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Karyological and genome size insights into cardoon (*Cynara cardunculus* L., Asteraceae) in Tunisia

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Abstract — This study contributes the first genome size data for wild populations of *Cynara cardunculus*, the presumed progenitor of artichoke and cultivated cardoon. C-values estimated by flow cytometry are 2C = 1.98–2.14(3.03) pg for wild cardoon (ten populations), 2C = 2.10–2.11 pg for cultivated cardoon (two accessions) and 2C = 2.05 pg for artichoke (one accession). Chromosome counting (carried out for all material except the artichoke) establishes diploidy. In order to provide a phylogenetic framework for Tunisian populations, internal transcribed spacer (ITS) region was sequenced and analysed together with previously published *Cynara* sequences. Our results show the wild and crop cardoons to present similar karyological features and genome sizes despite strong morphological differentiation, with the single exception of a Tunisian population (from Tajerouine), which exhibits a 42–53% higher genome size. Along with Sicilian individuals, Tunisian wild *C. cardunculus* appear genetically closer to artichoke and cardoon than to studied wild relatives from the remaining distribution. This highlights the crucial importance of taking into consideration the North African territory in deciphering the history of *C. cardunculus* crop domestication.

Key words: Artichoke, C-value, crop cardoon, *Cynara*, domestication, ITS, somatic chromosome number

Introduction

Efforts to resolve the systematics of cultivated cardoon and artichoke have eventually converged in determining the origination from *Cynara cardunculus* L. (reviewed in Sonnante *et al.* 2007b). The intraspecific evolutionary history of this species of considerable economic importance −positive− mainly as food crop species (Christaki *et al.* 2012) and −negative− as noxious weed (Global Invasive Species Database 2005) is still in question. The last comprehensive revision of the species was done by Wiklund (1992) and was based on morphological data. This author circumscribed two infraspecific entities, the subsp. *cardunculus*, distributed in the central and north-eastern Mediterranean region, and the subsp. *flavescens* Wiklund, distributed in the Iberian Peninsula and Macaronesia (Wiklund 1992). Sicily and North Africa were described as the contact zone of these two morphs, with individuals showing different combinations of the appendix bract characters that define the subspecies (Wiklund 1992). Taxonomic sampling of subsequent studies was strongly biased toward cultivated *C. cardunculus*, and was restricted to wild relatives from Sicily and the northern part of the species' distribution (e.g. Greece, Italy and Spain; Aquadro *et al.* 2005; Sonnante *et al.* 2007a, 2008). The fact that authors assume that Arabs, who dominated the southern Mediterranean during the Middle Ages, were likely involved in *C. cardunculus* crop domestication (Sonnante 2007b; Wright 2012) advocates for the study of wild African material. Recently, Khaldi *et al.* (2012a, 2012b) have carried out morphological and genetic studies of Tunisian wild populations, however, North African material has still not been considered in the attempts to establish the evolutionary origin of crop *Cynara*.

Genome size data are providing valuable insights in the understanding of plant domestication (e.g. in *Gossypium* L., the cotton, Grover *et al.* 2004; in *Artemisia*

arborescens L., Garcia *et al.* 2006). They constitute a valuable tool in the evolutionary studies of the Asteraceae family (Garnatje *et al.* 2011, and references therein). The genus *Cynara* L. has never been the subject of a genome size survey and C-value was assessed only once, for the artichoke (Marie and Brown 1993) that presents a very low C-value (1C ≤ 1.4 pg). Such very low C-values were also inferred for the close ancestors of *Cynara* (Vallès *et al.* 2013).

The present study aims to extend our knowledge of Tunisian populations to genomic aspects by: (1) investigating whether polyploidy and other chromosome number changes might be involved in the evolution of the species, so far known to present only the diploid level and $2n = 34$ chromosomes, as for the whole genus *Cynara* (Watanabe 2002, 2004); (2) establishing the cytogenetic and genetic patterns of the Tunisian *C. cardunculus* populations and proceeding to their comparison with other wild and crop populations; (3) discussing the evolution of *C. cardunculus* genome in a phylogenetic framework.

Material and methods

The plants were grown from cypselae collected in the field in the Tunisian localities indicated in Table 1 and Figure 1.

Chromosome counts were made from root tip meristems either from cypselae germinated on wet filter paper in Petri dishes at room temperature or from plants cultivated in a greenhouse. Root tip meristems were pre-treated in 0.002 M 8-hydroxyquinoline for 2.5–3 h at 16 ºC, fixed in absolute ethanol and glacial acetic acid (3:1) and stored in the fixative at 4 ºC. Samples were hydrolysed in 1 N HCl for 10–12 min at 60 ºC, stained with 1% aqueous aceto-orcein for 30 min minimum, and

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squashed into a drop of 45% acetic acid-glycerol (9:1) on slides. Metaphase plates were photographed with a digital camera (Zeiss AxioCam HRm) mounted on a Zeiss Axioplan microscope, and images were analysed with Axio Vision Ac version 4.2.

