RESEARCH ARTICLE

# Molecular insights on the conflicting generic boundaries in the *Carduncellus-Carthamus* complex (Compositae)

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**Abstract** The *Carthamus-Carduncellus* complex is formed by approximately 50 taxa that are widely distributed throughout the Mediterranean basin and western Asia. The generic delineation of this complex has always been controversial. Currently, there are three widely diverging taxonomical proposals, suggesting that the complex is formed by (1) a single genus, *Carthamus*; (2) two genera, *Carduncellus* and *Carthamus*; and (3) four genera, *Carduncellus*, *Carthamus*, *Femeniasia*, and *Phonus*. The generic classification has varied depending on the importance assigned to some morphological characters, i.e., the morphology/structure of pappus, achenes, and involucral bracts. All these generic changes have complicated the nomenclature of the complex, leading to species wandering between four different genera. To objectively assess the taxonomic delimitation of this complex, we carried out phylogenetic analyses using nuclear (external and internal transcribed spacers of rDNA) and plastid (*ndhF*, *trnH*, *rpl32*, *trnT*) data, as well as multispecies coalescent model analyses on near-complete sampling. Phylogenetic reconstructions resolved two monophyletic groups, *Carthamus* s.str. and *Carduncellus* s.l. Within the latter, some groups could be differentiated, such as the monophyletic genus *Phonus* and the monospecific *Femeniasia*. Our multispecies coalescent analyses strongly support a classification based on four genera in the complex *Carthamus-Carduncellus*. In contrast, classifications based on only one or two genera lack relevant support. However, the absence of synapomorphic morphological characters that define the *Carduncellus* lineage (*Carduncellus*, *Phonus*, *Femeniasia*), and the possible hybridization in the ancestral lineages detected by incongruences between plastid and nuclear markers make it difficult to define clear generic boundaries. We propose maintaining the hypothesis of four genera, which was the first classification supported by molecular evidence, pending a broader study (both molecular and morphological) to reach a more definitive delineation and avoid more unfounded generic changes.

**Keywords** Cardueae; generic delimitation; homoplasy; incongruence; multispecies coalescent model; phylogeny; taxonomy

**Supporting Information** may be found online in the Supporting Information section at the end of the article.

## ■ INTRODUCTION

The classification of living organisms has been a central part of biology since the Linnaean times and provides a framework in which knowledge can be organized (Mishler, 2009; Vences & al., 2013). This organization has multiple purposes, both biologically and practically (herbarium, Floras, GenBank, etc.; Nickrent & al., 2010). Biological classification also permits linking additional information (i.e., it allows to know some attributes of taxa not studied; Stevens, 1985, 1987; Pfeil & Crisp, 2005) and establishes stable boundaries and relationships between taxa (Orthia & al., 2005). However, all biological classifications are human constructs and therefore have a subjective character. There is a broad consensus within the scientific community that the classification of organisms should try to reflect their phylogenetic relationships as closely as possible and be composed of monophyletic clades (Stevens, 1985, 1987; Orthia & al., 2005; Pfeil & Crisp, 2005; Humphreys & Linder, 2009; Nickrent & al., 2010; Vences & al., 2013). However, the concept of monophyly has been subject to intense discussions among scientists (Rieseberg & Brouillet, 1994). Some of them defend the term in the strict sense following Hennig (1966), while others accept or support proposals to delimit taxa that are in fact paraphyletic (Mayr & Bock, 2002).

Changes in the classification, particularly at the generic level, entail significant implications because the generic name is part of the Linnaean binomial specific name (Orthia & al., 2005; Vences & al., 2013). Vences & al. (2013) advocate minimizing taxonomical changes because of the impact of these changes. In addition to monophyly, there is another set

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of recommendations to modify existing classifications and rename taxa, such as clade and nomenclature stability and phenotypic diagnosability (Backlund & Bremer, 1998; Vences & al., 2013). Some authors have also proposed secondary criteria, such as the size of the genera (i.e., suitability of a large or monospecific genus; Humphreys & Linder, 2009) or equivalence between sister taxa (Orthia & al., 2005). Problems arise when one recommendation fails or the priority between them is not clear, i.e., it varies depending on the author, which increases the subjectivity of the generic definition/delimitation. Taxonomical and systematic discrepancies usually rely on the selection of morphological characters (synapomorphies) that allow the separation of different genera (Clayton, 1983). However, some of these discrepancies could originate because the morphological characters are often under multigenic control of nuclear genes with complex patterns of expression and regulation, and they often present high levels of homoplasy (Hörandl & Stuessy, 2010). The genus concept is a complex idea that combines parallel lines (phylogeny vs. morphology) that do not always coincide (Clayton, 1983).

An example of a taxonomic group with historical discrepancies in generic boundaries is the complex *Carthamus-Carduncellus* (Centaureinae-Cardueae, Compositae). This complex comprises about 50 species distributed in the Mediterranean and Irano-Turanian regions, ranging from the Iberian Peninsula and North Africa to western Asia. Many attempts have been made to delineate the genera that are included within the complex, including morphological (Hanelt, 1963; Dittrich, 1969; López González, 1990, 2012; Vilatersana, 2008), karyological (López González, 1990; Vilatersana & al., 2000b), palynological (Vilatersana & al., 2001) and molecular (Vilatersana & al., 2000a; Mihoub & al., 2017) studies.

The tribal and subtribal position of the *Carthamus-Carduncellus* complex was also conflictive in the past, given the presence of plesiomorphic or ancestral characters in the tribe/subtribe, notably, the spinescence of the plants (see Vilatersana & al., 2000a, and references therein). However, recent studies have concluded that the complex belongs to the subtribe Centaureinae and, despite its spinescent habit, it is close to the derived Centaureinae clade (Garcia-Jacas & al., 2001; Susanna & al., 2006; Barres & al., 2013; Herrando-Moraira & The Cardueae Radiations Group, 2019).

Previous molecular studies (Vilatersana & al., 2000a; Mihoub & al., 2017) detected two different lineages usually accepted by most of the authors, with obvious differences in morphology, karyology and biogeography. The first lineage is formed by the genus *Carthamus* L. (hereafter *Carthamus* s.str.) with 18 annual species and the base chromosome numbers $x$ = 10, 11, and 12 (López González, 1990; Vilatersana & al., 2000b), distributed in the Eastern Mediterranean and Irano-Turanian regions, excluding some weedy hybrids such as *Cart. lanatus* L. and *Cart. creticus* L. (Vilatersana & al., 2007). *Carthamus* is a well-studied group (Hanelt, 1963; Knowles, 1980; Vilatersana & al., 2005; Garnatje & al., 2006; Bowles & al., 2010; Agrawal & al., 2013; Tarıkahya-Hacıoğlu & al., 2014) because of the economic importance of *Cart. tinctorius* L. as an oilseed crop, a more affordable substitute for saffron (Dempewolf & al., 2008; Emongor, 2010), as well as for its medicinal properties (Asgarpanah & Kazemivash, 2013). The second lineage, *Carduncellus* (hereafter *Carduncellus* s.l.), comprises approximately 30 perennial species, typically hemicryptophytes and less often chamaephytes, with the base chromosome number $x$ = 12 (López González, 1990; Vilatersana & al., 2000b); it is distributed in the western Mediterranean region, mainly the Iberian Peninsula and North Africa.

The morphological characters most frequently used in the classification of the group are achene anatomy, morphology/structure of the pappus, appendage of the involucral bracts, and life form (Table 1). The marked differences among classifications are due to the relative importance assigned to each character by different authors, which leads to the paradoxical wandering of some species among four different genera (suppl. Table S1) (Vilatersana & al., 2000a). A summary of the troubled history of generic classifications and delimitations is further detailed in suppl. Table S1.

Historically, Boissier (1875) and other French botanists (Battandier, 1890; Jahandiez & Maire, 1934; Quézel & Santa, 1963) divided the complex into two genera, *Carthamus* and *Carduncellus* Adans., but with the peculiarity that they included within *Carthamus* a group of species described as *Lamottea* by Pomel (1860) (suppl. Table S1) on the basis of carpological characters. Later on, Hanelt (1963), in an extensive morphological study, divided the complex also in two genera, but he included *Lamottea* species within the genus *Carduncellus* due to the presence of appendages in the involucral bracts (suppl. Table S1). Next, López González (1990) based his classification mainly on the anatomy of the pericarp and the structure of the pappus. His classification recognized four genera, *Carthamus*, *Carduncellus*, *Lamottea* and *Phonus* Hill. *Lamottea* was segregated as an independent genus from *Carduncellus*, and *Phonus* was promoted to genus from *Carthamus* sect. *Thamnacanthus* (DC.) Šostak together with two species of *Carduncellus* with undifferentiated pericarp, namely *Card. fruticosus* (Maire) Hanelt and *Card. mareoticus* (Delile) Hanelt (suppl. Table S1). Vilatersana & al. (2000a) performed the first molecular study of the complex and showed that *Lamottea* do not form a monophyletic clade that could be segregated from *Carduncellus* and that *Phonus* (= *Carthamus* sect. *Thamnacanthus*) is a lineage separate from *Carthamus* and sister of a monotypic genus, *Femeniasia* (Susanna, 1988). Given the difficulties of morphologically separating genera in the complex, Greuter (2003) resorted to the idea that the complex is formed by a single genus, *Carthamus*, as pointed out by Linnaeus (1753, 1763). Finally, López González (2012) recognized two genera (suppl. Table S1) – *Carthamus* and *Carduncellus*. According to that study, *Carthamus* includes annual species, and is well defined carpologically and biogeographically, coinciding with Vilatersana & al. (2000a); *Carduncellus* comprises perennial species and is morphologically more divergent because it includes all members of the *Carduncellus* s.l. lineage (*Carduncellus* s.str., *Phonus*, *Femeniasia*).

In this moment, the last three conflicting and widely divergent generic delineations in the group coexist. The difficulties in achieving a widely accepted classification stress the need for a more comprehensive approach that should be used in the context of evolutionary patterns in the complex. In this study, we used sequences of two nuclear and four plastid regions analyzed rigorously aiming to (1) investigate the phylogenetic relationships in the *Carthamus-Carduncellus* complex, and (2) re-evaluate the taxonomic treatment based on our molecular analyses and morphological data.

## ■ MATERIALS AND METHODS

**Taxon sampling. —** Sampling was designed to include 60 accessions of 49 taxa from the *Carthamus-Carduncellus* complex, representing approximately 94% of the recognized species in the complex. These account for all the species of the complex except for three North African *Carduncellus* endemic species, namely *Card. chouletteanus* (Pomel) Batt., *Card. ilicifolius* Pomel, and *Card. multifidus* (Desf.) Coss., which we could not obtain. Also, five outgroups were selected (*Centaurea cyanus* L., *Cent. scabiosa* L., *Phalacrachena inuloides* (Fisch.) Iljin, *Psephellus persicus* (DC.) Wagenitz, *Volutaria crupinoides* (Desf.) Maire), in accordance with Garcia-Jacas & al. (2001) and Barres & al. (2013). The initial generic delimitation was based on Vilatersana & al. (2000a). Voucher data, source of sampled material, and GenBank accession numbers are provided in Appendix 1.

