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The monophyly and evolution of *Cynara* L. (Asteraceae) *sensu lato*: evidence from the Internal Transcribed Spacer region of nrDNA

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Abstract. The monophyly and evolution of *Cynara* was investigated using ITS sequence data. Parsimony analysis supports the monophyly of *Cynara* sensu lato, i.e. including the distinctive taxa *C. humilis* and *C. tournefortii*. This contradicts the recent decision to create a new monotypic genus *Arcyna* for *C. tournefortii*. A hypothesised close relationship between *C. tournefortii* and *Silybum* Adans. is also refuted. Four of the five species of *Cynara,* for which multiple accessions were sequenced, were shown to be monophyletic but *C. baetica* was found to be non-monophyletic. Free energy estimates for ITS1 secondary structure and conservation of the 5.8S region suggest that this is not due to the occurrence of pseudogenes. Hybridisation is a plausible explanation but evidence for the likely parents involved in such an event is inconclusive.

Key words: Asteraceae, Carduinae, *Cynara*, *Arcyna*, molecular phylogeny, ITS, pseudogenes, hybridisation.

Cynara L. (Asteraceae) is distributed in the Mediterranean and adjacent regions where it forms a common and conspicuous component of the summer flora. Ten species of *Cynara* have been recognised including the cultivated globe artichoke (*Cynara scolymus* L.) and the cardoon (*Cynara cardunculus* L.) (Table 1). All are stout perennial herbs, typically with large pinnate or pinnatisect spiny leaves, large capitulas borne singly or in lax corymbs, blue, purplish or white corollas, and bracts that terminate in stout apical spines.

Cynara has traditionally been placed in the subtribe Carduinae of the tribe Cardueae, together with other genera that possess a ring of hairs on the shaft beneath the style branches (Bremer 1994). However, both morphological (Bremer 1994) and molecular (Susanna et al. 1995, Garcia-Jacas et al. 2002) parsimony analyses have demonstrated that the subtribe Carduinae is paraphyletic to the Centaureinae. Within paraphyletic Carduinae, *Cynara* has been resolved as sister group to either *Ptilostemon* Cass. (Bremer 1994, Häffner 2000), a clade that comprises *Ptilostemon* and *Galactites* Moench. (Häffner and Hellwig 1999) or a large clade comprising a number of Carduinae genera including the *Carduus* L.-group and the genus *Echinops* L. of subtribe *Echinopinae* (Garcia-Jacas et al. 2002). However, the relationships suggested by these studies are not been well supported.

The precise generic boundaries of *Cynara* and the infrageneric classification of the genus have also proved contentious. Cosson (1849),

Table 1. Species of *Cynara*, their distribution and classification by Franco (1976) and Wiklund (1992)

Species	Distribution	Franco (1976) taxonomy	Wiklund (1992) taxonomy
C. algarbiensis Coss. ex Mariz	Western Mediterranean (Iberia and Morocco)	Sect. *Cynara*	Clade I
C. baetica (Spreng.) Pau	Western Mediterranean (Iberia and Morocco)	Sect. *Cynara*	Clade II
C. cardunculus L.	Western-Central Mediterranean area, Canary Islands, Madeira	Sect. *Cynara*	Clade II
C. cornigera Lindley	Eastern Mediterranean	Sect. *Cynara*	Clade I
C. scolymus L.**	Cultivated	Sect. *Cynara*	Clade II
C. humilis L.	Western Mediterranean (Iberia and Morocco)	Sect. *Bourgaea* (Cosson) Franco	Clade I
C. tournefortii Boiss. & Reuter.	Western Mediterranean narrow endemic to Spain	Sect. *Acaulon* Franco	*Arcyna****
C. auranitiaca Post.*	Eastern Mediterranean	–	Clade II
C. cyrenaica Maire & Weiller	Eastern Mediterranean Libya	–	Clade I
C. syriaca Boiss.	Eastern Mediterranean	–	Clade II
C. humilis × *C. algarbiensis*	SW Spain	–	–
C. humilis × *C. baetica* subsp. *moroccana*	Morocco	–	–
C. humilis × *C. tournefortii*	Portugal	–	–

*conspecific with *C. syriaca*;
** Included by Wiklund (1992) in *C. cardunculus*;
*** Monotypic genus suggested by Wiklund (1992) and formally published by Wiklund (2003).

for example, considered that the winged tetragonal achenes of *C. humilis* L. were sufficiently distinct from those of all other species then known to treat this species in a separate monotypic genus *Bourgaea* Coss. Willkomm (1852) subsequently proposed that *C. tournefortii* Boiss. should also be included in *Bourgaea* although the samples he observed were already dried and he could only predict the shape of the achenes.

