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Authors: Jiménez-Mejías, Pedro, Hahn, Marlene, Lueders, Kate, Starr, Julian R., Brown, Bethany H., et al.

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# Megaphylogenetic Specimen-level Approaches to the *Carex* (Cyperaceae) Phylogeny Using ITS, ETS, and *matK* Sequences: Implications for Classification

## The Global *Carex* Group

Pedro Jiménez-Mejías,<sup>1,21</sup> Marlene Hahn,<sup>2</sup> Kate Lueders,<sup>2</sup> Julian R. Starr,<sup>3</sup> Bethany H. Brown,<sup>2</sup> Brianna N. Chouinard,<sup>3</sup> Kyong-Sook Chung,<sup>4</sup> Marcial Escudero,<sup>5</sup> Bruce A. Ford,<sup>6</sup> Kerry A. Ford,<sup>7</sup> Sebastian Gebauer,<sup>8</sup> Berit Gehrke,<sup>9</sup> Matthias H. Hoffmann,<sup>8</sup> Xiao-Feng Jin,<sup>10</sup> Jongduk Jung,<sup>11</sup> Sangtae Kim,<sup>11</sup> Modesto Luceño,<sup>12</sup> Enrique Maguilla,<sup>12</sup> Santiago Martín-Bravo,<sup>12</sup> Mónica Míguez,<sup>12</sup> Ana Molina,<sup>13</sup> Robert F. C. Naczi,<sup>14</sup> Jocelyn E. Pender,<sup>3</sup> Anton A. Reznicek,<sup>15</sup> Tamara Villaverde,<sup>12</sup> Marcia J. Waterway,<sup>16</sup> Karen L. Wilson,<sup>17</sup> Jong-Cheol Yang,<sup>18</sup> Shuren Zhang,<sup>19</sup> Andrew L. Hipp,<sup>2,20,21</sup> and Eric H. Roalson<sup>1,21</sup>

<sup>1</sup>School of Biological Sciences, Washington State University, Pullman, Washington, U. S. A.

<sup>2</sup>The Morton Arboretum, Lisle, Illinois, U. S. A.

<sup>3</sup>University of Ottawa, Ottawa, Ontario, Canada

<sup>4</sup>Jungwon University, Goesan, Chungbuk, Republic of Korea

<sup>5</sup>Department of Plant Biology and Ecology, University of Seville, Seville, Spain

<sup>6</sup>University of Manitoba, Winnipeg, Manitoba, Canada

<sup>7</sup>Allan Herbarium, Landcare Research, Lincoln, New Zealand

<sup>8</sup>Martin Luther University Halle-Wittenberg, Halle, Germany

<sup>9</sup>Johannes Gutenberg-Universität, Mainz, Germany

<sup>10</sup>Hangzhou Normal University, Hangzhou, Zhejiang, China

<sup>11</sup>Department of Biology, Sungshin Women's University, Seoul, Republic of Korea

<sup>12</sup>Universidad Pablo de Olavide, Seville, Spain

<sup>13</sup>University of León, León, Spain

<sup>14</sup>New York Botanical Garden, Bronx, New York, U. S. A.

<sup>15</sup>University of Michigan Herbarium, Ann Arbor, Michigan, U. S. A.

<sup>16</sup>Plant Science Department, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada

<sup>17</sup>National Herbarium of New South Wales, Sydney, New South Wales, Australia

<sup>18</sup>Division of Forest Biodiversity, Korea National Arboretum, Gyeonggi-do, Republic of Korea

<sup>19</sup>Institute of Botany, Chinese Academy of Sciences, Beijing, China

<sup>20</sup>The Field Museum, Chicago, Illinois, U. S. A.

<sup>21</sup>Authors for correspondence (pjimmej@gmail.com, ahipp@mortonarb.org, eric\_roalson@wsu.edu)

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**Abstract**—We present the first large-scale phylogenetic hypothesis for the genus *Carex* based on 996 of the 1983 accepted species (50.23%). We used a supermatrix approach using three DNA regions: ETS, ITS and *matK*. Every concatenated sequence was derived from a single specimen. The topology of our phylogenetic reconstruction largely agreed with previous studies. We also gained new insights into the early divergence structure of the two largest clades, core *Carex* and *Vignea* clades, challenging some previous evolutionary hypotheses about inflorescence structure. Most sections were recovered as non-monophyletic. Homoplasy of characters traditionally selected as relevant for classification, historical misunderstanding of how morphology varies across *Carex*, and regional rather than global views of *Carex* diversity seem to be the main reasons for the high levels of polyphyly and paraphyly in the current infrageneric classification.

**Keywords**—Homoplasy, paraphyly, polyphyly, supermatrix, taxonomy.

*Carex* L. (Cyperaceae) is one of the largest angiosperm genera (Reznicek 1990). *Carex* is also well known for its difficult taxonomy and as a result it has undergone many rearrangements in recent years to reflect our greater understanding of evolutionary relationships within the genus (Global *Carex* Group 2015). Despite more than 15 yr of phylogenetic investigation (e.g. Starr et al. 1999; Roalson et al. 2001; Hendrichs et al. 2004a, b; Waterway et al. 2009; Starr et al. 2015), the phylogeny is still imperfectly known. This is, at least in part, because even the most comprehensive previous phylogenetic studies have sampled at most 30% of the species richness (67–550 of ca. 2000 species; Roalson et al. 2001; Gehrke and Linder 2009; Waterway et al. 2009; Starr et al. 2015).

During the past five years, the focus of many caricologists has been on assessing relationships among closely related species in specific clades and/or sections, rather than on the

whole *Carex* phylogeny (Dragon and Barrington 2009; Escudero and Luceño 2009; Gehrke et al. 2010; Ford et al. 2012; Jiménez-Mejías et al. 2012b; Yano et al. 2014; Gebauer et al. 2015; Maguilla et al. 2015; Villaverde et al. 2015). A common finding in these studies is that most traditionally recognized groups (subgenera and sections) are not natural. While these studies have provided insights into fine-scale relationships within these groups, they have made only a small contribution to the understanding of broader *Carex* lineage relationships. A larger proportion of the species have been sampled in North America and Europe than elsewhere (e.g. Roalson et al. 2001; Ford et al. 2006; Hipp et al. 2006; Waterway and Starr 2007; Waterway et al. 2009; Ford et al. 2012; Gebauer et al. 2014; Maguilla et al. 2015); but sampling in Africa, Asia, and Australasia is increasing (Escudero and Luceño 2009; Gehrke et al. 2010; Yano et al. 2014; Starr et al. 2015). Progress toward defining clades of *Carex* for a more

natural sectional classification is still hampered by incomplete global sampling, a deficit we address in the work presented here.