**DNA extraction, amplification, and sequencing. —** The total genomic DNA was extracted from silica-dried leaves collected in the field or from herbarium specimens following the CTAB method, as stated by Doyle & Dickson (1987) and modified by Cullings (1992).

We sequenced two nuclear regions, the nrITS1 + nrITS2 (hereafter ITS) and nrETS spacers. We added four plastid DNA regions: *5′trnT-trnL* (hereafter *trnT*), *rpl32-trnL*$^{(UAG)}$ (hereafter *rpl32*), and *trnH-psbA* (hereafter *trnH*) intergenic spacers, and the 3′ end portion of the *ndhF* encoding region (hereafter *ndhF*). For 21 samples (18 *Carduncellus* and 3 *Carthamus* accessions, marked in Appendix 1), cloning of the ETS polymerase chain reaction (PCR) products was necessary because of the presence of double bands. The PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, California, U.S.A.) following the manufacturer's instructions and the protocol described by Vilatersana & al. (2007). The primers used and detailed PCR profiles are listed in Table 2. The *trnH* intergenic spacer presented a 25 bp inversion in the two *Phonus* species. To avoid over- or underestimation of phylogenetic information, we replaced the inversion with its reverse complement to maximize homology (Whitlock & al., 2010).

All PCR reactions were performed in 25 µl volumes with 10% 10× AmpliTaq buffer, 10% 25 mM $MgCl_2$, 10% of

**Table 1.** Summary of morphological, karyological and habitat characters employed in generic delimitation of *Carthamus-Carduncellus* complex.

| Taxon | Habit | Karyology | Polyploidy | Habitat | Distribution | Pericarp differentiated | Bolster cell in apical plate cypsela | Pappus | Appendage cucullate middle bractees |
|---|---|---|---|---|---|---|---|---|---|
| *Carthamus* | Therophytes | | | Open/ anthropogenic areas | | Yes | | Paleaceous/ persistent/ absent in external cypselas | No |
| sect. *Atractylis* | | $x = 10, 11, 12$ | Yes | | Eastern Mediterranean | | Yes | | |
| sect. *Carthamus* | | $x = 12$ | No | | Irano-Turanian | | Rudimentary | | |
| *Carduncellus* | Hemicryptophytes | $x = 12$ | Yes | Natural areas | Iberian Peninsula-North Africa | Yes | No | Linear/persistent or deciduous with basal ring | Yes/no |
| *Card. fruticosus* | Chamaephyte | | No | | North Africa | No | | Linear/persistent | No |
| *Card. mareoticus* | Chamaephyte | | No | | North Africa | No | | Linear/deciduous with basal ring | No |
| *Femeniasia* | Chamaephyte | $x = 12$ | No | Natural areas | Balearic Islands | No | No | Linear/deciduous with basal ring | No |
| *Phonus* | Chamaephytes | $x = 12$ | No | Natural/ open areas | Iberian Peninsula-North Africa | No | No | Linear/deciduous, no basal ring | No |

2 mM dNTP mix, 4% of each primer at 5 µM, 1 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California, U.S.A.), and 2 µl of template DNA of an unknown concentration. For the amplification of the plastid regions, 1 µl of 400 ng/µl BSA (New England Biolabs, Ipswich, Massachusetts, U.S.A.) was added to the reaction, while 0.5 µl DMSO (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) was added for the amplification of nuclear regions. A total volume of 25 µl was filled with distilled water.

The PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio, U.S.A.). Sequencing of the amplified DNA segments was performed using BigDye Terminator Cycle Sequencing v.3.1 (Applied Biosystems), following the manufacturer's recommended protocol. Nucleotide sequencing was carried out at the University of Florida ICBR Core Facility on an ABI 3730xl capillary sequencer (Applied Biosystems).

**Phylogenetic, recombination, and hybridization analyses. —** Nucleotide sequences were edited using BioEdit v.7.0.0 (Hall, 1999) with subsequent visual inspection and manual revision. The more variable 5′ ETS end was excluded from this analysis because of difficulties in aligning it with the outgroups. Indels were treated as missing data after sequence alignment (alignment files available as suppl. Appendices S1–S6). Phylogenetic analyses were performed with the following datasets: Dataset one consisted of the concatenated ITS plus the conserved 3′ ETS (hereafter ETS) regions. Dataset two used concatenated plastid DNA (*ndhF*, *trnH*, *rpl32*, *trnT*). The details of the datasets and analyses are presented in Table 3 and the individual phylogenetic trees for each region are available in suppl. Fig. S1.

Plastid and nrDNA regions were analyzed separately because of phylogenetic incongruences that were found between both datasets (see Results). Phylogenetic analyses of both datasets were performed using the Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP) methods. The BI analyses were implemented in MrBayes v.3.2.6 (Ronquist & al., 2012). The best-fit model of nucleotide substitution for each region for the BI analyses was evaluated with jModelTest v.0.1 (Posada, 2008) using the Akaike information criterion (Table 3). Two simultaneous and independent analyses of four Metropolis-coupled Markov chain Monte Carlo simulations were run for $10 \times 10^6$ generations and sampled every 1000 generations. After checking the convergence of the runs and ensuring that the effective sample

**Table 2.** Primers used in each DNA region in the study of the *Carthamus-Carduncellus* complex.

| Region | Primer name | Source or sequence (5′–3′) | Annealing temperature (°C) |
|---|---|---|---|
| nrITS | 1406F | Nickrent & al. (1994) | 55°C(1) |
| | ITS4 | White & al. (1990) | |
| | ITS1 | White & al. (1990) | |
| | 17SE | Sun & al. (1994) | |
| | 26SE | Sun & al. (1994) | |
| | ITS2 | White & al. (1990) | |
| | ITS3 | White & al. (1990) | |
| | 307R | Soltis & Kuzoff (1995) | |
| nrETS | ETS1f | Linder & al. (2000) | 48°C(2) |
| | 18S-2L | Linder & al. (2000) | |
| | 18S-ETS | Baldwin & Markos (1998) | |
| | *ETS-Car1 | TTCGTATCGTTCGGT | |
| Cloning | T7 | TOPO TA Cloning kit manual | 55°C(3) |
| | M13R | TOPO TA Cloning kit manual | |
| *trnH-psbA* | trnH(GUG) | Hamilton (1999) | 53°C(4) |
| | psbAR | Hamilton (1999) | |
| *5′trnT-trnL* | trnA2 | Cronn & al. (2002) | 52°C(5) |
| | trnL-b | Taberlet & al. (1991) | |
| | *rps4G-F | GGAACGCGATTGGTTTCTAAG | |
| | *trnT-B2R | AGCCTGCTTAGCTCAGAGGTT | |
| *rpl32-trnL*(UAG) | rpl32F | Shaw & al. (2007) | 54°C(6) |
| | trnL(UAG) | Shaw & al. (2007) | |
| *ndhF* (3′ end) | 3′F | Eldenäs & al. (1999) | 46°C(7) |
| | 1783R | Barres & al. (2013) | |
| | 1626F | Barres & al. (2013) | |
| | +607 | Kim & Jansen (1995) | |

Primer names, source, and annealing temperature (°C) are included for each primer. The asterisk (*) denotes the new primers designed in this study. PCR conditions following (1) Garcia-Jacas & al. (2006), (2) Sanz & al. (2008), (3) Vilatersana & al. (2007), (4) Hamilton (1999), (5) López-Vinyallonga & al. (2009), (6) Hilpold & al. (2011), (7) Barres & al. (2013).

size was higher than 200 using Tracer v.1.6.0 (available at http://beast.bio.ed.ac.uk/Tracer), data from the first 25% generations were discarded as the burn-in period. The 50% majority-rule consensus phylogeny and Bayesian posterior probability (BPP) of nodes were calculated from the remaining samples. Nodes with a BPP ≥ 0.95 were considered significantly supported.

The ML analyses were performed in RAxML v.7.2.8 (Stamatakis, 2006) using raxmlGUI v.1.5b2 (Silvestro & Michalak, 2012); the general time-reversible model with gamma-distributed rate heterogeneity (GTRGAMMA) was applied for nuclear analyses and GTRGAMMAI was applied for the plastid DNA dataset following the results of each individual region (Table 3). All ML analyses were conducted with 100 random addition replicates and 1000 bootstrap replicates (BS, Felsenstein, 1985) for each concatenated dataset. Nodes with a $BS_{ML}$ ≥ 75% were considered significantly supported. The MP analyses were conducted with PAUP* v.4.0a150 (Swofford, 2003) using a heuristic search with 1000 replicates of random taxon addition with MulTrees in effect and tree-bisection-reconnection (TBR) branch swapping. The most parsimonious trees were saved. The parsimony-uninformative positions were excluded from the analysis. After computing the strict consensus tree, BS analyses were performed in accordance with Lidén & al. (1997), using 1000 replicates of heuristic search, random taxon addition with 10 replicates per replicate, and no branch swapping. Nodes with $BS_{MP}$ ≥ 75% were considered significantly supported. The consistency index (CI) and retention index (RI) were calculated excluding uninformative characters (Table 3). Trees of all the phylogenetic analyses were visualized with FigTree v.1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree).

Evidence for recombination in the nuclear sequences (excluding outgroups) was checked using the RDP4 v.4.100 package (Martin & al., 2015) following the default settings with *P*-values of 0.05 and Bonferroni correction. Screening was performed using eight methods included in the package (RDP, GENECONV, MaxChi, Chimaera, BootScan, SiScan, Phylpro, 3Seq). Only the results supported by at least two methods were accepted.

To identify contradictory signals and detect the main spots of incongruence among tree topologies, we performed a consensus network (Holland & al., 2004) under method RECOMB2007 with SplitsTree4 v.4.15.1 (Huson & Bryant, 2006) from the BI 50% majority-rule consensus tree of each dataset (nuclear and plastid) from each independent lineage. Branches of the topologies with BPP < 0.90 support were collapsed in the input trees to reduce phylogenetic noise. We excluded from the analyses species of suspected hybrid origin, some of which showed divergent sequences in previous analyses, with the final purpose of reducing the complexity of the network. In *Carduncellus* s.l., we excluded the lineage of *Card. hispanicus* Boiss. ex DC. subsp. *intercedens* (Degen & Hervier) G.López, *Card. monspelliensium* All., and *Card. pectinatus* DC., whereas in *Carthamus* s.str., we excluded the allopolyploid lineage of *Cart. creticus* and *Cart. turkestanicus* Popov (Vilatersana & al., 2007).