Franco (1976), in contrast, recognised a broadly circumscribed *Cynara,* i.e. including *C. humilis* and *C. tournefortii*, whilst reflecting the morphological distinctiveness of these species in his sectional classification of the European representatives of the genus (Table 1). Thus, section *Bourgaea* (Cosson) Franco was characterised by pinnatisect leaves and included only *Cynara humilis* and section *Acaulon* Franco, distinguished by a stemless habit, included only *C. tournefortii*. The remaining species were placed in section *Cynara* characterised by a distinct stem and pinnatifid rather than pinnatisect leaves.

Wiklund (1992, 2003), in a taxonomic revision of *Cynara,* observed that most species possess thick coriaceous involucral bracts without a discernible midrib and long basal hairs borne ventrally on the inner pappus bristles. *Cynara tournefortii* however, only occasionally possesses these characteristics and as a result, Wiklund (1992) excluded this species from *Cynara*, later creating the new monotypic genus *Arcyna* for it (Wiklund 2003). Wiklund (2003) also suggested that *Arcyna* was closely related to either *Cynara* or *Silybum* Adans., the hypothesised relationship with the latter based on similarities in bract

morphology. However, as Wiklund (2003) noted, phylogenetic analyses of *Carduinae* have not included *Arcyna* and therefore the putative relationship between *Arcyna* and *Silybum* remains to be examined in an explicit phylogenetic context.

The results of Wiklund's (1992) morphological cladistic analysis *of Cynara* s.str. (i.e excluding *C. tournefortii*) were incongruent with the sectional classification of Franco (1976) and suggested two main clades within *Cynara* s.str. (Fig. 1). The first clade (Fig. 1 & Table 1, Clade I), comprised *C. algarbiensis, C. cornigera*, *C. cyrenaica,* and *C. humilis* and was supported in Wiklund's (1992) analysis by (1) leaf spines < 6 mm long, (2) leaf abaxial surface densely woolly and (3) leaf veins not forming large arches. The second clade (Fig. 1 & Table 1, Clade II), comprised *C. auranitiaca, C. baetica*, *C. cardunculus* (including the cultivated *C. scolymus*) and *C. syriaca* and was supported by the following synapomorphies: (1) basal spine clusters on the leaves, (2) indistinctly resolute leaf margins and (3) a densely glandular abaxial leaf surface. However, the results of this study should be treated with some caution as *C. tournefortii* was excluded from the analysis without explicit phylogenetic grounds for doing so and the characters were polarised *a priori*.

It is therefore apparent that the circumscription of *Cynara* and the relationships of species within the genus remain to be rigorously investigated. In this paper we use ITS data to examine these issues.

Materials and methods

Taxon sampling. Thirty six accessions were included in the analysis. All eight wild species of *Cynara,* as recognised by Rottenberg and Zohary (1996), were represented in the analysis with multiple accessions included for four species (Table 2). The cultivated globe artichoke (*C. scolymus*) and accessions suspected by Wiklund (1989) to be of putative hybrid origin were not sampled.

Silybum marianum (L.) Gaertner was included to test the putative relationship between *C. tournefortii* (= *Arcyna*) and *Silybum*. Representatives of related genera within Carduinae (*Carduus* L., *Cirsium* L., *Galactites* Moench., *Jurinea* Cass., *Notobasis* Cass., *Onopordum* L., *Ptilostemon* Cass. and *Tyrimnus* Cass.) were also included together with accessions representing three genera of