For nuclear DNA content estimation, fresh young leaves of *Cynara* individuals were chopped in a plastic Petri using a razor blade dish, together with an internal standard in 1200 µl of LB01 buffer with 0.5% Triton X-100 (Doležel *et al.* 1989) supplemented with 100 µg/ml of ribonuclease A (RNase A, Boehringer). *Pisum sativum* L. 'Express Long' (2C = 8.37 pg; Marie and Brown 1993) was used as internal standard. Nuclei were filtered through a 60-µm nylon filter in order to eliminate cell debris before the addition of 36 µg/ml of propidium iodide (1 mg/ml, Sigma-Aldrich Química, Alcobendas, Madrid, Spain). Samples were kept for 20 min on ice before measurement. Five individuals per population were analysed. Two samples of each individual were extracted and measured independently. Fluorescence analysis was carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Fla.) at the Centres Científics i Tecnològics de la Universitat de Barcelona, with the instrument set up with the standard configuration as described in Garnatje *et al.* (2004). The total nuclear DNA content was calculated by multiplying the known DNA content of the standard by the quotient between the 2C peak positions of the target populations and the standard in the histogram of fluorescence intensities. This follows the assumption that there is a linear correlation between the fluorescent signals from stained nuclei of the unknown specimen, the known internal standard and the DNA amount.

The ITS rDNA region was amplified and sequenced following the procedure described in Susanna *et al.* (2006), considering one individual of each of the wild populations of *Cynara cardunculus*. Resulting sequences were manually aligned with

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MacClade 4.08 (Maddison and Maddison 2005) together with sequences of *Cynara* gathered from GenBank. *Lamyropsis carpini* Greuter and *Ptilostemon stellatus* (L.) Greuter were selected as outgroups in accordance with the results of Vilatersana *et al.* (2010). Bayesian inference was carried out with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), using the SYM+G model of nucleotide substitution selected through the Akaike and Bayesian information criteria with jModelTest 0.1.1 (Posada 2008; Guindon and Gascuel 2003). Four Markov chains were analysed simultaneously for $5x10^6$ generations with tree sampling every 500 generations. The 50% majority rule consensus tree and posterior probabilities (PP) of nodes were calculated from the pooled samples, after discarding data from the first 1,000 generations as the *burn-in* period. Most of the variability of *Cynara cardunculus* sequences concentrates on polymorphic positions, leading us to perform a network analysis restricted to the species, using the neighbour-net (NN) algorithm implemented in SplitsTree4 (Huson and Bryant 2006) and the polymorphic positions recoded as pairs of monomorphic characters as described in Muratovic *et al.* (2010). Bootstrap values (BS) of 1000 replicates were calculated.

Results and discussion

Karyological data — Metaphase plates are presented in Figure 2. Chromosome counts were carried out in the ten wild populations and the two cardoon crop accessions, which all display the diploid level and $2n = 34$ chromosomes. This chromosome number is consistent with previous records, which state the diploid level and 34 chromosomes in both wild and cultivated *Cynara cardunculus* (Watanabe 2002, 2004).

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Genome size data — Data on nuclear DNA content are presented in Table 1. Genome size in the Tunisian wild populations ranges from 2C = 1.98–2.14(3.03) pg, while the genome size of cultivated cardoon is 2C = 2.10–2.11 pg and 2C = 2.05 pg for the artichoke. The mean HPCV (half-peak coefficient of variation) is 5.13% and 2.08% for the target plant and the standard, respectively (for details see Table 1).

ITS analyses — Results of phylogenetic analyses are presented in Figure 3. Bayesian analysis (Figure 3A) shows the *C. cardunculus* clustered together without significant support due to the weak association of artichoke with remaining accessions (PP = 0.66). In turn, wild and cultivated cardoons form a robust clade (PP = 1). The neighbour-net network (Figure 3B) better helps resolving the relationships within *C. cardunculus*.