Why is the section, a sub-subgeneric taxonomic category, an important taxonomic level in *Carex*? The high species diversity, disparate geographic distribution, and technically difficult morphology (due to character reduction and/or recurrent homoplasy) make visual identification of species difficult, even for specialists. As a result, there has long been an emphasis on sectional assignment as a gateway to species identification in *Carex*: to identify a particular specimen the first focus is on its subgeneric placement, then on choosing the correct section. This identification procedure is mirrored in floras with a large number of *Carex* species, which present keys that lead the reader to sections, and then to a much shorter key to species within a section, as a mechanism to divide keys into manageable pieces (Egorova 1999; Ball and Reznicek 2002; Dai et al. 2010, among many others). Other floras present keys that lead directly to species, but often still list species under sections, which allows the reader to compare a specimen to species with apparent morphological similarity (e.g. Chater 1980; Luceño et al. 2008). This sectional approach is useful in organizing the diverse morphological variation in *Carex*, thus easing identification for botanists not familiar with the genus.

A number of botanists have attempted infrageneric classifications (subgenera and sections) for the genus *Carex* since the 19th century (see Robertson 1979; Reznicek 1990). During the 20th century, three large monographic works have provided the sectional arrangement of the genus as we know it today: Kükenthal's (1909) world revision, Mackenzie's (1935) flora treatment for North America, and Egorova's (1999) treatment for the former USSR. The early Kükenthal classification is a valuable piece of work, notable since it established the basis for the infrageneric systematics of *Carex* that is still in place today. His treatment is clearly of evolutionary inspiration, although markedly phenetic. Kükenthal emphasized a few reproductive features to characterize the segregation of genera within the tribe Cariceae, and subgenera within *Carex*; for example inflorescence structure, morphology of cladophylls, whether perigynia were open or closed to form a utricle, and whether the rachilla was present, and if so whether it developed into a male spikelet or formed a hook (see Global *Carex* Group 2015 for an extended discussion on *Carex* inflorescence morphology, and Jiménez-Mejías et al. 2016a for terminology). Using largely these few characters, Kükenthal (1909) classified tribe Cariceae into four genera (*Carex*, *Kobresia* Willd., *Schoenoxiphium* Nees, and *Uncinia* Pers.), which persisted until very recently (Global *Carex* Group 2015). Within *Carex* he recognized four subgenera (*Psyllophora* (Degl.) Peterm., *Vignea* (P. Beauv ex T. Lestib.) Peterm., *Vigneastra* (Tuck.) Kük., and *Carex*; in this same order but using synonym names), which are still used today but are in need of reconfiguration. In turn, within each subgenus, numerous sections, defined on the basis of utricle morphology and indumentum, and morphology of the spikes, were distinguished. Remarkably he ordered groups within *Carex* from simpler inflorescences to more complex (compound) ones and from gynoecea with two stigmas to those with three, presumably to reflect an evolutionary progression (see Egorova 1999). Mackenzie (1935) provided the sectional treatment that was largely followed by the Flora of North America treatment (Reznicek 2001).

While clearly inspired by Kükenthal's work, Mackenzie departed from this earlier work in a number of aspects. First of all, Mackenzie did not recognize subgenera, and *Carex fraseriana* Ker Gawl. was placed into its own genus (*Cymophyllus* Mack.). In addition, sections were more narrowly defined, a reflection of philosophical differences between the two authors and the more regional scope of Mackenzie's work. It was not until the arrival of Egorova's (1999) treatment that an explicitly phylogenetic perspective was applied to *Carex* classification. She not only considered morphological characters to develop her infrageneric classification, but she also polarized these characters, hypothesizing primitive and more evolved states, and used this reasoning to arrange the sections. Interestingly, she relied on the same basic characters that Kükenthal did, although she sometimes gave them different relative importance. Thus, she maintained part of Kükenthal's scheme but also made significant innovations, such as adding a new subgenus (*Kreczetoviczia* Egor.) that was never widely accepted and splitting a number of sections (e.g. section *Holarrhenae* (Döll) Pax, split from section *Ammoglochin* Dumort.; some of the subsections within section *Acutae* Fries (= *Phacocystis* Dumort.) raised to section level, such as *Praelongae* (Kük.) Nelmes and *Forficulae* Franch. ex Kük.).

The goal of the Global *Carex* Group project is to develop a revised lineage-based sectional classification, using a large, international collaborative effort to more broadly sample *Carex* diversity, and then to apply a phylogenetic framework to revise the sectional classification of this ca. 2000 species lineage and create a refreshed phylogeny-based taxonomic scheme. The first outcome of this collaboration was the nomenclatural revision of *Carex* to formally include all satellite genera in the tribe Cariceae (*Cymophyllus*, *Kobresia*, *Schoenoxiphium*, and *Uncinia*) within a more broadly circumscribed *Carex* (Global *Carex* Group 2015). We are here continuing this process by presenting the first large-scale phylogenetic hypotheses for *Carex* as newly defined, using the largest species sampling to date and three gene regions: the nuclear ribosomal DNA (nrDNA) internal transcribed spacers (ITS), the nrDNA external transcribed spacer (ETS), and the chloroplast DNA (cpDNA) *maturase K* (*matK*) gene. We present and discuss the application of these DNA regions to the development of large (>1,000 samples) phylogenetic hypotheses and the methodological approaches used. We compare an updated classification hypothesis (i.e. Kükenthal's treatment with modifications mainly from Mackenzie and Egorova; see below) with the resulting phylogenetic hypotheses, as a step toward a future revised sectional classification.

## MATERIALS AND METHODS

**Study Group**—We will refer to the phylogenetic structure of *Carex* according to the four major clades detected in previous studies (see Waterway et al. 2009; Starr et al. 2015): 1) *Siderostictae* clade, sister to the rest of the genus; 2) core *Carex*, including the majority of subgenus *Carex* species; 3) *Vignea*, grouping nearly all species traditionally placed in subgenus *Vignea*; and 4) the Caricoid clade, a heterogeneous set of species belonging to *Carex* and the former Cariceae satellite genera *Cymophyllus*, *Kobresia*, *Schoenoxiphium*, and *Uncinia*. The Caricoid clade is composed of two main subclades, the Unispicate clade, and the *Schoenoxiphium* clade (Waterway and Starr 2007; Starr and Ford 2009), which have not always been recovered as a single monophyletic group (e.g. Waterway and Starr 2007; Gehrke et al. 2010; Starr et al. 2015). Supplemental files for this paper are available from the Dryad Digital Repository at <http://dx.doi.org/10.5061/dryad.k05qb>.