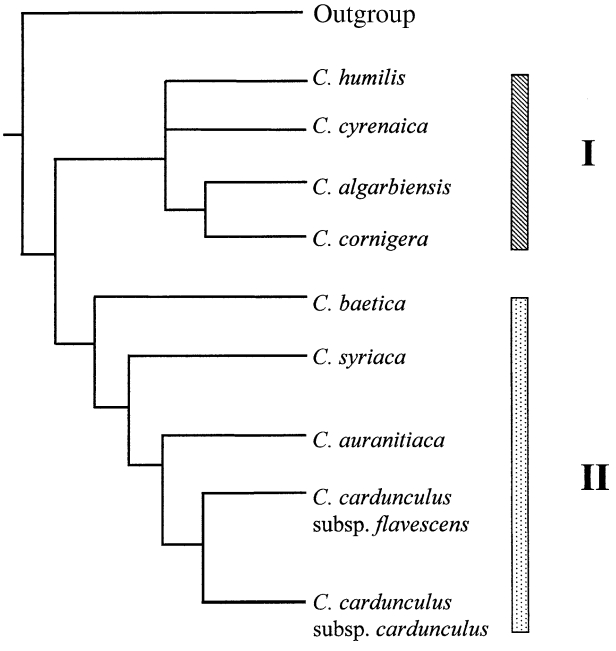

Fig. 1. A: Strict consensus tree from the morphological cladistic analysis of *Cynara* s.str. of Wiklund (1992). Clades I and II are referred to in Table 1 and in the text

Table 2. Voucher information, Genbank accessions numbers and length and G+C% of the ITS1 region for taxa included in the ITS analysis

Species	Voucher information	GenBank accession no.	ITS1 Length bp	ITS1 G + C %
Cynara algarbiensis	L. Robba S12, Spain (PAL)	AY780392	259	59.85
Cynara baetica 1	L. Robba S7, Spain (PAL)	AY780393	259	59.85
Cynara baetica 2	F. Pina, s.n.,Spain (SEV)	AY780394	259	59.85
Cynara baetica 3	L. Robba S6, Spain (PAL)	AY780395	259	59.85
Cynara baetica 4	L. Robba S8, Spain (PAL)	AY780396	259	62.16
Cynara cardunculus 1	L. Robba I2, Italy (PAL)	AY776173	260	62.69
Cynara cardunculus 2	L. Robba I3, Italy (PAL)	AY776174	258	62.79
Cynara cardunculus 3	L. Robba I4, Italy (PAL)	AY776175	259	62.55
Cynara cardunculus 4	s.col. 14522 Spain, (BM)	AY776176	259	62.55
Cynara cornigera 1	L.Robba 1G, Greece (PAL)	AY780397	258	62.40
Cynara cornigera 2	L. Robba s.n., Greece (PAL)	AY780398	258	62.40
Cynara cyrenaica	s. col. 3721 Cyprus (BM)	AY780399	258	63.18
Cynara humilis 1	L. Robba S2, Spain (PAL)	AY780410	259	61.39
Cynara humilis 2	L. Robba S1, Spain (PAL)	AY780411	259	61.39
Cynara humilis 3	L. Robba S9, Spain (PAL)	AY780412	259	61.39
Cynara humilis 4	L. Robba S8, Spain (PAL)	AY780413	259	61.39
Cynara syriaca 1	Wheeler Haines 1513 207*3, Iraq, (E)	AY780414	258	62.40
Cynara syriaca 2	T. Baytop 18242 Iraq, (E)	AY780415	258	62.40
Cynara syriaca 3	Wheeler Haines, s.n., Iraq, (E)	AY780416	259	61.78
Cynara tournefortii	M.P. Graells Spain, (BM)	AY780417	259	59.46
Carduus corymbosus Ten.	s. col. 1064, Romania (BM)	AY780400	256	64.06
Carduus nutans L.	s.col. s.n., Yugoslavia Croatia, (BM)	AY780401	259	52.51
Centaurea carolipauana Fern. Casas & Susanna	Garcia-Jacas et al. 2001	AY012278 – AY012314	259	50.19
Cirsium oleraceum (L.) Scop.	s. col. 2334, Slovenia (BM)	AY780402	259	54.05
Echinops spinosissimus Turra	Garcia-Jacas et al. 2002	AF319075 – AF319129	257	63.42
Galactites tomentosa Moench.	s.col., s. n., Malaga, (BM)	AY780403	258	56.20
Jurinea mollis (L.) Rchb.	s.col., s. n., Greece, (BM)	AY780404	256	59.38
Notobasis syriaca (L.) Cass.	s.col. 82 Alghero, (BM)	AY780405	257	57.20
Onopordum illyricum L.	s.col. 1219, Spain (BM)	AY780406	257	56.81
Ptilostemon afer (Jacq.) Greuter	E. Stamatiadou 15512 Greece Tessalia, (BM).	AY780407	258	55.43
Ptilostemon hispanicus (Lam.) Greuter	M. F. & S. G. Gardner 1182 Cadiz Spain, (BM)	AY780408	255	55.69
Ptilostemon niveus (Presl) Greuter	4103 Basilicata Italy, (BM)	AY780409	257	54.86
Klasea grandifolia (P.H. Davis) Greuter & Wagenitz	Garcia-Jacas et al. 2001	AY012296 – AY012332	255	52.94