**Generic delimitation procedures. —** The most likely hypotheses of generic boundaries were inferred by

**Table 3.** Dataset information and tree parameters of the phylogenetic and generic delimitation analyses.

| | Dataset one: nrDNA | Dataset two: plastid DNA | Dataset three-A: Generic delimitation | Dataset three-B: Generic delimitation |
|---|---|---|---|---|
| No. of taxa ingroup (outgroup) | 49 (5) | 49 (5) | 48 (2) | 48 (2) |
| No. of sequences | 70 | 65 | 51 | 51 |
| Regions | ETS / ITS | *ndhF* / *trnH* / *rpl32* / *trnT* | ETS / ITS | ETS / ITS / *ndhF* / *trnH* / *rpl32* / *trnT* |
| Parsimony analysis | | | | |
| No. of characters (individual regions) | 1030 (551 / 479) | 3235 (1338 / 426 / 910 / 561) | 1027 (550 / 477) | 4216 (550 / 477 / 1335 / 425 / 899 / 530) |
| Parsimony-informative characters | 194 | 96 | – | – |
| No. of most parsimonious trees | 219,516 | 731 | – | – |
| Tree length | 462 | 159 | – | – |
| Consistency index | 0.536 | 0.622 | – | – |
| Retention index | 0.855 | 0.889 | – | – |
| Homoplasy index | 0.464 | 0.379 | – | – |
| Bayesian inference | | | | |
| Models of molecular evolution | GTR+Γ / SYM+Γ | GTR+Γ+I / GTR+Γ+I / GTR+Γ / HKY+Γ | GTR+Γ / SYM+Γ | GTR+Γ / SYM+Γ/ GTR+Γ+I / GTR+Γ+I / GTR+Γ / HKY+Γ |

comparing the marginal likelihood using the Bayes factor delimitation (BFD) approach on five genera delimitation scenarios (Table 4) following Grummer & al. (2014). This method compares objectively a species tree model or hypotheses in which sequences are assigned a priori to a different number of lineages, between one and four genera, depending on each hypothesis (Table 4). We generated two new datasets to perform these analyses. Dataset three-A included only nuclear data (ETS, ITS), and dataset three-B included all six loci (two nuclear and four plastid DNA data; Table 3). Both datasets included only one accession of each taxon, except *Femeniasia balearica* (J.J.Rodr.) Susanna, for which we included two accessions owing to the software requirement of multiple samples per lineage (Heled & Drummond, 2010).

For each one of the five hypotheses (generic delimitation scenarios; Table 4), we performed a Bayesian reconstruction of the species tree using *BEAST (Heled & Drummond, 2010) following the multispecies coalescent (MSC) model included in the BEAST v.1.8.3 package (Drummond & Rambaut, 2007). *BEAST analyses were performed following the conditions of Grummer & al. (2014) and Hotaling & al. (2016). In all analyses, we included two species of the sister genus *Centaurea* (*Cent. cyanus*, *Cent. lingulata* Lag.) because *BEAST requires at least two lineages. Models of sequence evolution were applied to each locus as described above (Table 3). A gene tree was constructed using a relaxed uncorrelated lognormal clock and a birth-death model for the species tree prior. The population size model was set to piecewise linear and constant root, and we allowed differences in ploidy between plastid and nuclear genomes. For each hypothesis, four replicates of *BEAST analyses using different random starting trees were performed by applying $200 \times 10^6$ generations sampling every 5000 generations for dataset three-B and $50 \times 10^6$ generations sampling every 1000 generations for dataset three-A via the CIPRES Science Gateway v.3.3 (Miller & al., 2010). The convergence of the analyses was determined using the Tracer program. Each replicate run was combined using LogCombiner v.1.8.3 (part of the BEAST package) after discarding the first 25% of the trees from each run as burn-in. Finally, the genera tree was constructed using TreeAnnotator v.1.8.3 (part of the BEAST package). To visualize the results of our *BEAST analyses, we generated cloudograms using the program DensiTree v.2.2.1 (Bouckaert & Heled, 2014).

Marginal likelihood estimators (MLEs) were estimated using the path sampling (PS; Lartillot & Philippe, 2006) and stepping-stone (SS; Xie & al., 2011) methods with 100 path steps, a chain length of $10^6$ generations and likelihoods saved every 1000 generations. We averaged the MLEs across runs to generate a single PS and SS and calculated the Bayes factors (BFs) using the modification introduced by Kass & Raftery (1995) (i.e., twice the difference between the ln harmonic mean likelihoods of the two models). Values for 2lnBF greater than 2, 6, and 10 indicate positive, strong, and decisive support, respectively, for the generic hypothesis with a minor marginal likelihood.

## ■ RESULTS

**Phylogenetic analyses and cytonuclear incongruences. —** The phylogenetic reconstruction using the concatenated nrDNA regions (ETS, ITS) is summarized in Fig. 1 and Table 3. The three analyses developed with different algorithms (BI, ML, MP) were highly concordant. The nuclear phylogeny corroborated previous studies (e.g., Vilatersana & al., 2000a; Mihoub & al., 2017), but the branches were more supported; one relevant result is the recovered monophyly of the complex *Carthamus-Carduncellus* even though it was only supported by BI (BPP = 0.98). *Carduncellus* s.l. (which includes *Carduncellus* s.str. + *Femeniasia* + *Phonus*) showed high support values (BPP = 1, $BS_{ML}$ = 95 and $BS_{MP}$ = 88). The clade formed by

**Table 4.** The five generic classifications tested using the Bayes factor delimitation (BFD).

| Hypothesis | Generic delimitation | N | Author/based on |
|---|---|---|---|
| H1 | ***Carthamus*** (= *Carduncellus* + *Carthamus* + *Femeniasia* + *Phonus*) | 1 | Greuter (2003) |
| H2 | ***Carthamus***<br>***Carduncellus*** (= *Carduncellus* + *Femeniasia* + *Phonus*) | 2 | López González (2012) |
| H3 | ***Carthamus***<br>***Carduncellus***<br>***Phonus*** (= *Femeniasia* + *Phonus*) | 3 | Petit & al. (2001) (palynological data) |
| H4 | ***Carthamus***<br>***Carduncellus***<br>***Femeniasia***<br>***Phonus*** | 4 | Vilatersana & al. (2000a) |
| H5 | ***Carthamus***<br>***Carduncellus*** (= *Carduncellus* + *Phonus*)<br>***Femeniasia*** | 3 | Plastid DNA results (this paper) |

The genera included in each hypothesis are shown in bold, and those following Vilatersana & al. (2000a) when the generic treatment was not equivalent are in parenthesis. In all hypotheses, two accessions of *Centaurea* were used as an outgroup. N = number of genera tested.

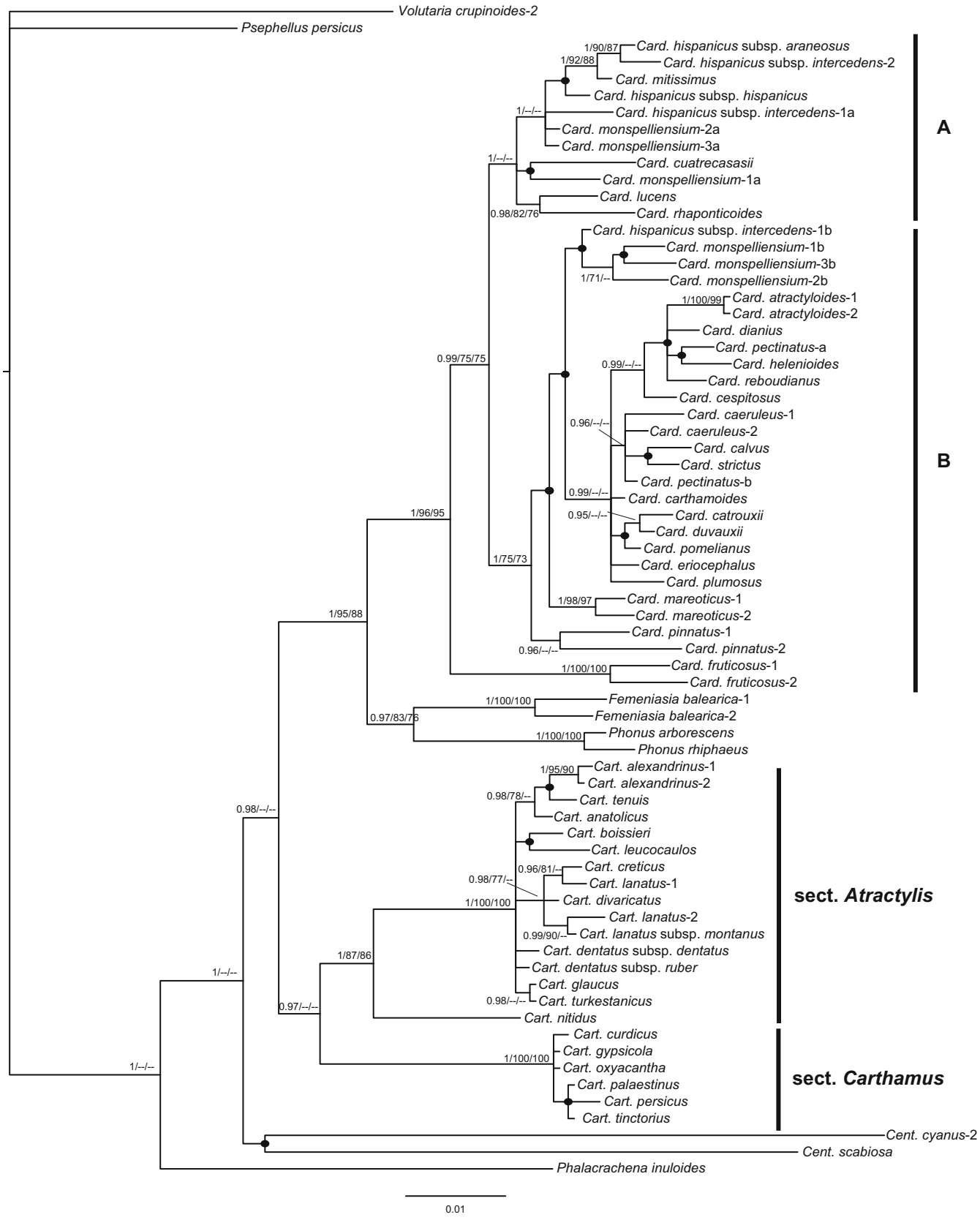


**Fig. 1.** Phylogram inferred from two concatenated nrDNA regions (ETS, ITS). Numbers associated with each node indicate Bayesian posterior probabilities (BPP)/maximum likelihood (ML)/maximum parsimony (MP) bootstrap (BS) values. Only BPP ≥ 0.95 and BS ≥ 70% are shown. Black dots indicate no significant support for the node. The length of the branches follows the BI phylogram.

*Phonus* plus *Femeniasia* (BPP = 0.97, $BS_{ML}$ = 83 and $BS_{MP}$ = 76) was sister to the *Carduncellus* genus (BPP = 1, $BS_{ML}$ = 96 and $BS_{MP}$ = 95). In the *Carduncellus* genus, *Card. fruticosus* was sister to the rest of the *Carduncellus* species. *Carduncellus* was divided into two subclades that mainly follow geographical distribution; specifically, clades B and A are found in North Africa and the Iberian Peninsula, respectively (Fig. 1). The clade formed by *Carthamus* was supported only by BI (BPP = 0.97). This clade was subdivided into two highly supported subclades, which correspond to *Cart*. sect. *Carthamus* (BPP = 1, $BS_{ML}$ and $BS_{MP}$ = 100) and sect. *Atractylis* Rchb. (BPP = 1, $BS_{ML}$ = 0.87 and $BS_{MP}$ = 0.86; Fig. 1).