Taxonomy of the sampled species predominantly follows the World Checklist of Cyperaceae (Govaerts et al. 2015), with occasional modifications according to the expertise of the members of the Global *Carex* Group. We have also included a few undescribed putative species and some unidentified accessions. Preliminary assignment to section for each species (Supplemental Appendix 1) primarily followed the schemes provided by the four largest *Carex* monographic treatments to date: Kükenthal (1909), Egorova (1999), Ball and Reznicek (2002), and Dai et al. (2010). We modified the sectional assignment for cases in which a published phylogenetic hypothesis supported a treatment different from the standard treatments listed above (see Supplemental Appendix 1). Sectional affiliation for species not listed in these four treatments was sought in alternative floras/treatments and the protologues. If no section was previously assigned, we considered the most probable section to be the one in which putatively closely related species have been placed in other treatments. If no information was found after this exhaustive search, we consulted the existing imaged type material in the online repository JSTOR Global Plants (<https://plants.jstor.org>) and section and subgenus affiliation was assigned by morphological affinity of the type material whenever it reasonably fit the standard characters for the alleged section. The reader should be aware that this is not a new proposal of sectional arrangement, but simply an updated compilation of what has been published to allow comparison to the results of the new phylogenetic hypotheses presented here.

**Taxon Sampling**—Materials from a total of 2153 individuals belonging to 996 of the 1983 accepted species (50.23%) from 110 of the 126 recognized sections (92.06%) as well as from four formerly recognized genera *Cymophyllus*, *Kobresia*, *Schoenoxiphium*, and *Uncinia* (now merged in *Carex*, Global *Carex* Group 2015) and from around the world were provided by the co-authors of this paper (Supplemental Data 1). In addition, materials from the herbaria FT, H, LEB, M, MSB, P, SOM, and UPOS were also included. The taxonomic expertise of the co-authors of this paper should minimize potential species misidentifications and ensure a high standard for taxonomic quality in our dataset. *Eriophorum vaginatum* L., *Scirpus polystachyus* F. Muell., *Trichophorum alpinum* (L.) Pers. and *Trichophorum cespitosum* (L.) Hartm. sequences obtained from GenBank for the three selected markers were used as an outgroup (Léveillé-Bourret et al. 2014). It should be noted that, given the large amount of material processed, we aimed to include more than one sequence per taxon to be able to identify possible misidentifications, mislabelings, and contaminations (see below).

**Data Matrix Construction**—Total genomic DNA was extracted using a modified CTAB procedure (Doyle and Doyle 1987). ITS was amplified using the primers ITSa and ITS4 (Blattner 1999; White et al. 1990) for all the samples, except for herbarium materials, for which we used a nested PCR approach where a first PCR was performed using the primers 17SE and 26SE (Sun et al. 1994), and the product of this was used as template for a second PCR using the primer pair ITSa-ITS4. ETS was amplified according to Starr et al. (2003). For *matK*, we used primers *matK*-2.1f and *matK*-5r with PCR conditions following Starr et al. (2009) for freshly collected silica-gel dried materials. For herbarium material we used a nested PCR with primers *matK*-2.1 and *matK*-5r for the first step, and for the second step a specifically designed primer pair *matK*f-61 (5'-BTTYAAAGAAATCGGTTTCTATATTCTC) and *matK*r-673 (5'-BAAA TCTGTCCAGATCGGCTTACTAGTAGG); the annealing temperature in both steps was increased to 54°C. Cleaned products were sequenced using BigDye Terminator v. 3.1 (Applied Biosystems, California). All lab work was performed in the labs of four authors (ALH, EHR, JRS, MJW) and cleaned cycle-sequencing products were run at the Field Museum (Chicago), WSU Molecular Biology and Genomics Center, Canadian Centre for DNA Barcoding at the University of Guelph and Canadian Museum of Nature, or McGill University and Genome Quebec Innovation Centre, respectively. Sequences were edited in the program Sequencher v. 4.10.1 and automatically aligned using MUSCLE (Edgar 2004). Three independent matrices were compiled, each one containing sequences of only one DNA region (ITS, ETS, or *matK*). A preliminary set of maximum likelihood analyses was performed as implemented in RAxML v. 8.2 (Stamatakis 2009; see below for details) using these raw matrices to see which species were placed into each of the major clades found in *Carex* s. l. (see above). We then partitioned each matrix into three sub-matrices, each containing sequences recovered in one of the three major clades: 1- core *Carex*, 2- *Vignea*, and 3- Caricoid clade. In the third group we also included sequences belonging to the *Siderostictae* clade, as well as the outgroup accessions. Each matrix was re-aligned again using MUSCLE and manually corrected. The goal of splitting the matrix in sub-matrices was to ease the alignment of homologous positions across more similar sequences in the smaller subsets. The curated

matrices were then re-merged using the profile-profile option of MUSCLE and manually corrected again. A taxonomic disparity index (TDI) was calculated to discover possible sources of mislabeling or contamination, so those sequences could be removed from the curated matrices. TDI calculates the difference between the number of samples for a particular taxon rank, and the number of samples in the smallest clade that includes all the samples of this taxon (Global *Carex* Group 2016). We calculated TDI for species, and each case was evaluated individually. It allowed us to discriminate between high disparity scores due to actual mislabeling/contamination, evidenced by the different accessions of a particular species being placed in different well-supported clades, from those with high disparity scores that reflected lack of resolution, such as, when the accessions of a particular species are placed in a polytomy with other species. In the case of actual contamination or mislabeling, the discordant accessions were removed from the matrix, while those that appeared to be cases of poor resolution were kept in the matrices.

Two different concatenated matrices were prepared with the three markers: 1) the all-nrDNA matrix, in which we considered sequences of ITS, ETS and *matK* but only for those accessions that successfully amplified for both ITS and ETS markers, regardless of whether *matK* did or not; and 2) the all-sequences matrix, in which all the obtained sequences were included. Disparity analysis was performed a second time on the all-nrDNA matrix to find any additional cases of mislabeling/contaminations involving different markers amplified from the same accession. Those sequences found to be in conflict were removed from both the final all-nrDNA and the all-sequences matrices. The three separate gene alignment matrices may be found in Supplemental Data 2-4.