Table 2. (Continued)

Species	Voucher information	GenBank accession no.	ITS1 Length bp	ITS1 G + C %
Silybum marianum (L.) Gaertner	Garcia-Jacas et al. 2002	AF319094 – AF319148	251	54.58
Tyrimnus leucographus (L.) Cass.	Garcia-Jacas et al. 2002	AF319097 – AF319151	258	61.24
Volutaria crupinoides (Desf.) Maire	Garcia-Jacas et al. 2001	AY012304 – AY012340	256	55.47

Centaureinae (*Centaurea* L., *Klasea* Cass. and *Volutaria* Cass.) as this subtribe is known to be nested within a paraphyletic Carduinae (Bremer 1994, Susanna et al. 1995, Garcia-Jacas et al. 2002). *Echinops spinosissimus* Turra (Echinopsidinae) was included as an outgroup taxon based on the analyses of Bremer (1994) and Susanna et al. (1995). Details of all accessions included in the analysis are provided in Table 2.

Molecular methods. Total genomic DNA was extracted from $\sim$ 0.1 g of dried leaf material (silica gel-dried or herbarium specimens) using a modified CTAB micro-extraction protocol (Doyle and Doyle 1987). Extracted DNA was further purified, without precipitation, using QIAquick columns.

Standard polymerase chain reaction (PCR) procedures were applied to amplify the ITS region together with part of the 17S and 26S gene region using primers 17SE and 26SE (Sun et al. 1994). Betaine was added to prevent the formation of secondary structures in difficult templates. PCR reactions were carried out using a Techne Techne-gene Thermal Cycler (30 cycles: 1 min. of denaturation at 94 °C, 3 min of annealing at 49 °C, 1 min of extension at 72 °C, 8 min final extension at 72 °C). Amplification products were purified using QIAquick columns following the manufacturer's protocols.

Dideoxy cycle sequencing (28 cycles: 30 sec. of denaturation at 95 °C, 15 sec of annealing at 50 °C, 4 mins of extension at 60 °C) with big dye terminators was performed in 10 μl volumes using PCR primers on a Hybaid Omnigene Thermal Cycler. Excess dye-labelled nucleotides from the sequence reactions were removed by standard ethanol/sodium acetate precipitation. Sequence products were subsequently re-suspended and run on a Perkin Elmer ABI 377 DNA sequencer.

Sequence alignment and phylogenetic analyses. Sequence data were edited and assembled using DNAStar Lasergene Navigator. Verified sequences were then aligned by eye in Se-Al (ver. 1.0al; Rambaut 1996) prior to phylogenetic analysis. Gaps were not used as characters for analysis because many were overlapping and consequently problematic for coding. The data matrix is available from the authors on request.

Phylogenetic analyses of the data were performed using PAUP* β version 4.0b5 (Phylogenetic Analysis Using Parsimony; Swofford 2001). All parsimony analyses were simultaneous and unconstrained with character state changes unordered and weighted equally. One thousand random replicate searches were performed with ACCTRAN, MULPARS and TBR options. Trees were rooted on *Echinops spinosissimus* which is the most distal outgroup according to the analyses of Bremer (1994) and Susanna et al. (1995).

The ensemble consistency index (CI; Kluge and Farris 1969) and retention index (RI; Farris 1989) were calculated in PAUP*. The robustness of clades in the strict consensus tree was evaluated by non-parametric bootstrap analysis (Felsenstein 1985). Bootstrap values were determined from 100 bootstrap replicates each comprising 1000 random stepwise addition heuristic searches with TBR branch swapping.

Free-energy values associated with predicted secondary structures of the ITS1 region were estimated with the minimum-free energy (MFE) algorithm (Zuker 1989). Fold prediction were made at the Michael Zuker, Rensselaer Polytechnic Institute (BiC) web site (http://www.bioinfo.rpi.edu/applications/mfold/) by use of the mfold program version 3.0. Foldings were performed at a temperature of 37 °C (Zuker 2003, Mathews et al. 1999).

Results

The aligned data matrix is 628 bp long of which 361 bp were constant, 89 uninformative and 178 informative for parsimony analysis. If ingroup taxa alone are considered, 569 characters are constant, 18 parsimony uninformative and 41 parsimony informative.