Discussion

***Cynara cardunculus* exhibits a considerable intraspecific variability with regard to genome size of wild populations** — Genome size of *C. cardunculus* wild populations ranges between 2C = 1.98 and 2.14 pg except the population of Tajerouine, at the South-West extreme of the studied area (Figure 1), which has a genome size 42–53% higher than other ones despite sharing the same chromosome number (Table 1, Figure 2). This raises the intraspecific genome size variation of *C. cardunculus* up to 1.53-fold. Such intraspecific variation at a same ploidy level is considerable if compared to remaining Cardueae, which never reaches even 1.2-fold. For example, within the genera *Carthamus* L., *Centaurea* L., *Cheirolophus* Cass., *Cirsium* Mill., *Colymbada* Hill and *Cyanus* Mill., the intraspecific range of 1C*x*-values (monoploid genome size) peaks to

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1.06-fold in *Carthamus oxyacantha* M.Bieb. (two accessions, 2*x*), 1.06-fold in *Colymbada orientalis* (L.) Holub (four accession, 2*x* and 4*x*), 1.09-fold in *Cirsium arvense* (L.) Scop. and *C. palustre* (L.) Scop. (two accessions each, 2*x*), 1.13-fold in *Cyanus triumfetti* (All.) Dostál ex Á.Löve & D.Löve (five accessions, 2*x*, two subspecies), 1.14-fold in *Cheirolophus intybaceus* (Lam.) Dostál (37 accessions, 2*x*) and 1.18-fold in *Centaurea stoebe* L. (six accessions, 2*x* and 4*x*) (calculated using data from GSAD database, www.asteraceaegenomesize.com, release 2010, Garnatje *et al.* 2011). It is noteworthy that maximum ranges are found in species showing several ploidy levels, subspecies differentiation or a wide geographic coverage.

Whether *C. cardunculus* population from Tajerouine represents an isolated case of genome size divergence or is the witness of a more general genome size transition in the considered area remains to determine. Interestingly, this population appears in the network inference as isolated from remaining Tunisian populations and positioned in between a cluster of populations from Tunisia (Beja, Enfidha and Masakin) and the group of accessions from Northern distribution (Figure 3B). Given that Tunisia is thought to be inhabited by both *C. cardunculus* subspecies (Wiklund 1992), the cytogenetic differentiation, coupled with genetic divergence, might be indicative of a taxonomic heterogeneity. This being the case, genome sizes would be of great help to geographically circumscribe the *C. cardunculus* intraspecific entities. Alternatively, the genome size divergence displayed by Tajerouine population might also result from hybridisation events between *C. cardunculus* and other *Cynara* species.

Domestication of *Cynara cardunculus* leading to artichoke and cardoon proceeded within the range of genome size established for wild populations — Genome sizes of

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artichoke and crop cardoon are inscribed into the range of wild population C-values, even when discarding the divergent value of Tajerouine population, this result stating for a genome size constancy −or moderate reshuffling− during the domestication process of *C. cardunculus*. Indeed, genome size of artichoke remained apparently unaltered (Table 3) despite a strong morphological and genetic divergence with respect to wild relatives (see the distance-based network, Figure 3B). Bayesian inference well-illustrates this genetic differentiation, as it shows artichoke excluded from a significantly supported clade which groups all wild and cultivated cardoons (Figure 3A). Such a configuration, previously described by Sonnante *et al.* (2007a), was related by these authors to the domestication syndrome.

There is little insight into genome size trend with domestication, even in a family such as the Asteraceae, which includes many economically important species (Garnatje *et al.* 2011). In this sense, our study significantly increases the pool of data available on this topic, adding genome size information for wild and crop *C. cardunculus* to previous data on e.g. *Artemisia arborescens*, *Dahlia variabilis* Desf., *Helianthus annuus* L. and *Lactuca sativa* L. (Garnatje *et al.* 2011, and references therein). In *Artemisia arborescens*, domestication was accompanied by a slight decrease of genome size with respect to wild populations, which was more accentuated in cultivars (-8.68%) than in cultivated plants (-4.60%). Our results allow discarding a drastic genome size reshuffling with *Cynara cardunculus* domestication, however, it cannot be ruled out that analysing a wider sample of crop and wild *C. cardunculus* might shed light on a significant variation −although subtile−, as found in *Artemisia arborescens* (Garcia *et al.* 2006).

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Origin of artichoke and cardoon should be seek amongst North African *Cynara cardunculus* — The network obtained (Figure 3B) is fully consistent with the phylogeny of Sonnante *et al.* (2007a), and shows a strong geographic cohesion, with the accessions from Northern distribution fully segregated from those of Southern distribution and crops. The only exception is the placement of the Spanish population AJ831533 amongst crop cardoon accessions, which was previously noticed by Sonnante *et al.* (2007a) on this and other Iberian accessions. These authors provided three alternative hypotheses to explain such positioning: Spanish populations belong to a genepool different from the one of Italian and Greece populations, there is a gene flow with crop cardoons, or these particular Spanish populations are crop cardoons escaped from cultivation (Sonnante *et al.* 2007a). Our results tend to point for the last explanation because they show the AJ831533 accession indistinguishable from crop cardoons, while other Spanish population (AY776176, from Robba *et al.* 2005) appears perfectly nested amongst the Northern group of wild populations (Figure 3B).