**Phylogenetic Analyses**—We used a supermatrix total evidence approach to test the phylogenetic hypotheses in *Carex*. Such an approach is considered appropriate for large-scale phylogenetic analyses (Soltis et al. 2013; Hinchliff and Roalson 2013). Trees were built using maximum likelihood (ML) as implemented in RAxML. To assess topology uncertainty we performed 100 non-parametric bootstraps (BS). The deleterious effect of the rogue sequences (sequences with highly variable positions in the tree set) was avoided by identifying them using RogueNaRok (Aberer et al. 2013). All the identified rogues were excluded from the all-nrDNA matrix and transferred to the all-sequences matrix. The only exception was made with a sequence of *C. erictorum* Pollich, which after removal from the all-nrDNA matrix caused an odd placement of the very closely related *C. melanocarpa* Cham., so it was kept in the all-nrDNA matrix. Similar obvious consequences were not observed with other sequences after removal of any other rogues. ML analysis was run again on the all-nrDNA matrix after removal of the rogues. The phylogenetic placement of all excluded sequences (rogues and sequences lacking either ITS or ETS) was tested building a “query tree” using the evolutionary placement algorithm (EPA; Berger et al. 2011) as implemented in RAxML. The analysis was performed using as reference tree the best tree yielded by the ML analysis of the all-nrDNA matrix. The all-sequences matrix was used as the source of all the other sequences to be tested against the reference tree topology. The nonparametric Shimodaira-Hasegawa implementation of the approximate likelihood-ratio test (aLRT; Anisimova and Gascuel 2006) was performed to evaluate branch support (Roalson and Roberts 2016). TDI based on sections instead of species was performed using the reference and query trees to quantify the polyphyly of the different sections and compare results between analyses.

Our analyses represent a scaffolding approach, in which we use a “dominant” dataset where some of the selected DNA regions are represented for all the taxa. Similar strategies have already been used in Cyperaceae with satisfactory results in terms of resolution and support (Hinchliff and Roalson 2013).

## RESULTS

**Sequence Characteristics**—The number of sequences and aligned lengths for each data matrix were: 1588 sequences and 873 aligned bp for ETS; 1809 sequences and 1011 bp for ITS; and 1278 sequences and 888 bp for *matK*. The final all-sequences matrix and all-nrDNA matrix were 2772 bp long, containing sequences from 2150 and 1322 samples respectively. The all-nrDNA matrix, from which the reference tree was built, contained 36.32% missing data (including gaps).

**Phylogenetic Analyses**—The topology revealed by the all-nrDNA matrix (reference tree; Supplemental Data 5) was

largely congruent with previous analyses. Four of the five main clades recovered in previous studies were strongly supported in our tree: *Siderostictae* clade (100% BS), *Vignea* (95% BS), core Unispicate (89% BS) and *Schoenoxiphium* clade (89% BS). The fifth clade containing the core *Carex* was poorly supported (63% BS). Relationships among the five main clades were not strongly supported, except for the sister relationship of the *Siderostictae* clade to the rest of *Carex* (100% BS). In contrast to several previous analyses, the Core Unispicate and *Schoenoxiphium* clades were recovered as distinct clades rather than as sister clades within the larger Caricoid clade. Major subclades within each of these main clades were also recovered by our phylogenetic reconstruction, although it mostly failed to find hierarchical relationships among them, with the striking exception of *C. gibba* Wahlenb., the only member of section *Gibbae* Kük. and well known to be sister to the rest of *Vignea* (100% BS; Ford et al. 2006, 2012).

Because the query tree (Supplemental Data 6; Figs. 1–14) is constrained by the reference tree, their large-scale topologies are identical, though support values varied between the two and additional (non-reference) taxa are only found in the query tree. The aLRT support values for the main clades were as follows (Fig. 1): *Siderostictae* clade (aLRT=91%), core *Carex* (aLRT=100%), *Vignea* (aLRT=97%), core Unispicate (aLRT=97%), and *Schoenoxiphium* clade (aLRT=98%). Relationships among major clades were more strongly supported than in the reference tree: the *Siderostictae* clade was sister to a clade containing the rest of the sections (aLRT=86%), clade *Schoenoxiphium* was sister to the remaining clades (aLRT=86%), followed by the core Unispicate clade sister to core *Carex* plus *Vignea* (aLRT=85%). It should be noted that these relationships are not very strongly supported: critical aLRT values recommended for interpretation as strong support are >85% (see Anisimova et al. 2011). Nevertheless, relationships within core *Carex* and *Vignea* clades experienced a significant increase in resolution and support, especially in the early-diverging branches, compared to the reference tree.

Values for TDI comparing taxonomic sections rather than species exhibit a distribution in three clusters (Fig. 15; Supplemental Appendix 2), reflecting three levels of sectional polyphyly. The highest TDI scores were found when species of the same section were nested in different major clades of genus *Carex*. The lowest scores (mostly grouped in the “0 to 100” category but still 24 of them higher than zero) are found in sections that become paraphyletic due to the nesting of members of other sections within them. Figure 16 summarizes the placement of the sections on the tree. Sections with the highest TDI are displayed in the outermost ring, whereas sections with the lowest values are displayed in the innermost ring.