The phylogenetic reconstruction using the four concatenated plastid regions (*ndhF*, *rpl32*, *trnH*, *trnT*) is presented in Fig. 2, and the results are summarized in Table 3. The plastid phylogeny recovered the *Carthamus-Carduncellus* complex as monophyletic (BPP = 1, $BS_{ML}$ = 98 and $BS_{MP}$ = 90), but there are many important incongruences between the plastid and nuclear phylogenies, mainly at later diverging lineages (i.e., species level; Figs. 1, 2). At a specific level, the hybridization networks showed widespread cytonuclear discordance, especially in *Carduncellus* (Fig. 3B), where a complex network was detected in the core of *Carduncellus* s.str. In contrast, *Carthamus* s.str. presents only incongruence at specific level between the species of the southern Levant zone (*Cart. alexandrinus* (Boiss. & Heldr.) Bomm., *Cart. anatolicus* (Boiss.) Sam., *Cart. nitidus* Boiss., and *Cart. tenuis* (Boiss. & C.I.Blanche) Bornm.; Fig. 3A), and between *Cart. divaricatus* Bég. & Vaccari and *Cart. lanatus*. However, two relevant incongruences between plastid and nuclear markers were detected at the supraspecific level. One was located in the *Carduncellus* s.l. clade, where the two species of *Phonus* are included in the *Carduncellus* clade with high support in all the phylogenetic analyses (BPP = 1, $BS_{ML}$ = 78 and $BS_{MP}$ = 79, Fig. 2), and it was represented as a network between the early diverging taxa (*Femeniasia*, *Phonus*, *Card. fruticosus*) of the lineage in the hybridization network (Fig. 3B). The second one was found in the *Carthamus* genus, where the two accessions of *Cart. alexandrinus* were included in the clade formed by the species of *Cart*. sect. *Carthamus*, instead of sect. *Atractylis*, with high support in the plastid gene tree (BPP = 1, $BS_{ML}$ and $BS_{MP}$ = 100; Fig. 2); this was also detected in the hybridization network (Fig. 3A).

Three putative recombination events were identified (Table 5), all of which were located in the ETS region. The first evolved from the two sequences of *Carduncellus fruticosus* and an unknown sequence as the minor parent. The second was related to the two clones of *Card. monspelliensium* (population 1) and *Card. calvus* Boiss. & Reut. as the minor parent. A third event pointed to *Cart. nitidus* as the recombinant sequence, and the major and minor parents were *Cart. tenuis* and *Cart. persicus* Desf. ex Willd., respectively.

**Generic delimitation analyses. —** The better result of the BFD using both nuclear datasets and plastid and nuclear datasets corresponds to hypothesis H4, which recognizes four genera: *Carduncellus*, *Carthamus*, *Femeniasia*, and *Phonus*, as suggested by Vilatersana & al. (2000a). However, in the nuclear dataset, the BF results were not significant when H4 was compared to H3 and H5 (Tables 4 and 6). In any case, the BFD strongly rejects hypotheses H1 (all species of the complex belong in only one genus, *Carthamus*; Greuter, 2003) (Table 4) and H2 (only two genera are recognized that correspond to the *Carduncellus* s.l. lineage and *Carthamus* genus; López González, 2012) (Table 4). The best genera trees in both datasets and the respective cloudograms are shown in Fig. 4.

## ■ DISCUSSION

**Phylogenetic and hybridization analyses of the *Carthamus-Carduncellus* complex. —** We present a more comprehensive molecular phylogenetic hypothesis obtained for the *Carthamus-Carduncellus* complex. The present study, including 94% of all the taxa, retrieved a similar topology to earlier phylogenies (e.g., Vilatersana & al., 2000a). We identified two well-supported clades in the nuclear and plastid gene trees that recovered the species of *Carthamus* s.str. and *Carduncellus* s.l. (*Carduncellus* s.str. + *Femeniasia* + *Phonus*), respectively, although support values tended to decrease towards the later diverging lineages, especially at close species relationships. The monophyly of the *Carthamus-Carduncellus* complex has been questioned previously (Vilatersana & al., 2000a), however it is confirmed using the concatenated nuclear and plastid DNA markers (Figs. 1, 2). The close relationship between both lineages is clear based on morphological characters, especially because they share some exclusive characters considered plesiomorphic or ancestral in the modern Centaureinae: spiny habit, ecaveate pollen, long stigmas, and absence of outer specialized flowers in the capitulum (Garcia-Jacas & al., 2001; Vilatersana & al., 2001; Susanna & Garcia-Jacas, 2009). The more conflicting species in *Carduncellus* s.str. are *Card. fruticosus* and *Card. mareoticus*, which have been considered the earliest diverging species within the genus (Hanelt, 1963; López González, 1990; Vilatersana & al., 2000a) owing to some unusual morphological characters in the genera (e.g., prickly shrublet habit and undifferentiated pericarp). Our current gene trees confirm that *Card. fruticosus* is a sister species to the core of *Carduncellus* s.str. However, *Card. mareoticus* is not the successive sister species, as has been suggested in previous molecular analyses (Vilatersana & al., 2000a). In our analyses, it is included in a more internal polytomy that contains a large number of *Carduncellus* species in both gene trees (nuclear and plastid; Figs. 1, 2). This supports the previous idea that the "primitive" morphological characters present in the complex have appeared several times as reversals and are presumably related to extreme aridity, and this reversion occurred in *Card. mareoticus*.

An important aspect of our analyses is the lack of concordance between the nrDNA and cpDNA gene trees (Figs. 1, 2). Similar patterns have also been detected in other groups in the Cardueae (e.g., *Centaurea*, Hilpold & al., 2014; *Cheirolophus*, Vitales & al., 2014; *Rhaponticoides*, Bozkurt & al., in

prep.; *Volutaria*, Calleja & al., 2016). Several processes could generate disagreement between gene trees, such as technical causes (e.g., sequence assembly or taxon sampling) or more often the effect of two main evolutionary processes: hybridization/introgression and persistence of ance stral polymorphisms via incomplete lineage sorting (ILS; Wendel & Doyle, 1998). Any of these could be contemporary or ancient events, and they are not mutually exclusive. Discriminating between both processes might be difficult because both of them leave the same incongruence footprint.

In *Carthamus* s.str., the more obvious incongruence is the position of *Cart. alexandrinus*. In the concatenated cpDNA gene tree (Fig. 2), it belongs to *Cart*. sect. *Carthamus*, whereas in the nuclear gene tree (Fig. 1) it is placed in sect. *Atractylis*; this position is more in accordance with morphological and karyological studies. Currently, no wild *Cart*. sect.

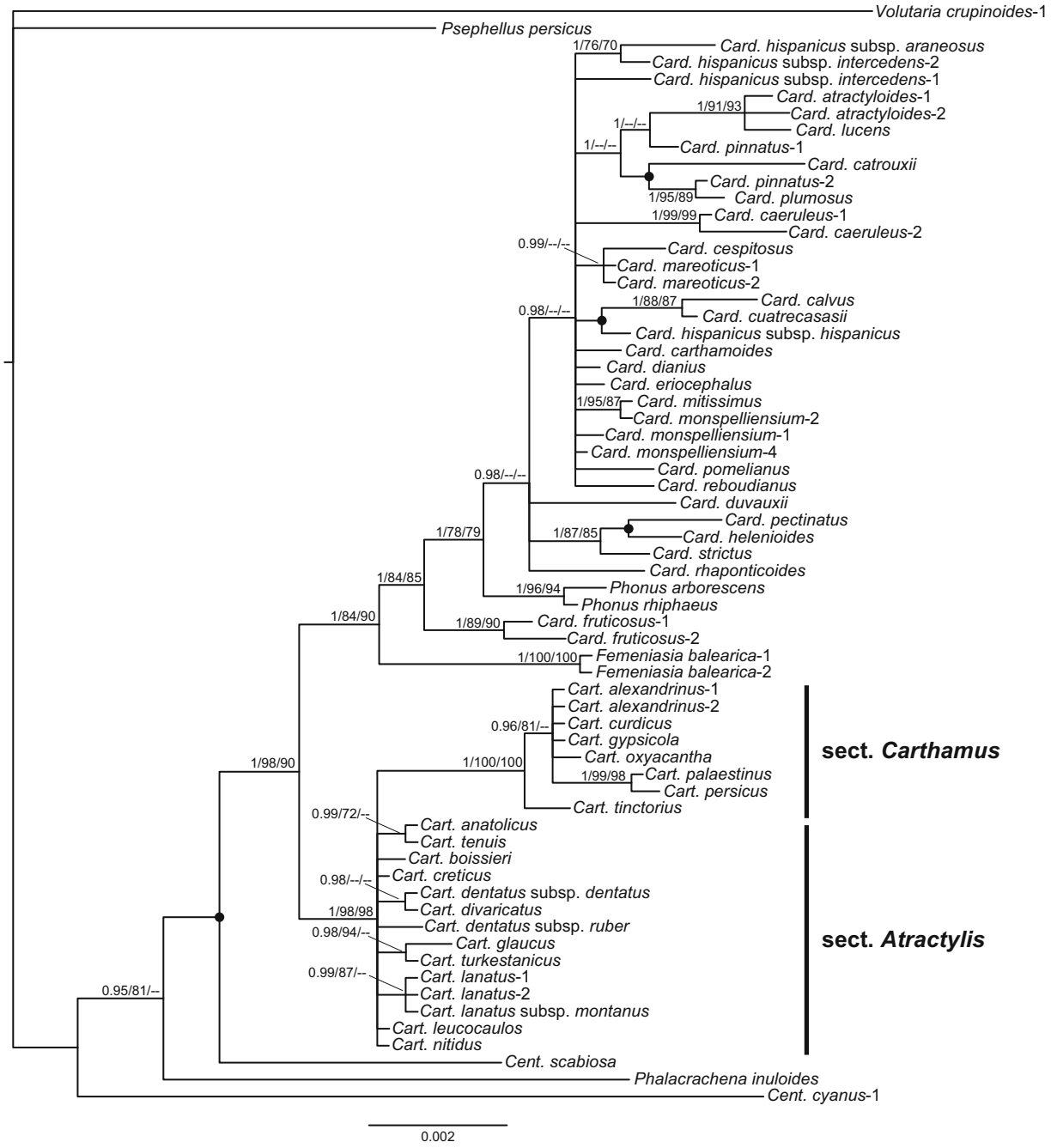


**Fig. 2.** Phylogram inferred from four concatenated plastid DNA regions (*ndhF*, *rpl32*, *trnH*, *trnT*). Numbers associated with each node indicate Bayesian posterior probabilities (BPP)/maximum likelihood (ML)/maximum parsimony (MP) bootstrap (BS) values. Only BPP ≥ 0.95 and BS ≥ 70% are shown. Black dots indicate no significant support for the node. The length of the branches follows the BI phylogram.