Although ITS accessions of both artichoke and cardoon crop form a cohesive cluster, they exhibit definitively divergent patterns in the network phylogeny (Figure 3B). The six artichoke accessions share a long-single-branch (BS = 99.9%), suggesting that domestication leading to this crop may have occurred once in the time and no further exchange of genetic material happened with other *C. cardunculus*. However, this assumption should be regarded as preliminary only, because the number of genetic groupings does not always reflect the domestication history (Morrell *et al.* 2012). In turn, crop cardoon accessions group together through reticulations without significant statistical support, and appear closer to wild plants than are the artichokes. At the origin of this pattern, several −no exclusive− reasons might be evoked: multiple domestication

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processes leading to crop cardoons happened through times, domestication impacted crop cardoon to a lesser extent than artichoke, and/or gene flow occurred with wild plants after domestication. It is to note that these results are congruent with morphological divergence, more pronounced between artichoke and wild plants than between wild and crop cardoon.

Archaeological record implicates Arabs in *C. cardunculus* crop domestication (Sonnante 2007b; Wright 2012), which makes plausible the assumption of a North African origin for artichoke and cardoon. Our phylogenetic reconstruction includes for the first time African populations (Figure 3B). Interestingly, but not surprisingly, it evidences a tightest relationship of *Cynara* crops with Tunisian and Sicilian populations than with populations from the Northern area (Greece, Italy and Spain), these being isolated in their own cluster (BS = 81.6%, Figure 3B). This result suggests that *Cynara* crops likely evolved from a genepool present in the Southern distribution of the species. The high plasticity of *C. cardunculus* is thought to have promoted its domestication, naturalisation and invasiveness (Sonnante 2007a). In this sense, it is not surprising that the regions of higher intraspecific diversity, such as Tunisia and Sicily (Wiklund 1992), are those genetically closest to the *C. cardunculus* crops. Further studies emphasising the North African distribution of the species would help to clarify its evolutionary history.

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Table 1. Origins of the populations studied, genome size data and GenBank references of ITS sequences. 2C: nuclear content [mean ± standard deviation (SD) of 10 measurements, two replicates for five individuals each]; 1C*x*: monoploid genome size (DNA content per basic chromosome set); 1 pg = 978 Mbp (Doležel *et al.* 2003).

***Cynara cardunculus* L. Locality in Tunisia**	**2C (pg)**	**1Cx (pg)**	**2C (Mbp)**	**HPCV sample (%)**	**HPCV standard (%)**	**ITS GenBank number**
• Wild						
Sousse: Enfidha, *Khaldi 1*(BC)	1.98 ± 0.01	1.00	1936.44	5.16	1.98	HG798953
Jendouba: Oued Mliz, *Khaldi 2* (BC)	1.99 ± 0.03	1.00	1946.22	4.97	1.91	HG798954
El Kef: Tourief, *Khaldi 3* (BC)	2.02 ± 0.07	1.01	1975.56	4.91	1.62	HG798955
Zarghouan: Zriba, *Khaldi 4* (BC)	2.03 ± 0.06	1.01	1985.34	4.67	1.14	HG798956
Sousse: Bouficha, *Khaldi 5* (BC)	2.05 ± 0.01	1.03	2004.90	4.63	2.47	HG798957
Tunis: Beja road, *Khaldi 6* (BC)	2.08 ± 0.04	1.04	2034.24	4.95	1.94	HG798958
El Kef: Bahra, *Khaldi 7* (BC)	2.09 ± 0.04	1.05	2044.02	5.61	2.96	HG798959
Jendouba: Bou Salem, *Khaldi 8* (BC)	2.13 ± 0.05	1.07	2083.14	5.80	2.84	HG798960
Sousse: Masakin, *Khaldi 9* (BC)	2.14 ± 0.05	1.20	2092.92	5.09	2.81	HG798961
El Kef: Tajerouine road, *Khaldi 10* (BC)	3.03 ± 0.03	1.51	2963.34	5.26	1.78	HG798962
• Cultivated						
Artichoke 'Violet d´Hyères', *Khaldi 11* (BC)	2.05 ± 0.03	1.03	2004.90	5.63	1.94	
Cardoon 1, *Khaldi 12* (BC)	2.10 ± 0.04	1.05	2053.80	4.37	2.19	
Cardoon 2, *Khaldi 13* (BC)	2.11 ± 0.03	1.06	2063.58	5.46	2.45	

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Figure 1. Geographical locations of the Tunisian populations of *Cynara cardunculus* L. studied. 1: Enfidha, 2: Oued Mliz, 3: Tourief, 4: Zriba, 5: Bouficha, 6: Beja, 7: Bahra, 8: Bou Salem, 9: Masakin, 10: Tajerouine.