## DISCUSSION

### *Validation of Specimen-level Supermatrix Approach—*

Our study provides the most comprehensive phylogenetic evaluation to date of the systematics of *Carex*. The use of three DNA regions yielded a topology that is largely consistent with previous phylogenetic reconstructions (Starr et al. 2009, 2015; Waterway et al. 2009) and that recovered most of the widely recognized clades based on reconstructions of particular species groups even when different combinations of DNA regions were used (e.g. Starr et al. 1999, section

*Phyllostachyae* Tuck. ex Kük., Fig. 3; Roalson et al. 2001, section *Acrocystis* Dumort., Fig. 8; Ford et al. 2006, subgenus *Vignea* (P. Beauv. ex T. Lestib) Peterm., Figs. 4–6; Hipp et al. 2006, section *Ovales* Kunth, Fig. 5; Starr et al. 2008, former genus *Uncinia* Pers., Fig. 3; Dragon and Barrington 2009, section *Phacocystis*, Fig. 14; Escudero and Luceño 2009, section *Spirostachyae* (Drejer) L. H. Bailey, Fig. 11; Gehrke et al. 2010, *Schoenoxiphium* clade, Fig. 2; Jiménez-Mejías et al. 2012b, section *Ceratocystis* Dumort., Fig. 11; Gebauer et al. 2014, section *Vesicariae* (Heuff.) J. Carey, Fig. 12; Martín-Bravo et al. 2013, section *Syloaticae* Rouy, Fig. 11; Gebauer et al. 2015, section *Ramosae* G. Don, Fig. 10; Maguilla et al. 2015, section *Glareosae* G. Don, Fig. 6; Molina et al. 2015, section *Heleoglochis* Dumort., Figs. 4, 6; Villaverde et al. 2015, section *Capituligeriae* Kük., Fig. 2). Nevertheless, adding species to the clades that have already been studied completes the phylogenetic picture, helping to more clearly see and evaluate the monophyly of other sections that have not been the subject of detailed study yet. Our approach resolves clades finely enough to provide a roadmap for revision of sectional circumscriptions in the near future. However, the use of a more limited subset of genes than in many of the studies cited above did not allow us to reconstruct some of the deepest relationships with any confidence (see Soltis et al. 2013), thus pointing to the need for a backbone phylogeny with additional genes and a smaller taxon sampling to recover fine-scale resolution in particular clades (e.g. Hinchliff and Roalson 2013; Waterway et al. 2015).

The specimen-level approach in our phylogeny differs from other very large phylogenies focusing on particular plant groups. Phylogenies built using data-mining techniques (above 500 entries; Hinchliff and Roalson 2013; Soltis et al. 2013; Roalson and Roberts 2016) do not generally aim for fully supported resolution at the tips of the tree, but for understanding the hierarchical relationships of deeper nodes. Thus, such studies merge sequences regardless of whether they belong to different individuals or not in order to decrease the amount of missing data in the matrix (but cf. Global *Carex* Group 2016). We did not merge sequences from different individuals, allowing us to test species monophyly and explore intraspecific variation.

*Toward a Global Phylogeny of Carex: New Systematic Insights in a Broader View—*New systematic insights are given by our reconstruction. The early-diverging branch structure of the core *Carex* clade agrees in part with Starr et al. (2015), which includes fewer species but a greater diversity of sections traditionally placed in subgenus *Vigneastra*. Both trees have a single species as sister to the rest of the Core *Carex* clade, but they are not the same: *C. dissitiflora* Franch. (sect. *Mundae*) in the Starr et al. (2015) tree, and *C. bostrychostigma* Maxim. (sect. *Debiles* (J. Carey) Ohwi; Dai et al. 2010) in our query-tree (Fig. 7). *Carex bostrychostigma*'s placement is particularly surprising because it has traditionally been classified in subg. *Carex* (see below). The next split within Core *Carex*, between what Starr et al. 2015 called the “small Core *Carex*” clade, comprising species from sections *Decorae* (Kük.) Ohwi p. p., *Euprepes* Nelmes & Airy Shaw, *Graciles* Kük., *Indicae* Tuck. s. s., *Mapaniifoliae* Nelmes & Airy Shaw, and the “large Core *Carex*” clade with the remaining species, is not strongly supported in the query tree (Fig. 7). In our reconstructions, at least one species from sections *Decorae*, *Graciles*, *Indicae*, and *Mundae* were placed in a basal grade equivalent to Starr et al.'s (2015) “small Core *Carex*”