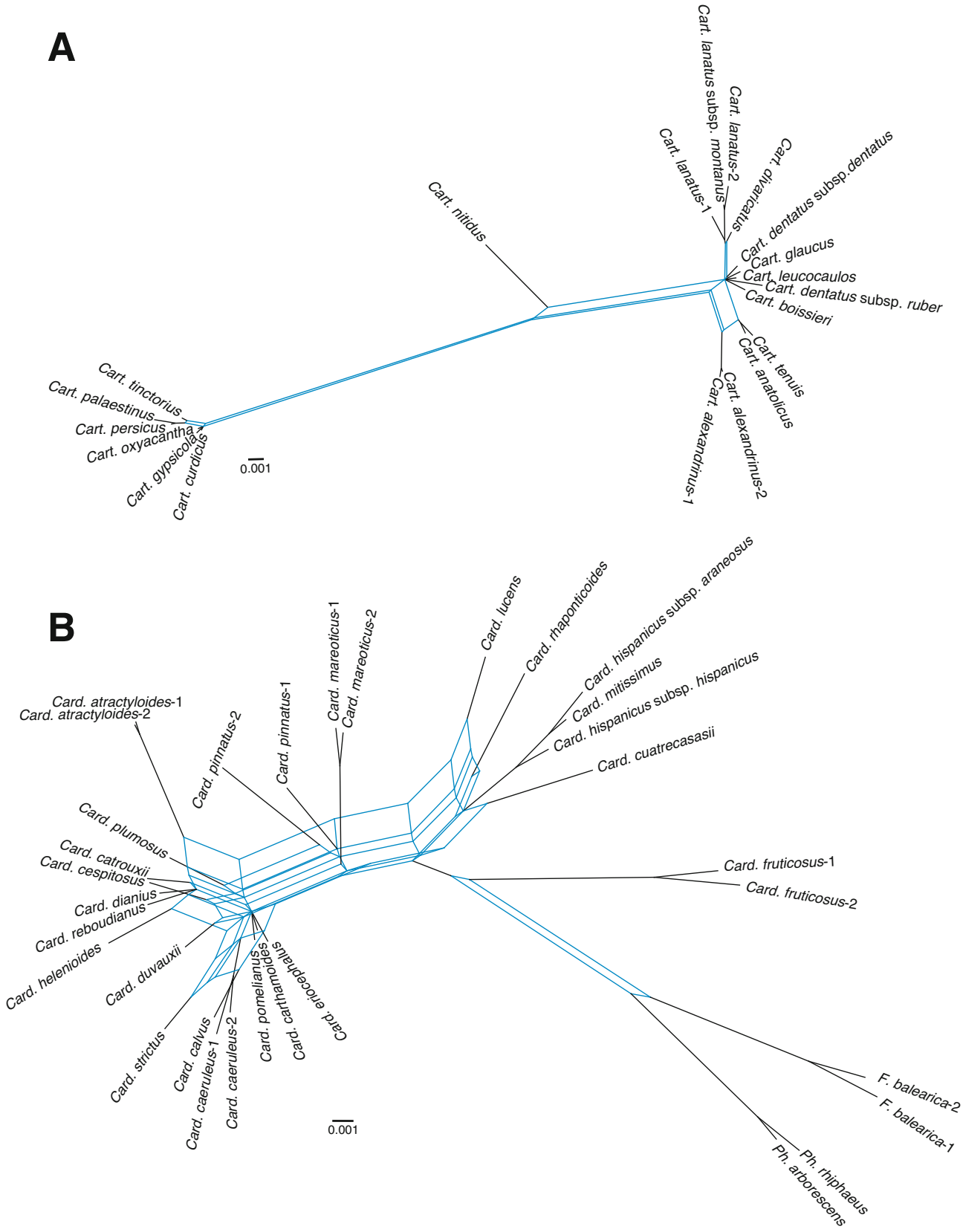


**Fig. 3.** Consensus (hybridization) network obtained from the BI 50% majority-rule consensus tree of the nuclear and cpDNA dataset of the *Carthamus-Carduncellus* complex, excluding allopolyploids generated with SplitsTree4. **A**, *Carthamus* s.str. lineage; **B**, *Carduncellus* s.l. lineage. Incongruences between markers are marked with blue lines.

*Carthamus* live in sympatry with *Cart. alexandrinus* (Alavi, 1983; Boulos, 2002). However, cultivated *Cart. tinctorius* could be the origin of potential introgression with wild relatives (McPherson & al., 2004). This discordance does not corroborate recent hybridization because it is found in samples from the extremes of the current distribution of *Cart. alexandrinus*. The observed pattern would be consistent with an ancient hybridization event or ILS and both hypotheses present similar expectations (Joly & al., 2009). At a specific level, the discordances are in the position of *Cart. divaricatus* and *Cart. nitidus* (Figs. 1, 2, 3A). Both could be reconciled by invoking recent hybridization events between species currently living in sympatry (Ashri, 1961; Vilatersana & al., 2007).

Hybridization in the *Carduncellus* lineage has been poorly evaluated; it has only been identified in some floristic works based on morphological evidence (López González, 1990) or in molecular studies of allopolyploid species (Vilatersana & al., in prep.). The causes of the incongruous cytonuclear position of *Phonus* (Figs. 1, 2) are unknown. We performed phylogenetic analyses on both datasets, excluding one taxon successively (*Card. fruticosus*, *Femeniasia*, or *Phonus*, data not shown) without any changes in the topology of the tree. Therefore, long-branch attraction did not influence the position of *Phonus* in the tree. Accordingly, as in the case of *Cart. alexandrinus*, the pattern detected in *Phonus* could have originated from an ancient hybridization (when the ancestors of the genus were sympatric), but ILS could not be rejected. The extensive discordance between plastid and nuclear phylogenetic trees found in the core *Carduncellus* (Fig. 3B) is compatible with a recurrent pattern of secondary contact induced by the repeat glacial and interglacial cycles of the

**Table 5.** Bonferroni-corrected *P*-values and sequences involved in the three recombination events detected in the nuclear ETS sequences using RDP4.

| Recombination test (methods included in RDP4 v.4.100) | Event 1 (*Carduncellus* s.l.) | | Event 2 (*Carduncellus* s.l.) | | Event 3 (*Carthamus* s.str.) | |
|---|---|---|---|---|---|---|
| | No. of sequences detected | *P*-value | No. of sequences detected | *P*-value | No. of sequences detected | *P*-value |
| RDP | – | – | – | – | 1 | $9.32 \times 10^{-3}$ |
| GENECONV | 1 | $5.54 \times 10^{-4}$ | – | – | – | – |
| BootScan | – | – | – | – | – | – |
| MaxChi | – | – | 1 | $4.21 \times 10^{-2}$ | 1 | $4.53 \times 10^{-2}$ |
| Chimaera | – | – | – | – | 1 | $2.22 \times 10^{-2}$ |
| SiScan | – | – | 1 | $2.27 \times 10^{-2}$ | 1 | $3.57 \times 10^{-5}$ |
| Phylpro | 1 | $1.32 \times 10^{-3}$ | 5 | $2.24 \times 10^{-1}$ | – | – |
| 3Seq | – | – | – | – | – | – |

**Table 6.** Bayes factor delimitation (BFD) results of generic delimitation using coalescent-based model and combined nuclear and plastid data or nuclear data alone.

| | PS | 2lnBF | SS | 2lnBF |
|---|---|---|---|---|
| Nuclear and plastid data | | | | |
| H1 (1 sp.) | −13531.48 ± 3.92 | 448.48 | −13533.63 ± 4.26 | 448.79 |
| H2 (2 sp.) | −13360.70 ± 2.79 | 106.92 | −13362.14 ± 4.43 | 105.82 |
| H3 (3 sp.) | −13321.26 ± 4.03 | 28.04 | −13323.31 ± 3.71 | 28.16 |
| H4 (4 sp.) | **−13307.24 ± 2.66** | – | **−13309.23 ± 2.98** | – |
| H5 (3 sp.) | −13325.36 ± 3.31 | 36.24 | −13327.01 ± 3.37 | 35.56 |
| Nuclear data | | | | |
| H1 (1 sp.) | −5446.38 ± 2.10 | 160.02 | −5446.79 ± 2.15 | 160.12 |
| H2 (2 sp.) | −5406.35 ± 1.82 | 79.96 | −5406.89 ± 1.95 | 80.33 |
| H3 (3 sp.) | −5366.49 ± 1.28 | 0.24[(1)] | −5366.87 ± 1.32 | 0.29[(1)] |
| H4 (4 sp.) | **−5366.37 ± 1.49** | – | **−5366.73 ± 1.54** | – |
| H5 (3 sp.) | −5366.41 ± 2.02 | 0.08[(1)] | −5366.74 ± 2.12 | 0.02[(1)] |

H1–H5 = Hypotheses as in Table 4; PS = Path sampling log marginal likelihood; SS = Stepping-stone log marginal likelihood. Best results are in bold. Bayes factor (BF) values above 10 indicate decisive evidence against a model as compared with the best result.
[(1)] BF values less than 2 indicate no significant support for a hypothesis with lower log marginal likelihood.