Parsimony analysis of the data resulted in 144 most parsimonious trees (length 603 steps, CI = 0.513 [excluding uninformative characters]; RI = 0.750).

Figure 2 shows the strict consensus tree from the parsimony analysis. The monophyly of *Cynara* s.l., i.e. including both *C. tournefortii* and *C. humilis* is strongly supported (Fig. 2; bootstrap = 100%). In the strict consensus tree, *Galactites tomentosa* is resolved as sister group to *Cynara* with *Onopordum illyricum* resolved as sister species to this clade although neither are well supported (Fig. 2). Within *Cynara*, the three *C. syriaca* accessions form a strongly supported group (bootstrap = 97%) that is weakly supported as sister group to one of the four *C. baetica* accessions (bootstrap = 56%). The four *C. cardunculus* accessions form a well supported monophyletic group (bootstrap = 100%; Fig. 2).

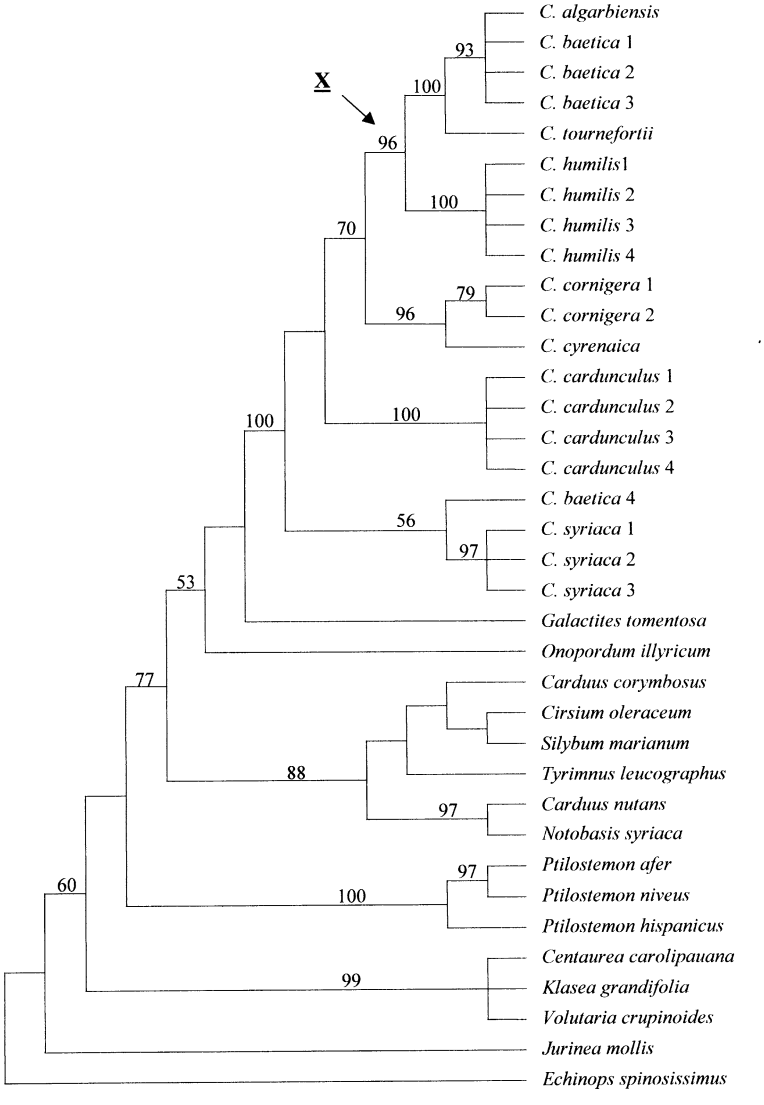

Fig. 2. Strict consensus tree from the ITS region analysis. Numbers above nodes indicate bootstrap values. The Clade marked X is discussed in the text

A large clade comprising the Eastern Mediterranean species *C. cyrenaica* and *C. cornigera*, the Western Mediterranean *C. humilis, C. tournefortii*, *C. algarbiensis* and three accessions of *C. baetica* is resolved in the strict consensus tree (bootstrap = 70%). Within this clade the Eastern- and Western-Mediterranean species are resolved separately. Within the East Mediterranean clade (bootstrap = 96%), the two accessions of *C. cornigera* form a monophyletic group (bootstrap = 70%) that is sister group to *C. cyrenaica*. In the Western Mediterranean clade (bootstrap = 96%), three *C. baetica* samples form a clade together with *C. algarbiensis* (bootstrap = 93%) that is sister group to *C. tournefortii* (bootstrap = 100%). This larger clade is sister to a monophyletic group comprising all of the *C. humilis* accessions included in the analysis (bootstrap = 100%).