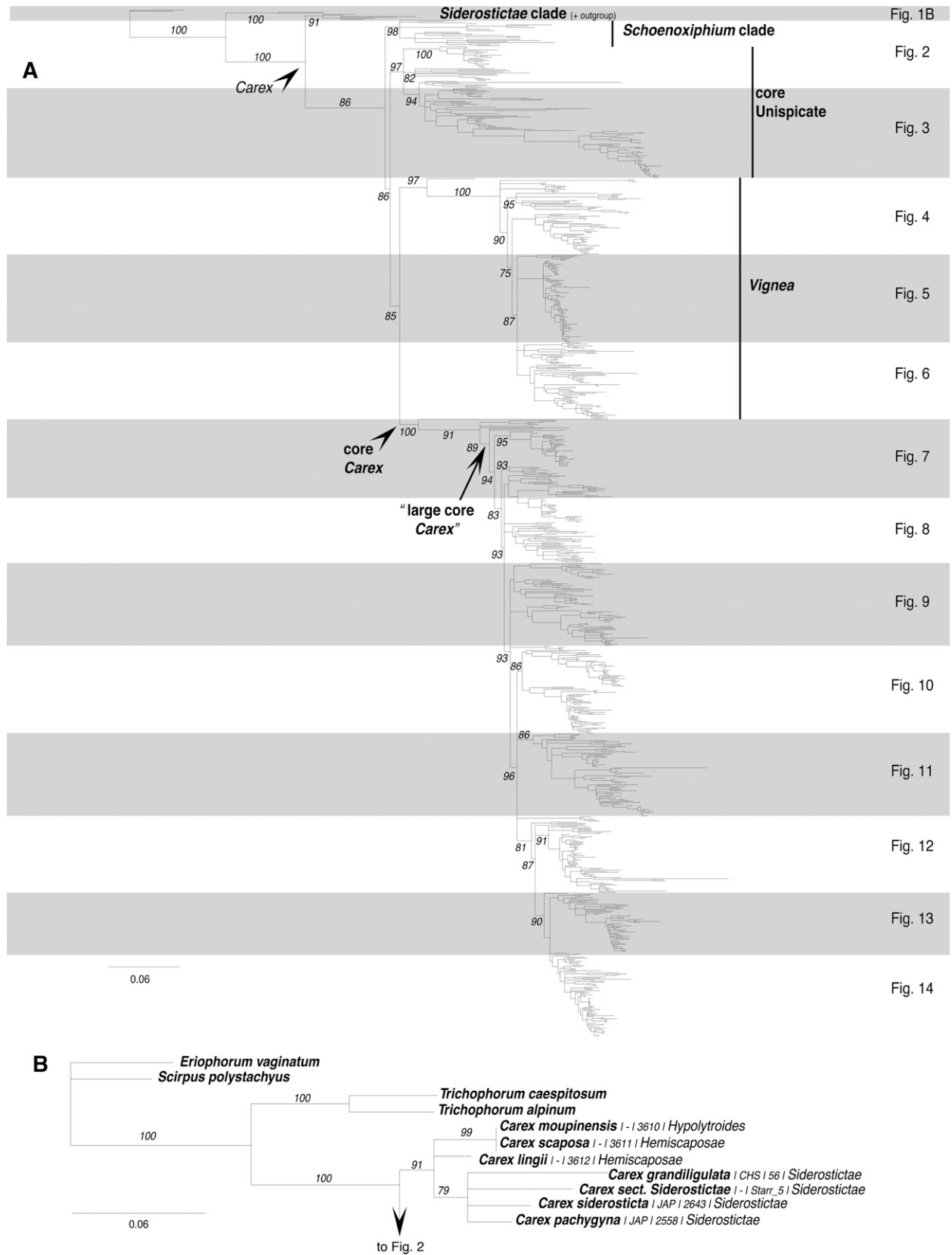


FIG. 1. A. Maximum likelihood phylogenetic hypothesis of *Carex* concatenated ETS, ITS, and *matK* gene regions. The tree from the all-nrDNA matrix has been used as reference tree. Sequences from the all-sequences matrix not included in the all-nrDNA matrix have been placed on the reference tree using the evolutionary placement algorithm. Major groups within the tree are named. Supports > 75 (aLRT) are given for major clades. The detailed view of each portion of the tree is shown in Figs. 2B–15 as explained at the right of the tree. B. Basal portion of the tree in Fig. 1A showing the outgroup plus the *Siderostictae*-clade. The labeling of the accessions is as follows: species | three-letter TDWG geographical code (as in Govaerts et al. 2015); occasionally we just provided two letters if the complete information for the sample was not in our database | accession number (or DNA isolation number if not available; see Supplemental Appendix 3) | section.

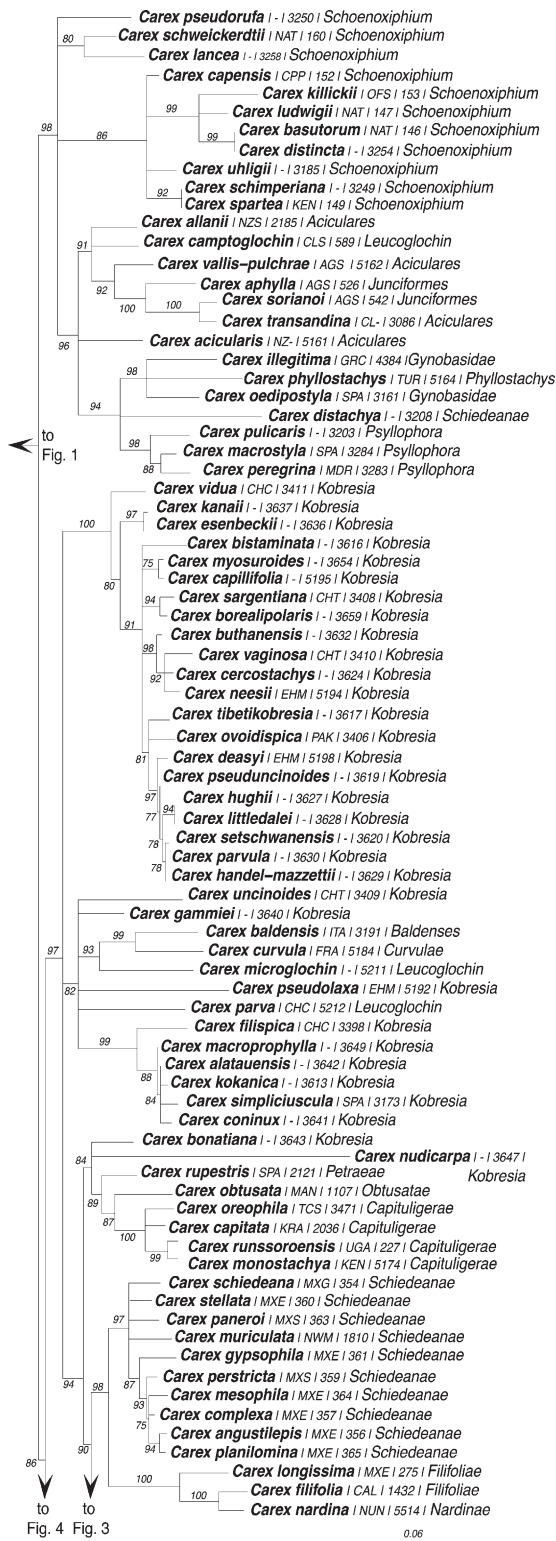


FIG. 2. Maximum likelihood phylogenetic hypothesis of *Carex* concatenated ITS, ETS, and *matK* gene regions (continued from Fig. 1 and continued in Figs. 3–14). The tree from the all-nrDNA matrix has been used as reference tree. Sequences from the all-sequences matrix not included in the all-nrDNA matrix have been placed on the reference tree using the evolutionary placement algorithm. Terminals have been pruned to show only one per included species. Supports > 75 (aLRT) are given next to the branches. The labeling of the accessions is as follows: species | TDWG geographical code (as in Govaerts et al. 2015) | accession number (or DNA isolation number if not available; see Supplemental Appendix 3) | section. An expanded version with all the included samples is presented in Figure S2.

clade, but other sampled species in section *Decorae* and the rest of the species of section *Indicae* were instead placed within an early-diverging lineage of the “large Core *Carex*” clade. The sections mentioned above in these early branching groups of *Carex* have generally been placed in subgenus *Vigneastra* and predominantly display paniculiform inflorescences, androgynous spikes, and utriculiform cladoprophylls harboring female flowers (although only in section *Mundae* does the achene eventually develop in the cladoprophylls; Dai et al. 2010). These characters have been regarded as plesiomorphic within the genus *Carex* (Reznicek 1990; Starr and Ford 2009). The assumption that this suite of characters is plesiomorphic in the subgenus *Vignea* has been previously refuted (Ford et al. 2006); however, having paniculiform inflorescences has been proposed as the most probable ancestral state in subgenus *Carex* (Molina et al. 2012), as it seemed to be supported by the topologies found to date (see trees in Waterway and Starr 2007; Starr and Ford 2009; Starr et al. 2015; among others). What is most surprising in our reconstructions (Figs. 1, 7) is that four species and groups of species with presumably derived morphology (racemiform inflorescences, unisexual spikes, and tubular cladoprophylls) are also found in these early-diverging lineages: 1) the aforementioned East Asian *C. bostrychostigma* (section *Debiles* (J. Carey) Ohwi; Dai et al. 2010), which was placed as sister to the rest of the core *Carex*; 2) the species of section *Albae* (Asch. & Graebn.) Kük. (*C. alba* Scop., *C. eburnea* Boott, *C. ussuriensis* Kom); 3) the Iberian endemic *C. durieui* Steud. ex Kunze (formerly section *Ceratocystis*; see Jiménez-Mejías et al. 2012b); and 4) *C. tristachya* Thunb. and *C. pseudotristachya* X. F. Jin & C. Z. Zheng (section *Mitratae* Kük.; Dai et al. 2010). These findings are significant in highlighting the uncertainty still surrounding the topology of the phylogenetic tree for core *Carex*, and suggest that caution is needed when evaluating characters as plesio- or apomorphic until a more complete picture of the entire genus is obtained.