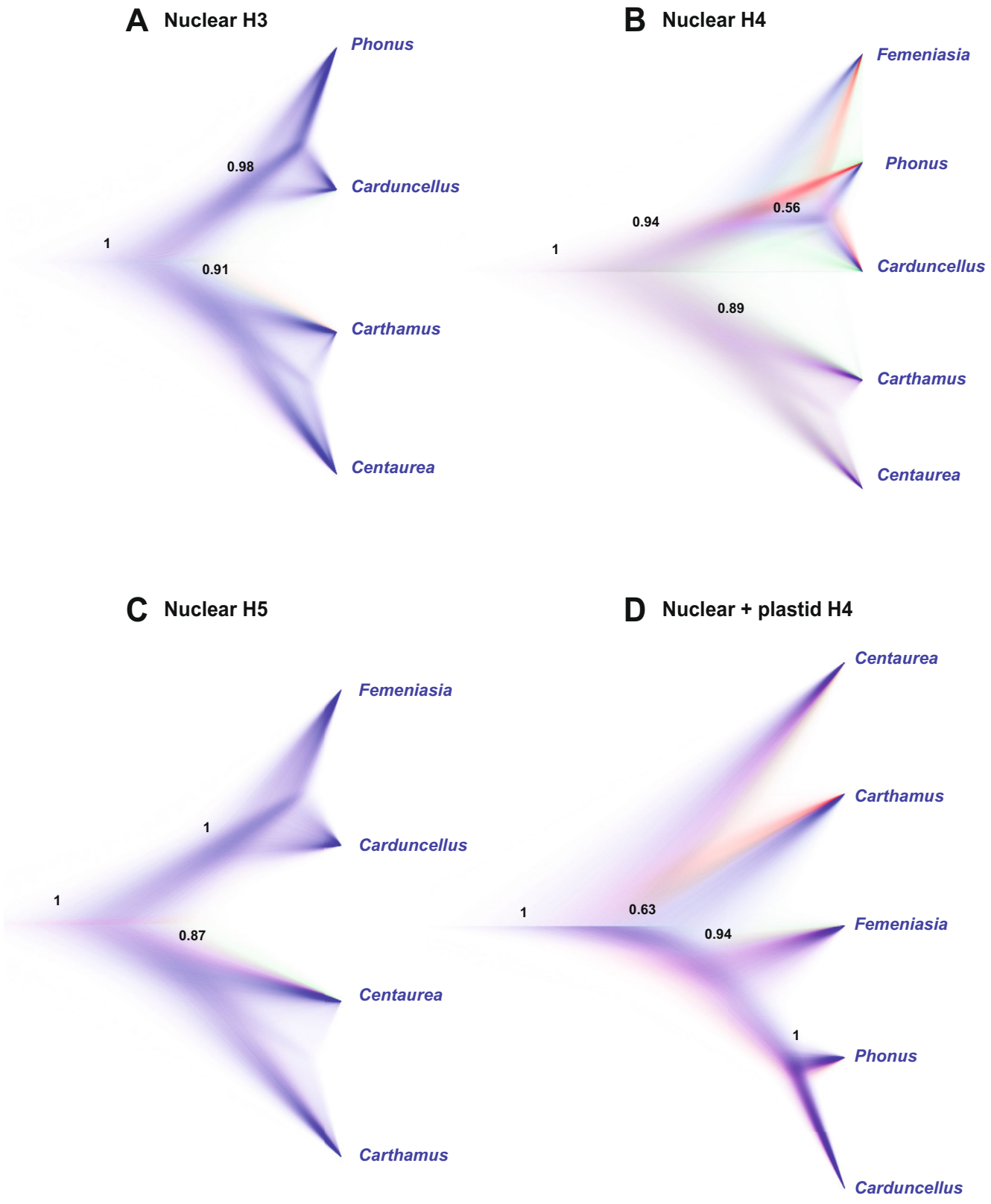


**Fig. 4.** Cloudograms of the better species tree obtained from Bayes factor delimitation (BFD) with *BEAST analyses for the *Carthamus-Carduncellus* complex using DensiTree. The root canal option was selected to highlight the topology of the maximum clade credibility (MCC) tree (in blue), and the next two most frequent topologies are drawn with red and green branches. **A–C**, Nuclear-only dataset results; **D**, Nuclear + plastid dataset results. H3 = hypothesis 3, H4 = hypothesis 4, and H5 = hypothesis 5 (see generic delimitation, hypotheses in Table 4). Numbers at nodes indicate Bayesian posterior probabilities (BPPs).

Pleistocene, which could promote hybridization or introgression between taxa (Thompson, 2005); this pattern is documented in genera close to the complex, such as *Centaurea* (Garcia-Jacas & al., 2009; Ben-Menni Schuler & al., 2019).

Based on our results, the role of recombination as a possible source of discrepancy in our study does not seem relevant, as none of the three recombinant events (Table 5) supports the incongruent topology of our analyses.

**Revised taxonomic treatment of the *Carthamus-Carduncellus* complex. —** The MSC model has been used mainly for the delimitation of species (e.g., Grummer & al., 2014; Hotaling & al., 2016) but has also been used at the supraspecific level (Song & al., 2012; Zhong & al., 2013; Lu & al., 2018). This statistical method objectively distinguishes the structure associated with the data (i.e., lineages), but it does not categorize them taxonomically (Heled & Drummond, 2010; Sukumaran & Knowles, 2017). The MSC model accommodates gene tree heterogeneity resulting from ILS as a potential source of discrepancy and assumes no horizontal gene transfer or admixture between individuals from different lineages or recombination within the loci (Heled & Drummond, 2010). In our study, the hybridization processes occur mainly within each lineage and therefore do not compound the objectives of the present work (i.e., generic delimitation). However, for a correct generic delimitation in the *Carthamus-Carduncellus* complex, the main stumbling block may be a possible ancient hybridization event involving *Femeniasia*, *Phonus*, and *Card. fruticosus*, which are currently allopatric lineages. Comparing the phylogenetic results obtained using concatenation or coalescence approaches, we found differences within the *Carduncellus* s.l. lineage. Especially relevant is the position of *Phonus* with respect to *Femeniasia* and *Carduncellus* s.str. Using a concatenation approach with nuclear regions, *Phonus* is classified as a sister genus to *Femeniasia* (Fig. 1); however, using the coalescent approach, *Phonus* is classified as sister to *Carduncellus* s.str. using nuclear alone and nuclear plus cpDNA datasets (Fig. 4A,D). The latter result would be more in agreement with the isolation of *Femeniasia* from the other species of the *Carduncellus* s.l. lineage. Similar differences have been observed between these methods in other phylogenetic studies (Xi & al., 2014; Herrando-Moraira & The Cardueae Radiations Group, 2018).

Our MSC results strongly support the presence of four lineages that would merit generic rank (*Carthamus*, *Carduncellus*, *Phonus*, *Femeniasia*) using nuclear plus plastid coalescence analyses, but without significant support using only nuclear datasets (ETS, ITS; Table 6). However, the model of a single *Carthamus* lineage (hypothesis H1) presents the lowest support next to the model of two genera, *Carthamus* and *Carduncellus* (hypothesis H2) for both datasets (Tables 4, 6). We will analyze in the following the three generic hypotheses currently in use.

Vilatersana & al. (2000a) (hypothesis H4, Table 4), suggested the first taxonomic classification of the complex based on molecular evidence. It redefined the genus *Carthamus* and divided the *Carduncellus* lineage (*Carduncellus* s.l.) in three genera: *Carduncellus* (*Carduncellus* s.str.), *Femeniasia*, and *Phonus*. At molecular level, this hypothesis is well resolved and is the most supported by our MSC analyses (Table 6). This definition of the genus *Carthamus* is well accepted by specialists in subtribe Centaureinae (e.g., Hellwig, 2004; Susanna & Garcia-Jacas, 2007, 2009). *Carthamus* is characterized by some apomorphic characters exclusive to the complex (see Table 1), such as biogeography (East Mediterranean and Irano-Turanian distribution), karyology (presence of a presumably descending dysploid series from $x = 12$ to $x = 10$), habit and ecological traits (annual plants mainly in human-disturbed habitats), and morphological and anatomical traits of achene and pappus, such as the presence of bolster cells in the apical plate (only rudimentary in *Cart*. sect. *Carthamus*), heterocarpy (pappus absent in the peripheral achenes), and presence of a double persistent pappus with paleaceous setae (Hanelt, 1963; Dittrich, 1969; López González, 1990, 2012; Vilatersana, 2008).

The partition of the *Carduncellus* s.l. lineage in three genera (H4) segregates *Carthamus* sect. *Thamnacanthus* as genus *Phonus* as previously suggested by López González (1990). The genus *Carduncellus* is defined according to Hanelt (1963) and the genus *Femeniasia* is also accommodated within the complex. This hypothesis presents morphological problems in the separation of the genera inside the *Carduncellus* s.l. lineage because the characters of the achenes or the bract appendages alone cannot solve the generic delimitation. However, the genera could be established by making convoluted combinations of morphological characters (Vilatersana, 2008). Given this dilemma, the generic hypothesis of Vilatersana & al. (2000a) opted for being nomenclaturally conservative.

Later on, Greuter (2003: 51) returned to the old Linnaean hypothesis (H1) stating: "A major merge is that of *Carthamus* L. with *Carduncellus* Adans., under the former name, which in my view and in the present state of our knowledge is the preferable alternative to recognising a number of additional, minor segregate genera such as *Phonus* Hill and *Femeniasia* Susanna." This classification involved a new generic ascription for 14 species of the *Carthamus-Carduncellus* complex (50 taxa) and caused taxonomic changes in 50% of the species from the *Carduncellus* s.l. lineage. In spite of fulfilling the criterion of monophyly, this hypothesis does not follow the taxon naming criteria because it violated the principle of economy of taxonomic changes (Vences & al., 2013). In our opinion, insufficient knowledge due to the lack of studies is not a good basis for making nomenclatural changes. Indeed, any taxonomic classification is likely to be replaced by a new one with the contribution of new evidence (e.g., new morphological or molecular studies) and accompanied by a substantial improvement in its definition (Stevens, 1997; Vences & al., 2013), but this is not the case. This principle of taxonomic stability has already been suggested by Linnaeus (see Vences & al., 2013: 209). More research is needed to definitively resolve a definitive classification. Dittrich (1969) defended the same taxonomic proposal when *Phonus* is included in the genus *Carthamus* as sect.

*Thamnacanthus*, a suggestion that impeded a morphological definition of the genus.

Finally, López González (2012) defended hypothesis H2 (Table 4, suppl. Table S1), which claims that the *Carthamus-Carduncellus* complex is formed by only two genera, *Carthamus* (*Carthamus* s.str.) and *Carduncellus* (*Carduncellus* s.l.). In this classification, the genus *Carthamus* is defined in the same way as hypothesis H4 (Vilatersana & al., 2000a), but it supports a wider genus concept in *Carduncellus* (*Carduncellus* s.l.). This lineage presents a clear definition on biogeographic grounds (Western Mediterranean distribution) and habit and ecological traits (perennial plants mainly from natural habitats), but it does not present any exclusive morphological apomorphy as a genus (Table 1). The characters of achene and pappus vary significantly within the broadly defined genus *Carduncellus* (*Carduncellus* s.l.) (Table 1) and they are established, in contrast to the more uniform morphological characters in *Carthamus* s.str. (Table 1). In this new taxonomical classification, taxonomic problems in *Carduncellus* s.l. are moved to the infrageneric level, i.e., sections (without any geographic or morphological description) and series, which are not monophyletic (López González, 2012). All these taxonomical changes do not solve the position of *Card. fruticosus*, *Femeniasia*, and *Phonus*, the most problematic taxa in the complex.

A major problem of morphologically defining the genera in the complex, especially the *Carduncellus* lineage, is the low number of morphological characters used and the high level of homoplasy, which contribute to the confusion surrounding the evolution of the group. Some of these characters, such as the undifferentiated pericarp, were considered symplesiomorphic within the group. However, because of this new phylogeny, the undifferentiated pericarp has been shown to be a homoplastic character after more extensive sampling and using more regions (Vilatersana & al., 2000a; López González, 2012). The patterns of morphological diversity in achenes and pappus within the lineage of *Carduncellus* s.l., together with the ecological diversity of their species, suggest that high homoplasy may be caused by convergence or reversion of these morphological features because many of these morphological characters are strongly adaptive (Stuessy & Garver, 1996). In the Compositae, it is usual to find groups where the boundaries between genera are still unresolved because of the presence of homoplasy or the low number of morphological characters. Two examples are the *Gerbera* complex (Pasini & al., 2016) and the *Carduus-Cirsium* complex (Ackerfield & al., 2020).