In contrast to all other species for which multiple accessions were included, *C. baetica* is found to be non-monophyletic: whilst three accessions of this species group together in the Western Mediterranean clade, the fourth is resolved as sister group to *C. syriaca* (bootstrap = 56%) (Fig. 2).

ITS1: Length, free energy values and G + C % content. The length and G + C% content for the ITS1 region of all samples included in the ITS analysis is shown in Table 2 and the ranges of free energy estimates obtained for each sample are summarised in Fig. 3. The length of the ITS1 region varies between 251 and 260 bp, although within *Cynara,* variation was slight with all sequences between 258 and 260 bp long. G + C% content varied between 52.51% and 64.06%, with a range of 59.46–63.18% within *Cynara*.

The maximum estimated free energy content of secondary structures of RNA transcripts ranged between 79.5 and 125 kcal/Mol, with *Cynara* samples ranging between 110.4 and 123 kcal/Mol (Fig. 3). The accession *C. cardunculus* 2 has the highest estimated free energy content of all *Cynara* accessions but the G + C% content of this accession was within the range of other accessions.

Discussion

The monophyly of *Cynara*. The molecular data presented in this paper provide strong support for a monophyletic *Cynara* sensu latu, i.e.

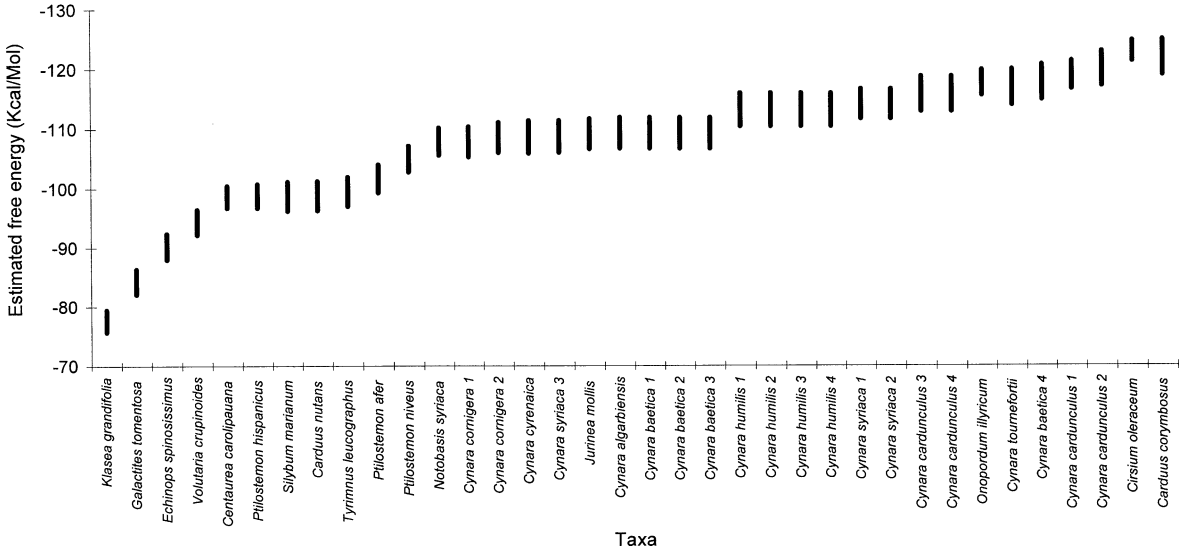

Fig. 3. Range of estimated free energy content (kcal/Mol) of the ITS1 region for taxa included in the ITS analysis, arranged by ascending maxima

including both *C. humilis* and *C. tournefortii* (Fig. 2; bootstrap = 100%). Whilst both of these species have distinctive morphologies that are not shared with other members of *Cynara,* the ITS analysis demonstrates that these characters are autapomorphic within the genus. The results therefore support the broad circumscription of *Cynara* proposed by Franco (1976). The results, however, do not support the infrageneric classification of Franco (1976) who recognised the monotypic sections *Bourgea* and *Acaulon* for *C. humilis* and *C. tournefortii*, respectively. The placement of these species in the analysis means that the recognition of these sections would result in a residual paraphyletic section *Cynara*.