Results for the *Vignea* clade were consistent with previous studies (Ford et al. 2006, 2012) in the strong support for the clade as a whole as well as for the placement of *C. gibba*, the sole member of section *Gibbae*, as sister to the rest of the *Vignea* clade. However, for the first time, representatives of the sections *Ammoglochin*, *Foetidae* (Tuck. ex L. H. Bailey) Kük., *Holarrrhenae*, *Phaestoglochin* Dumort., and *Physodeae* Christ ex Kük. formed a basal grade, whereas the rest of the members of subgenus *Vignea* were recovered in a well-supported clade (aLRT=97%; Figs. 1, 4). The species that are placed in this basal grade display spike-like inflorescences, which branch slightly or not at all at the base, and spikes that, when bisexual, are mostly androgynous (the only exception being *C. disticha* Huds., which sometimes bears female spikes with male flowers at the middle; Luceño et al. 2008). This possibly plesiomorphic state of androgynous spikes contrasts with the gynecandrous spikes of *C. gibba*. The suggested position of *C. satsumensis* Franch. & Sav., with androgynous spikes, as sister to the rest of *Vignea* (Starr et al. 2015) may play a critical role in evaluating the ancestral characters for this particular clade.

In any case, caution is still recommended in interpreting our results. The size of the dataset and nested-PCR approach to amplify poor quality herbarium samples appears to be very sensitive to contamination. Although we tried to ameliorate this risk by including more than one sample per species, it was not possible to do so for all included taxa.

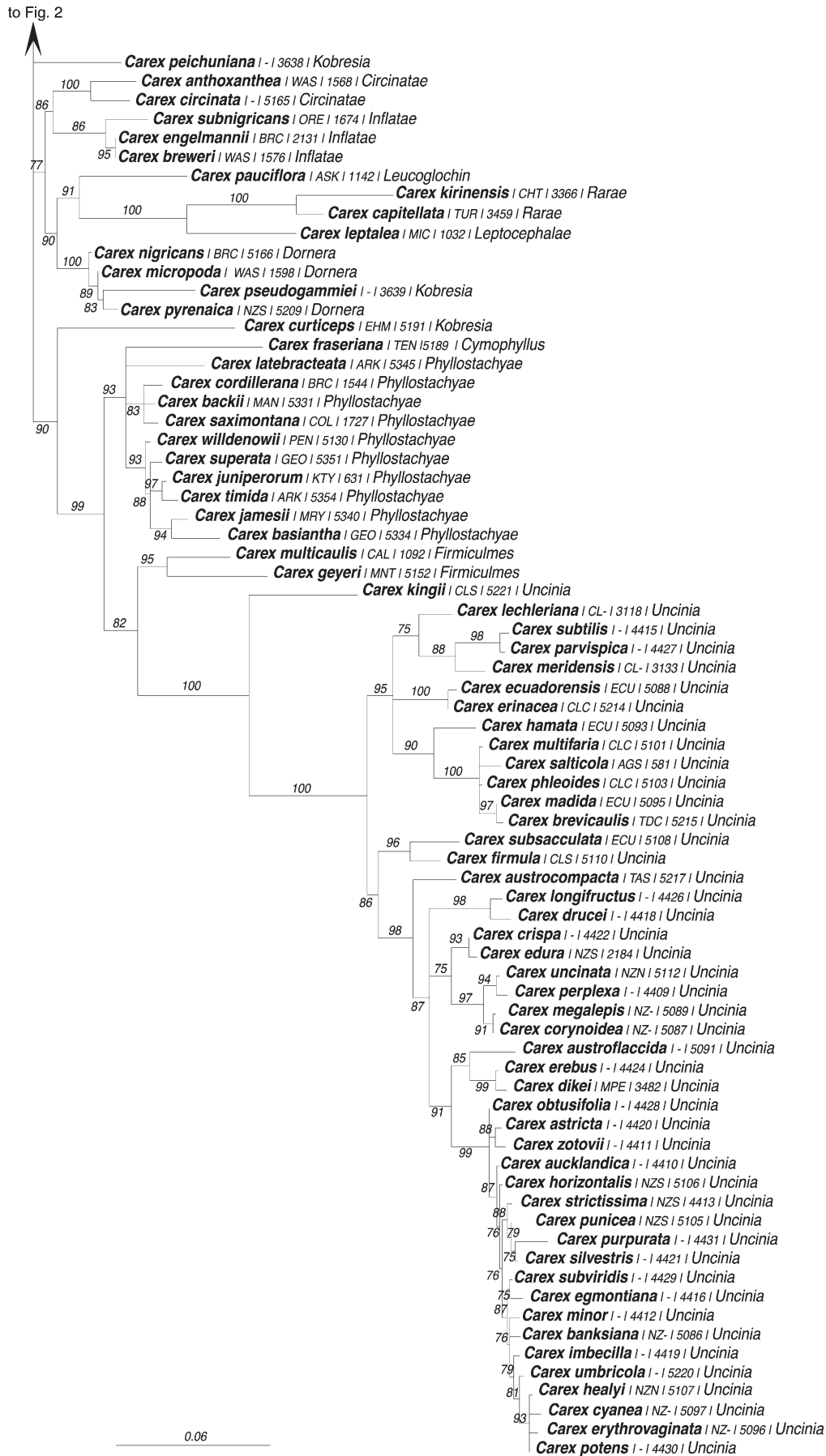


FIG. 3. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.