At this point, the most sensible option for the *Carthamus-Carduncellus* complex is to continue using the generic boundaries proposed in hypothesis H4 (four genera: *Carthamus*, *Carduncellus*, *Femeniasia*, and *Phonus*) following the molecular phylogeny (Vilatersana & al., 2000a), at least until more studies detect better apomorphies that help in their generic delimitation, especially in the *Carduncellus* lineage. Further phylogenomic studies including high-throughput sequencing will improve our understanding of the relationships within the *Carduncellus* lineage. After such studies, more definitive taxonomic proposals can be made, and we should avoid creating more disorder and implementing unnecessary generic changes. We should remember that in recent years some of the most problematic species of *Carduncellus* s.l. have been classified under *Carthamus*, *Carduncellus*, *Phonus* and *Femeniasia* (suppl. Table S1) (Petit & al., 2001).

In addition, the biogeographic isolation of *Femeniasia* and its uncertain position and relationship with *Phonus* and *Carduncellus* s.str. could boost the use of this nomenclaturally conservative hypothesis (H4), which favors keeping monotypic genera as indicative of phylogenetic uncertainty (Linder & al., 2010).

## ■ CONCLUSIONS

Our study offers new evolutionary insights into the *Carthamus-Carduncellus* complex using an almost complete taxon sampling aimed at untangling the controversial generic delimitation of this complex. Our results revealed two monophyletic lineages: the first was formed by the annual genus *Carthamus* (*Carthamus* s.str.) and the second by the perennial lineage (*Carduncellus* s.l.). The latter showed extensive incongruence between datasets (nuclear and plastid). Our MSC results statistically discard a classification based on only one genus (*Carthamus*) or two genera (*Carduncellus*, *Carthamus*) in the complex as the best solution. Instead, hypothesis H4 (presence of four genera, *Carthamus*, *Carduncellus*, *Femeniasia*, and *Phonus*) was the most supported. Nevertheless, the absence of morphological characters that define the *Carduncellus* lineage (*Carduncellus*, *Femeniasia*, *Phonus*) and the existence of homoplasies and frequent reversals of states of these characters make it very difficult to define the lineage morphologically. This paper elucidates the main challenges that hinder the generic classification of the complex, all of which lie within the *Carduncellus* s.l. lineage: the putative hybridization in the ancestral lineages and the absence of synapomorphic morphological characters, significantly compounding a clear definition of generic boundaries. More studies are needed to find some apomorphic morphological characters, as well as phylogenomic studies with next-generation sequencing to help in their generic delimitation before making a definitive proposal of the complex, aiming to avoid more unnecessary generic changes that add more taxonomic chaos to the group.

## ■ AUTHOR CONTRIBUTIONS

RV, NGJ, and AS designed the study. RV performed the molecular labwork. RV, JAC, and SHM analyzed all data. All authors interpreted the results. RV wrote the manuscript with comments from JAC, SHM, and AS. — RV, https://orcid.org/0000-0002-5106-8764; JAC, https://orcid.org/0000-0002-6586-0939; SHM, https://orcid.org/0000-0002-0488-5112; NGJ, http://orcid.org/0000-0003-1893-5122; AS, http://orcid.org/0000-0003-4717-9063

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**Appendix 1.** List of taxa used in this study.

Taxon name, geographic origin, collector, collection number, herbarium code and GenBank accession numbers (new data start with ON). Order: ITS (or ITS1/ITS2), ETS, *rpl32*, *ndhF*, *trnH*, *trnT*. The inserted symbols mean: "–" no sequence used for this specific region, "†" cloned ETS region (if applicable, multiple clones are separated by an hyphen).

***Carduncellus atractyloides*** Coss. & Durieu ex Pomel **Voucher 1**, Morocco, High Atlas Central, valley of Aït Bougmaze, Adrar Azourki, to Tizi-n-Tsalli-n-Imenaïn, *Romo 12752 & al.* (BC), ON320416/ON320461, ON398270, ON222900, ON304257, ON165549, ON165604; ***Carduncellus atractyloides*** **Voucher 2**, Morocco, Ouarzazate, valley of Dadès Superior, a long of road to Tizi-n-Ouano, N of Tilmi, 2900 m, *Staudinger 3073 & al.* (Herb. Staudinger), ON320417/ON320462, ON398271, ON222901, ON304258, ON165550, ON165605; ***Carduncellus caeruleus*** (L.) C.Presl. **Voucher 1**, Spain, Málaga: road C-344 between Coín and Tolox, *Susanna 1610 & al.* (BC), AF140442/AF140443, (†)ON398313, ON222902, ON304259, ON165551, ON165606; ***Carduncellus caeruleus*** **Voucher 2**, Morocco, Fes, Oued Zloul valley near Ahermoumou, *Susanna 1801 & al.* (BC), ON320418/ON320463, ON398272, ON222903, ON304260, ON165552, ON165607; ***Carduncellus calvus*** Boiss. & Reut., Morocco, Taza: S side of Djebel Bou Messoud, *J.M. Montserrat 3642 & al.* (BC 875418), ON320420/ON320465, ON398274, ON222905, ON304262, ON165554, ON165609; ***Carduncellus carthamoides*** (Pomel) Hanelt, Algeria, 10 km south of Tlemcen, 2000 m, *Dubois, Maurel & Rhamoun s.n.* (BC 837109), ON320423//ON320468, ON398275, ON222906, ON304263, ON165555, ON165610; ***Carduncellus catrouxii*** Emb. ex Maire, Morocco, High Atlas, M'goun, Ouzighimt valley, *Finckh & Staudinger 859* (HBG), MW209004/MW208954, (†)ON398314, ON222907, ON304264, ON165556, ON165611; ***Carduncellus cespitosus*** Batt., Morocco, Khenifra, Central Atlas, Plateau of Jebe Bougriy, near Kerrouchen, 2100 m, *Staudinger 2844* (Herb. Staudinger), ON320419/ON320464, ON398273, ON222904, ON304261, ON165553, ON165608; ***Carduncellus cuatrecasasii*** G.López, Spain, Jaén, Sierra de Mágina, between Mancha Real and Torres, *Susanna 1608 & al.* (BC), ON320424/ON320469, (†)ON398315, ON222908, ON304265, ON165557, ON165612; ***Carduncellus dianius*** Webb., Spain, Valencia, Cape San Antonio near Javea, *Susanna 1479 & al.* (BC), AF140440/ON320470, ON398276, ON222909, ON304266, ON165558, ON165613; ***Carduncellus duvauxii*** Batt. & Trab., Morocco, Al Hoceima: 8 km S of Tafraoute, *Gómiz s.n.* (BC 907085), AY826239, ON398277, ON222910, KC589930, ON165559, ON165614; ***Carduncellus eriocephalus*** Boiss., Morocco, Bouarfa: 100 km from Bouanane to Bouarfa, *Susanna 1785 & al.* (BC), ON320425/ON320502, ON398278, ON222911, ON304267, ON165560, ON165615; ***Carduncellus fruticosus*** (Maire) Hanelt **Voucher 1**, Morocco, Tinehir, gorges of the river Todhra, *J.M. Montserrat 2400 & al.* (BC 813805), ON320426/ON320471, ON398279, ON222912, ON304268, ON165593, ON165616; ***Carduncellus fruticosus*** **Voucher 2**, Morocco, Anti-Atlas, along road from Igherm to Souk-Tleta-de-Tagmoute, 5 km SE Igherm, 1600 m, *Hilpold AH20103029 & Calleja* (BC), ON320427/ON320472, ON398280, MK598504, ON304269, ON165594, ON165617; ***Carduncellus helenioides*** (Desf.) Hanelt, Algerie, M'sila: Ouanougha, *K. Rebbas s.n.* (Rebbas herbarium), ON320421/ON320466, (†)ON398334, ON222952, ON304306, ON165591, ON165658; ***Carduncellus hispanicus*** Boiss. ex DC. **subsp. *araneosus*** (Boiss. & Reut.) G.López, Spain, Toledo, between Valdecarábanos and Cabañas de Yepes, *Susanna 1603 & al.* (BC), ON320413/ON320458, (†)ON398309, ON222897, ON304253, ON165547, ON165601; ***Carduncellus hispanicus*** **subsp. *hispanicus***, Spain, Almería, Sierra de Gádor, near TV tower on the road Félix-Canjáyar, *Susanna 1614 & al.* (BC), ON320428/ON320473, ON398281, ON222913, ON304270, ON165561, ON165618; ***Carduncellus hispanicus*** **subsp. *intercedens*** (Degen & Hervier) G.López **Voucher 1**, Spain, Múrcia, Sierra de la Muela, Moratalla, to 2.8 km the camping "La Puerta", *Garnatje & Vilatersana 460* (BC), ON320414/ON320459, (†)ON398310–311, ON222898, ON304254, ON165599, ON165602; ***Carduncellus hispanicus*** **subsp. *intercedens*** **Voucher 2**, Spain, Granada, Almaciles, *Sanz & Vilatersana 473* (BC), ON320415/ON320460, (†)ON398312, ON222899, ON304255, ON165548, ON165603; ***Carduncellus lucens*** Ball, Morocco, High Atlas Mountains Tacheddirt, 2400 m, *Zerny s.n.* (W 1958/14848), MW209005/MW208955, ON398282, ON222914, ON304271, ON165562, ON165619; ***Carduncellus mareoticus*** (Delile) Hanelt **Voucher 1**, Egypt, Alexandria, road Alexandria-Marsah Matruh, km 106, *Susanna 1860 & Vilatersana* (BC), ON320429/ON320474, (†)ON398316, ON222915, ON304272, ON165563, ON165620; ***Carduncellus mareoticus*** **Voucher 2**, Egypt, Alexandria, 1 km N New Bourg-el-Arab, *Susanna 1846 & Vilatersana* (BC), ON320430/ON320475, (†)ON398318, ON222916, ON304273, ON165564, ON165621; ***Carduncellus mitissimus*** DC., Spain, Navarra, between Burgui and Navascués, *Carretero & Vilatersana 72* (BC), ON320431/ON320476, ON398283, ON222917, ON304274, ON165565, ON165622; ***Carduncellus monspelliensium*** All. **Voucher 1**, Spain, Almería, Sierra de Gádor, El Morrón, *del Rey & Vilatersana 1010* (BC), ON320432/ON320477, (†)ON398319–320, ON222918, ON304275, ON165595, ON165623; ***Carduncellus monspelliensium*** **Voucher 2**, Spain, Soria, Cabrejas del Pinar, *Sanz 306 & al.* (BC), ON320433/ON320478, (†)ON398321–322, ON222919, ON304276, ON165566, ON165624; ***Carduncellus monspelliensium*** **Voucher 3**, Spain, Tarragona: Monsant range, Grau del Carrasclet, *Vilatersana 18* (BC), ON320434/ON320479, (†)ON398323–324, –, –, –, –; ***Carduncellus monspelliensium*** **Voucher 4**, Spain, Tarragona, Benifallet, Cardó range, coll de Murtero, *Susanna 2672 & Vilatersana* (BC), –, –, ON222920, ON304277, ON165596, ON165625; ***Carduncellus pectinatus*** DC., Morocco, Fes, between Bir-Tam-Tam and Ahermoumou, 16 km to Ahermoumou, *Susanna 1800 & al.* (BC), MW209007/MW208957, (†)ON398325–326, ON222921, ON304278, ON165567, ON165626; ***Carduncellus pinnatus*** (Desf.) DC. **Voucher 1**, Morocco, Chefchaouen: between Bab Berred and Ketama, *Talavera 4154/94 & al.* (BC 828111), ON320435/ON320480, (†)ON398327, ON222922, ON304279, ON165568, ON165627; ***Carduncellus pinnatus*** **Voucher 2**, Italy, Palermo, eastern side of the Rocca Busambra, 2 km NNE Giardinello, *Vilatersana 1171 & al.* (BC), ON320436/ON320481, (†)ON398328, ON222923, ON304280, ON165566, ON165628; ***Carduncellus plumosus*** Pomel, Tunisia, between Kaserina and Argelian border, Jabel Hamra, Ain Senan, *Aldasoro 2953 & al.* (MA), ON320437/ON320482, (†)ON398329, ON222924, ON304281, ON165570, ON165629; ***Carduncellus pomelianus*** Batt., Morocco, Al Hoceima: Aknoul, Jbel