The close relationship between *C. tournefortii* and *Silybum* hypothesised by Wiklund (2003) is refuted. *Silybum* is resolved in a well supported clade with *Carduus*, *Cirsium*, *Tyrimnus* and *Notobasis* (Fig. 2; bootstrap = 88%), a placement that is consistent with the results from analysis of *mat*K and *mat*K + ITS by Garcia-Jacas et al. (2002). This result indicates that the similarity in bract morphology between *C. tournefortii* and *Silybum* noted by Wiklund (2003) is the result of convergent evolution.

The sister group of *Cynara*. In the ITS analysis, *Galactites* (represented by *G. tomentosa*) is resolved as sister group to *Cynara* and *Onopordum* (represented by *O. illyricum*) is resolved as sister group to (*Cynara* + *Galactites*). However, both relationships are weakly supported with bootstrap values of less than 50% and 53% respectively. There is very limited agreement concerning the precise relationships of *Cynara, Galactites* and *Onopordum* in cladistic analyses of these taxa to date (Bremer 1994, Susanna 1995, Häffner and Hellwig 1999, Häffner 2000, Garcia-Jacas et al. 2002). Deeper relationships within Carduinae remain poorly resolved and further sampling of both taxa and characters are necessary to gain further insights into these relationships and to understand the complex patterns of morphological evolution within the group.

Evolution and infrageneric classification of *Cynara*. Multiple accessions were included for five of the eight species of *Cynara* in the ITS analysis. Four of the species for which multiple accessions were included (*C. cardunculus*, *C. humilis*, *C. syriaca* and *C. cornigera*) were found to be monophyletic. However, the fifth species, the western Mediterranean endemic *C. baetica,* was non-monophyletic in the analysis. Three of the accessions of *C. baetica* formed a well-supported monophyletic group with *C. algarbiensis* (also from the western Mediterranean) but the fourth accession was placed as sister group to *C. syriaca* (Fig. 2). There are a number of possible explanations for this unexpected pattern.

The first possibility is the non-monophyly of *C. baetica* since *Cynara baetica* sensu Wiklund (1992) comprises two subspecies differing in the pigmentation of the middle involucral bracts and florets. However, all *C. baetica* accessions included in the ITS analysis are morphologically similar and belong to the same subspecies (subsp. *baetica*). Non-monophyly therefore does not provide a satisfactory explanation of the result observed.

The occurrence of pseudogenes in *Cynara* is a second possible explanation for the relationships observed. Methods for detecting the presence of pseudogenes include examination of the patterns of variation in the 5.8S region and comparison of secondary structure free-energy content of the ITS regions (Gernandt and Liston 1999, Denduangboripant and Cronk 2000, Hartman et al. 2001, Mayol and Rosseló 2001). Applying these methods to the data set presented in this study suggests that the pattern of relationships observed cannot be attributed to pseudogene activity. Indels in the 5.8 S region are considered indicative of pseudogene activity but none are detectable in the 5.8 S region in any of the accessions included in this analysis (not shown). Pseudogenes typically differ significantly from their functional counterparts in free-energy content, however in the present analysis there is no substantial difference in free energy estimates for the ITS1 region of any of the sequences

included in the study and no discrete gaps are detectable when the ranges of free energy estimates are plotted (Fig. 3). Furthermore, the difference between the maximum free energy estimate for *C. baetica* 4 and the remaining *C. baetica* accessions, (8.9 kcal/Mol; a difference of 8%) is significantly less than the highly divergent free energy estimates obtained for suspected functional/pseudogene pairs (e.g. 43.6 kcal/Mol, a difference of 41% in *Quercus rubra* L., Mayol and Rosselló 2001). Pseudogene activity in *Cynara* does not appear to be a plausible explanation for the non-monophyly of *C. baetica* accessions.

A further possible explanation for this result is hybridisation and introgression involving *C. baetica*. Some species of *Cynara* have been shown to be inter-fertile (Rottenberg and Zohary 1996, 2000) and Wiklund (1989, 1992) have demonstrated that *C. baetica* hybridises with *C. humilis* in the wild. At least two hybridisation scenarios are consistent with the ITS phylogeny presented in this paper.

In the first scenario: (i) *C. baetica* is the sister species of the western Mediterranean *C. algarbiensis* (a relationship suggested by three of the four accessions of *C. baetica*); (ii) hybridisation occurs between *C. baetica* and East-Mediterranean *C. syriaca*; (iii) post-hybridisation introgression with *C. baetica* coupled with intragenomic hybridisation between homologous ITS copies from each parent (e.g. Nieto Feliner et al. 2004) results in a hybrid that is morphologically indistinguishable from *C. baetica* but possesses an ITS sequence-type that shows similarities (but is not identical) to that of *C. syriaca*.

