*, given the fact that its sequence differences with *C. reticulatum* and *C. echinospermum* are limited to a single autapomorphic mutation in ITS2.

Given the economic importance of *C. arietinum*, the group to which it belongs has been studied in depth, and previous literature includes abundant references which investigate relationships between the three above-mentioned *Cicer* species and the rest of the genus. Kazan and Muehlbauer (1991), for example, reported a close relationships among *C. anatolicum* and *C. reticulatum*,

C. echinospermum and *C. arietinum*, which has been confirmed by Staginnus *et al.* (1999) on the basis of the structure and the localization of repetitive DNA, and by Choumane *el al.* (2000) by using STMS markers.

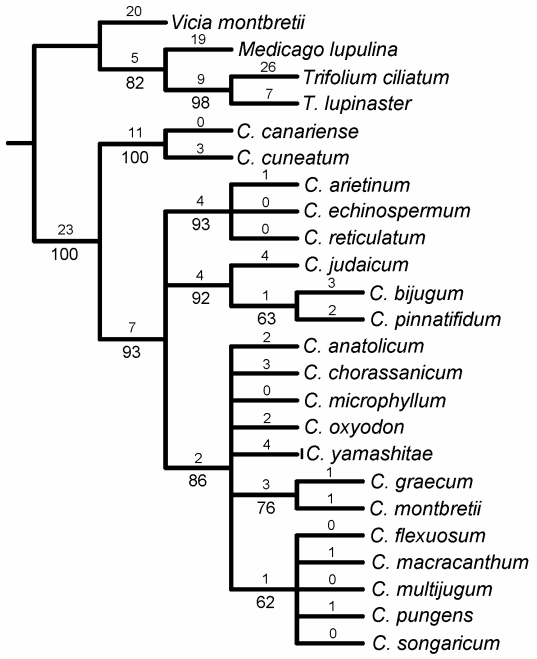

Fig. 2. Strict consensus among the three most parsimonious cladograms for the species in study (length 195, C.I. 0.67, R.I. 0.89; after removal of uniformative characters length 123, C.I. 0.82, R.I. 0.89). Numbers above branches indicate the number of apomorphic characters at the clade/terminal above; numbers below branches indicate the percentage of bootstrap replicates (out of 1000) in which the clade above occurs. Percentages below 50 % are not shown.

On the other hand, Rajesh *et al.* (2002) analysing seven perennials (five of which also present in our investigation) and six annuals, observed that the perennials *C. nuristanicum* and *C. pungens* clustered with *C. reticulatum*, *C. echinospermum* and *C. arietinum*. Neither of the above mentions suggested patterns of relationships is congruent with our data. Indeed, forcing the topology of Fig. 2 with *C. anatolicum* as sister group to the clade including *C. arietinum*, *C. echinospermum*, and *C. reticulatum* causes a two-step increase in the length of the most parsimonious (MP) cladograms, whereas forcing a clade in which *C. pungens* is sister group to the just mentioned clade causes a three-step increase.

Another clade composed only of annual species which emerges from our analysis is that including *C. judaicum*, *C. bijugum*, and *C. pinnatifidum*. This clade is congruent with previous data based on isozyme polymorphisms (Kazan and Muehlbauer 1991, Labdi *et al.* 1996, Tayyar and Waines 1996, Ahmad *et al.* 1992), and RAPDs (Ahmad 1999). However, this clade does not concur with the crossability groups (Ladizinsky *et al.* 1988) or with the ISSR-based results by Rajesh *et al.* (2002), as it does not include *C. yamashitae*. Forcing a clade in which *C. yamashitae* is sister group to the clade including *C. judaicum*, *C. bijugum*, and *C. pinnatifidum* causes a two-step increase as compared to the MP topologies. It may be mentioned in this regard that *C. bijugum*, according to part of previous literature (*e.g.* Van der Maesen 1987), was regarded as a close relative of the cultivated chickpea. Including *C. bijugum* in the clade to which *C. arietinum* belongs, would cause an increment of six steps in the MP trees.

The position of *C. chorassanicum* deserves special mention, as our data place this species and *C. yamashitae* in the same clade, in agreement with the results of Ahmad *et al.* (1992), Ahmad (1999), Kazan and Muelhbauer (1991) and Staginnus *et al.* (1999). In our study *C. chorassanicum* and *C. yamashitae* grouped with the perennials, in partial agreement with the results of Tayyar and Waines (1996) who, however, investigated only two perennials, *C. anatolicum* and *C. songaricum*. Forcing any of the two annual species outside the group of perennials would cost an increase of at least two steps in the MP topologies.

The placement of the annual *C. cuneatum* together with the perennial *C. canariense* in a very long branch (Fig. 2), showing the largest distance from the all the other investigated taxa (Fig. 1) is supported by the morphology of the species; indeed, they are the only *Cicer* species with a climbing growth habit, elongate pods, globular seed and non-conspicuous beak (Van der Maesen 1987). Previous literature data, based on enzyme data, RAPD markers as well as on the structure and localization of repetitive DNA (Kazan and Muehlbauer 1991, Ahmad and Slinkard 1992, Ahmad *et al.* 1992, Tayyar and Waines 1996, Ahmad 1999, Staginnus *et al.* 1999) placed *C. cuneatum* far away from all the other annual species. More recently Iruela *et al.* (2002) by using RAPD and ISSR markers, obtained a dendrogram in which *C. cuneatum* clustered with *C. canariense*, both of them very distant from the rest of species of *Cicer*.

In terms of higher-level phylogeny, our results indicate that annual species of *Cicer* are far from being monophyletic. Kazan and Muehlbauer (1991), on the contrary, suggested that all annuals are monophyletic. The rationale for their hypothesis was that all annual species share an isozyme duplication and, as gene duplications are rare events, they are likely to occur only once in the evolution of a small group. Our data, on the contrary, better match those by Tayyar and Waines (1996) and those by Rajesh *et al.* (2002), both indicating that wild annuals are not monophyletic in nature, and suggest that transition from perennials to annuals (or vice-versa) occurred more than once in the genus. However, we would suggest that the relevance of annuity in genus *Cicer* has been overestimated by previous literature.

The sequence data shown here do not concur with the sectional treatment of genus *Cicer* by van der Maesen (1987). In fact, neither of the two sections represented in our investigations (namely, sections *Monocicer* and *Polycicer*) is strictly monophyletic. In particular, representatives of section *Monocicer*, to which *C. arietinum* belongs, occur in all the clades of Fig. 2; moreover, the clade including all the investigated representatives of section *Polycicer* also contains *C. macracanthum*, *C. pungens* (sect. *Acanthocicer*), *C. chorassanicum* (sect. *Chamaecicer*), and *C. yamashitae* (sect. *Monocicer*). We restrain from proposing a novel sectional treatment, given the poor resolution of our cladogram and the absence of a large number of perennial species from our investigation. However, we would like to suggest that any section including the majority of the annuals should not include *C. chorassanicum*, *C. cuneatum*, and *C. yamashitae*. Further investigation, including other critical taxa (*e.g.*, *C. incisum*, regarded by previous literature as the closest perennial to the group of annuals), as well as the extension of the molecular dataset will cast better light on the phylogeny of the genus and will clarify whether at least the two clades entirely made of annuals in the cladogram of Fig. 2 can be regarded as monophyletic.

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