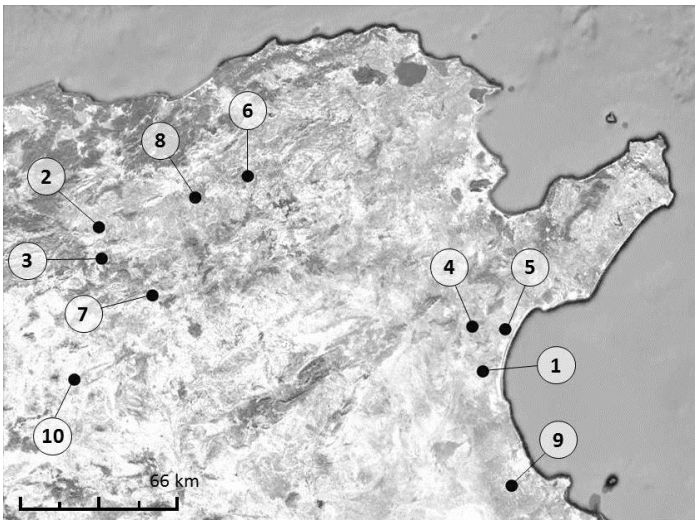

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Figure 2. Somatic metaphases of *Cynara cardunculus* L. showing $2n = 2x = 34$. **A-J.** Wild populations, **(A)** Zriba, **(B)** Bouficha, **(C)** Bahra, **(D)** Bou Salem, **(E)** Masakin, **(F)** Tajerouine. **G.** Cultivated cardoon 1. Scale bar: 10 µm.

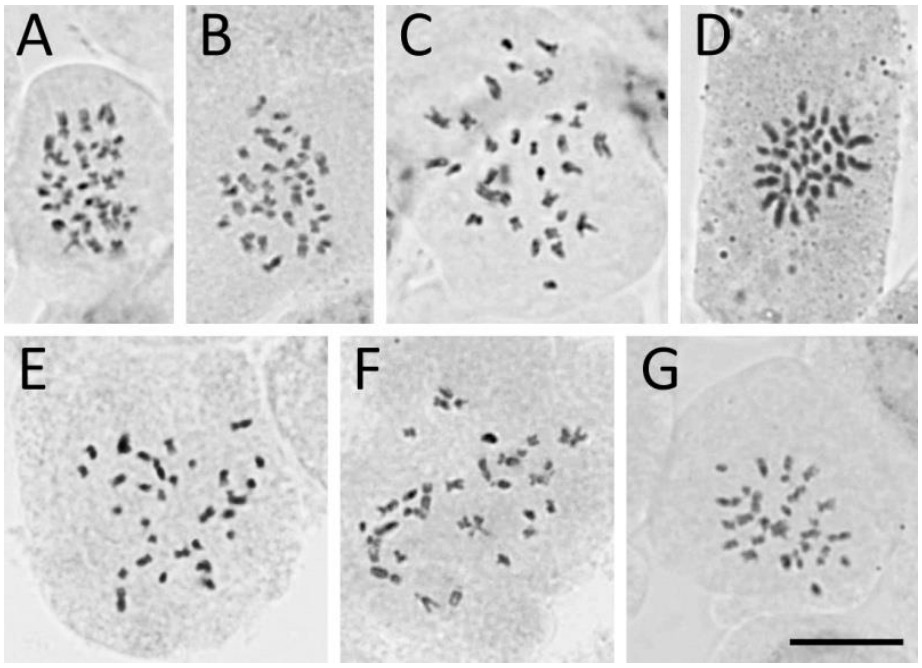

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Figure 3. Phylogenetic reconstructions based on ITS sequences. Accession numbers are provided for the sequences gathered from GenBank. **(A)** Bayesian inference applied to a dataset including *Cynara* species. Posterior probability values are indicated on branches. **(B)** Neighbour-net reticulate network of *C. cardunculus* sequences. Tunisian populations are labelled in grey. Numbers on network branches are bootstrap values. Artichoke (a) and cardoon (b) illustrations are from Coste (1901).