In the second scenario: (i) *C. baetica* and *C. algarbiensis* are not closely related within *Cynara* and the parental ITS type of *C. baetica* is that demonstrated by *C. baetica* 4 in the analysis; (ii) *C. baetica* hybridised with *C. algarbiensis*; (iii) the three *C. baetica* accessions that group with *C. algarbiensis* are the result of hybridisation between *C. algarbiensis* and *C. baetica*. In this scenario, introgression with *C. baetica* following hybridisation results in specimens that are morphologically indistinguishable from *C. baetica*. However, the ITS type found in these accessions is the result of homogenisation to the *C. algarbiensis* type.

The morphological cladistic analysis of Wiklund (1992) partitions the taxa such that *C. baetica* is closer to *C. syriaca* than to *C. algarbiensis* (Fig. 1). The group in the morphological analysis (Wiklund 1992) that comprises *C. algarbiensis, C. cornigera, C. cyrenaica* and *C. humilis* (Fig. 1) is also identical in composition to a clade in the molecular tree (Fig. 2; Clade X) if the three *C. baetica* accessions of suspected hybrid origin are excluded (i.e. *C. baetica 1–3*) and *C. tournefortii* is not considered. This may, therefore, lend some support to the hypothesis that hybridisation between *C. baetica* and *C. algarbiensis* explains the non-monophyly of *C. baetica*. Species ranges also suggest that hybridisation is more likely between *C. baetica* and *C. algarbiensis* (both western Mediterranean) than between *C. baetica* (Western Mediterranean) and *C. syriaca* (Eastern Mediterranean).

Crossing experiments between wild species of *Cynara* performed by Rottenberg and Zohary (2000) demonstrated that while some species of *Cynara* are interfertile, most are largely cross-incompatible. However, in the case of *C. algarbiensis* × *C. baetica* viable seeds were not obtained whereas crosses attempted between *C. baetica* and *C. syriaca* resulted in some viable seeds. Whilst this may be interpreted as support for a hybridisation event between *C. baetica* and *C. syriaca*, an alternative explanation is that the ability of these two species to interbreed is a plesiomorphic trait reflecting the recency of their common ancestry.

It is apparent that whilst the results of the ITS analysis provide robust support for the monophyly of *Cynara* s.l., infrageneric relationships remain to be adequately resolved. The results of this paper suggest either that hybridisation within *Cynara* may have been more widespread than was previously thought

or that ITS has evolved in a complex manner. Either way, further characterisation of ITS variation within the genus, coupled with the development of other marker systems is now necessary. Pending such an exhaustive study, it is perhaps somewhat premature to consider the implications of these data for the infrageneric classification of *Cynara*. Nevertheless, the morphological data (Wiklund 1992) and the ITS data do show some congruence if *C. baetica* 1–3 are considered of hybrid origin. In Wiklund's (1992) analysis, the group comprising *C. algarbiensis, C. humilis, C. cornigera* and *C. cyrenaica*, was diagnosed by three unambiguous synapomorphies: short leaf spines ($<$ 6 mm long); leaves lacking basal spine clusters and leaves abaxially densely woolly. In the molecular analysis, these four species form a clade together with the putative hybrid accessions *C. baetica* 1-3 and *C. tournefortii*. It is interesting to note that *C. tournefortii* also possesses the three synapomorphies identified for the clade in the morphological study.

The ITS region has been widely used to infer phylogenetic relationships in Asteraceae (e.g. Cerbah et al. 1998, Eldenäs et al. 1998, Fiz et al. 2002, Font et al. 2002, Garcia-Jacas et al. 2002, Oberprieler 2001, Susanna et al. 1995, Torrell et al. 1999) and has provided valuable insights into relationships within the family. The present study serves to re-emphasise the need for caution when using this particular marker. The ITS region is known to have evolved in a complex and unpredictable manner in several groups of plants (e.g. Wendel et al. 1995, Buckler and Holtsfort 1996, Hughes et al. 2002, Álvarez and Wendel 2003). Including multiple accessions of species in the present analysis, highlighted potentially complex patterns of ITS evolution in *Cynara* that now demand further research. Similar sampling strategies should routinely be adopted in other studies that attempt to utilise this marker to investigate species-level relationships.

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