**Appendix 1.** Continued.

Azrou Akechar, *Boratynski & Romo 8792* (BC 875419), ON320438/ON320483, ON398284, ON222925, ON304282, ON165571, ON165630; ***Carduncellus reboudianus*** Batt., Morocco, Meknes, 2 km to Oued Amesheguir in the road to Meknes, *Susanna 1796 & al*. (BC), MW209006/MW208956, (†)ON398330, ON222927, ON304284, ON165573, ON165632; ***Carduncellus rhaponticoides*** Coss. & Dur., cultivated in the Royal Botanical Garden of Madrid, Spain (López González 234/87), ON320439/ON320484, ON398285, ON222926, ON304283, ON165572, ON165631; ***Carduncellus strictus*** (Pomel) Hanelt, Algeria, Tikjda, 129 m, *Mihoub & Amirouche s.n*. (BC), ON320422/ON320467, (†)ON398333, ON222953, ON304307, ON165588, ON165659; ***Carthamus alexandrinus*** (Boiss. & Heldr.) Bornm. **Voucher 1**, Egypt, Alexandria, between El Amiriya and Bourg-el-Arab, *Susanna & Vilatersana 1835* (BC), ON320440/ON320485, ON398286, ON222928, ON304285, DQ917442, ON165633; ***Carthamus alexandrinus*** **Voucher 2**, Libya, Tobruk, *Susanna & Vilatersana 2686* (BC), ON320441/ON320486, (†)ON398331, ON222929, ON304286, ON165574, ON165634; ***Carthamus anatolicus*** (Boiss.) Sam., Israel, Kefar Shammai, Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, *Cart 53/76* (BC), ON320443/ON320487, ON398288, ON222930, ON304287, DQ917451, ON165635; ***Carthamus boissieri*** Halácsy, Greece, Crete, Rethymnon, road N-77 between Rethymnon and Armeni, *Carretero & Vilatersana 26* (BC), ON320443/ON320488, ON398287, ON222931, ON304288, DQ917450, ON165636; ***Carthamus creticus*** L., Morocco, Al Hoceima, 38 km S of Al Haceima on the road to Nador, *Susanna 1772 & al*. (BC), AY826247, (†)ON398332, ON222932, ON304289, DQ917448, ON165637; ***Carthamus curdicus*** Hanelt, Irak, conglomerate and mud hills 35 miles NE Baquba, *Stutz s.n*. (W 1962/11596), ON320444/ON320489, ON398289, ON222933, ON304290, ON165575, ON165638; ***Carthamus dentatus*** Vahl **subsp. *dentatus***, Greece, Chios, just west of megas Limnionas, along gravel roadside, *A. Brysting 05-7* (BC), ON320445/ON320490, ON398290, ON222934, ON304291, ON165597, ON165639; ***Carthamus dentatus*** **subsp. *ruber*** (Link) Hanelt, Greece, Crete, Rethymnon, road between Fouforas and Kourote, *Carretero & Vilatersana 33* (BC), ON320446/ON320491, ON398291, ON222935, ON304256, DQ917441, ON165640; ***Carthamus divaricatus*** Bég. & Vaccari, Lybia, 40 km W of Tekmar, *Susanna 2683 & Vilatersana* (BC), ON320447/ON320492, ON398292, ON222936, ON304292, ON165600, ON165641; ***Carthamus glaucus*** M.Bieb., Armenia, Ekhegnadzor, near Agarakadzor, *Susanna 1551 & al*. (BC 954170), ON320448/ON320493, ON398293, ON222937, ON304293, DQ917446, ON165642; ***Carthamus gypsicola*** Iljin, Armenia, Ararat, Vedi, *Susanna 1579 & al*. (BC), ON320449/ON320494, ON398294, ON222938, ON304294, ON165576, ON165643; ***Carthamus lanatus*** L. **Voucher 1**, Greece, Crete, Rethymnon, between road N-77 and necropolis Minois, *Carretero & Vilatersana 27* (BC), ON320450/ON320495, ON398295, ON222939, ON304295, DQ917444, ON165644; ***Carthamus lanatus*** **Voucher 2**, Lebanon, USDA, Western Regional Plant Introduction Station, Pullman, Washington, PI 243151, sub "*Carthamus glaucus*", ON320451/ON320496, ON398296, ON222940, ON304296, ON165577, ON165645; ***Carthamus lanatus*** **subsp. *montanus*** (Pomel) Gahand & Maire, Tunisia, golf of Tunis, Cedria Plage, IPK Gatersleben, *Cart 84/95* (BC), AJ969138/AJ969156, ON398297, ON222941, ON304297, ON165578, ON165646; ***Carthamus leucocaulos*** Sm., Greece, Crete, Hania, base of Mount Hrissokalitissas, *Carretero & Vilatersana 40* (BC), ON320452/ON320497, ON398298, ON222942, ON304298, DQ917447, ON165647; ***Carthamus nitidus*** Boiss., Israel, Negev Desert, Dead Sea, *R. Levy s.n*. (BC), ON320453/AF140483, ON398299, ON222943, ON304299, DQ917443, ON165648; ***Carthamus oxyacantha*** M.Bieb., Iran, Tehran, Sorkhehesar near Tehran, *Susanna 1626 & al*. (BC), AY826248, ON398300, ON222944, KC589940, ON165579, ON165649; ***Carthamus palaestinus*** Eig, Israel, USDA, Western Regional Plant Introduction Station, Pullman, Washington, PI 235663 (BC), GU969343, ON398301, GU990467, ON304300, ON165580, HM002848; ***Carthamus persicus*** Desf. ex. Willd., Turkey, Elaziğ, road to Elaziğ to Bingöl Ertuğ, *Susanna 2358 & al*. (BC), ON320454/ON320498, (†)ON398317, ON222945, ON304301, ON165581, ON165650; ***Carthamus tenuis*** (Boiss. & C.I.Blanche) Bornm., Israel, Binyamina, *R. Levy s.n*. (BC), AF140478/AF140479, ON398302, ON222946, ON304302, DQ917449, ON165651; ***Carthamus tinctorius*** L., seeds of Botanical Garden of Nancy, France (607/96) (BC), ON320455/ON320499, ON398303, ON222947, ON304303, ON165582, ON165652; ***Carthamus turkestanicus*** Popov, Armenia, Ararat, near Surenavan, *Susanna 1532 & al*. (BC), AY826249, ON398304, ON222948, KC589941, DQ917445, ON165653; ***Femeniasia balearica*** (J.J.Rodr.) Susanna **Voucher 1**, Spain, Balearic Islands, Minorca, Mongofre Vell, *J.M. Montserrat 2802* (BC), AY826284, ON398305, ON222949, KC589971, ON165583, ON165654; ***Femeniasia balearica*** **Voucher 2**, Spain, Balearic Islands, Minorca, Binimel-la, *Carretero & Vilatersana s.n*. (BC), ON320456/ON320500, ON398306, MK598503, ON304304, ON165584, ON165655; ***Phonus arborescens*** (L.) G.López, Spain, Almería: Sierra de Gádor near Félix, *J.M. Montserrat s.n*. (BC), ON320457/ON320501, ON398307, ON222950, ON304305, ON165585, ON165656; ***Phonus rhiphaeus*** (Font Quer & Pau) G.López, Morocco, Al Hoceima: Tleta Oued Laou between Tarerha and Azenti, *J.M. Montserrat 4360 & al*. (BC), AY826310, ON398308, ON222951, KC589988, ON165586, ON165657; ***Centaurea cyanus*** L. **Voucher 1**, France, Lozère: Causse de Sauveterre, cultivated fields between le Sec and l'Aumède, near Chanac, *Carretero & Vilatersana 51* (BC), –, –, ON222954, ON304308, ON165589, ON165663; ***Centaurea cyanus*** **Voucher 2**, Spain, Palencia, near Guardo, *Garcia-Jacas & Susanna 2076* (BC), AY826254, HQ147679, –, –, –, –; ***Centaurea lingulata*** Lag. **Voucher 1**, Spain, Jaén, Mancha Real, Sierra de Mágina, by the relay station on the Almadén Peak, *Susanna 1607 & al*. (BC), HQ147739/HQ147631, HQ147687, –, –, –, –; ***Centaurea lingulata*** **Voucher 2**, Spain, Teruel, Sierra del Pobo, stoneware upper part of TVE relay, 1700 m, *Litzler 72/702 E* (ZT), –, –, ON222955, ON304309, ON165590, ON165664; ***Centaurea scabiosa*** L., France, Lozère, between Le Sec and Aumède, near Chanac, *Carretero & Vilatersana 52* (BC), FJ4596927, FJ4596367, ON458153, ON458154, FJ4597267, ON458155; ***Phalacrachena inuloides*** (Fisch.) Iljin, Ukraine, Kherson, National Reserve Askania Nova, Plot N7, 06/07/2006, *Romaschenko 402 & Didukh* (BC), JF754816, JF754793, JF754889, JF754851, ON165592, ON165662; ***Psephellus persicus*** (DC.) Wagenitz, Iran, Hamadan, *Susanna 1716 & al*. (BC), AY826316, DQ310957, JF754893, JF754855, ON165587, ON165661; ***Volutaria crupinoides*** (Desf.) Maire **Voucher 1**, Morocco, *Vogt 11075 & Oberprieler* (B), –, –, JF754905, JF754867, ON165598, ON165660; ***Volutaria crupinoides*** **Voucher 2**, Morocco, Eastern Anti-Atlas, Agadir-Melloul, 1504 m, *T. Buira & J. Calvo 0421* (MA), KU309368, KU324117, –, –, –, –.