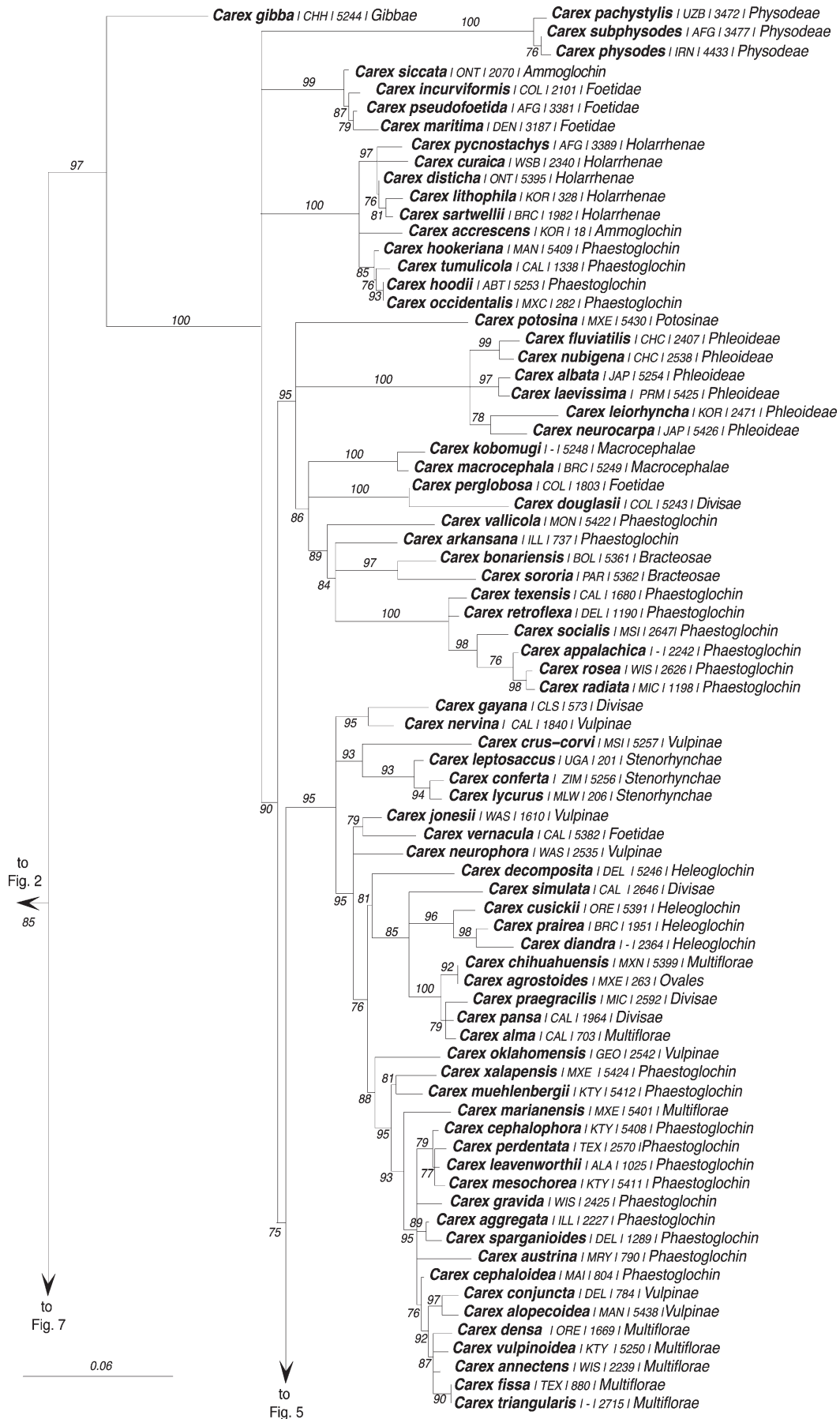


FIG. 4. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

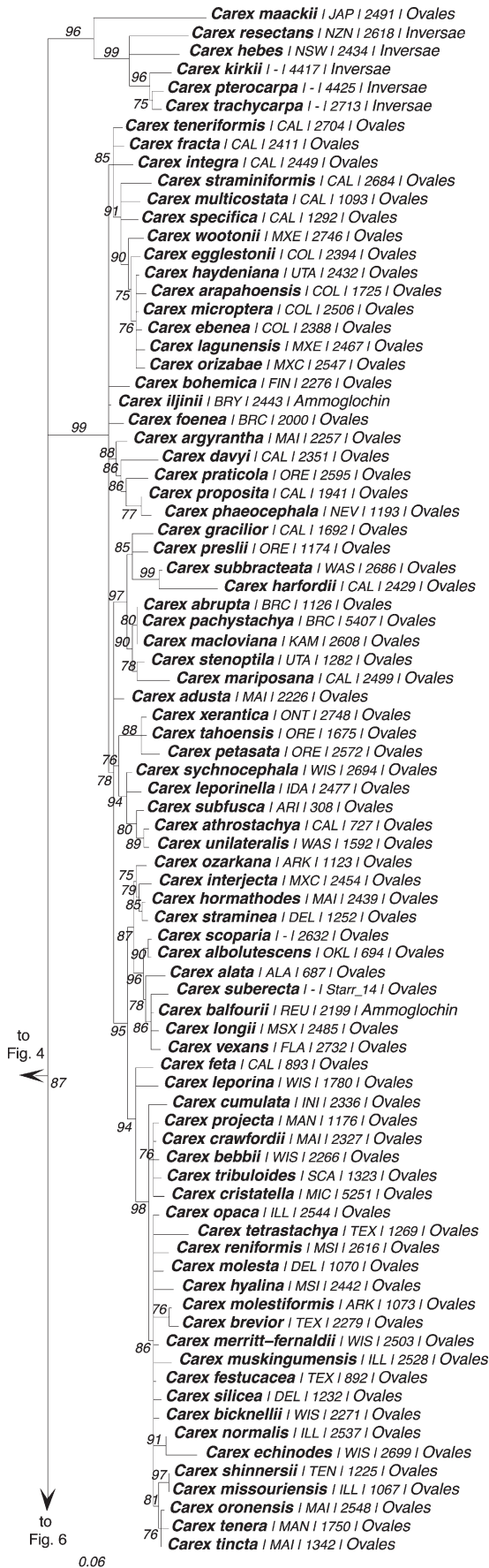


FIG. 5. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

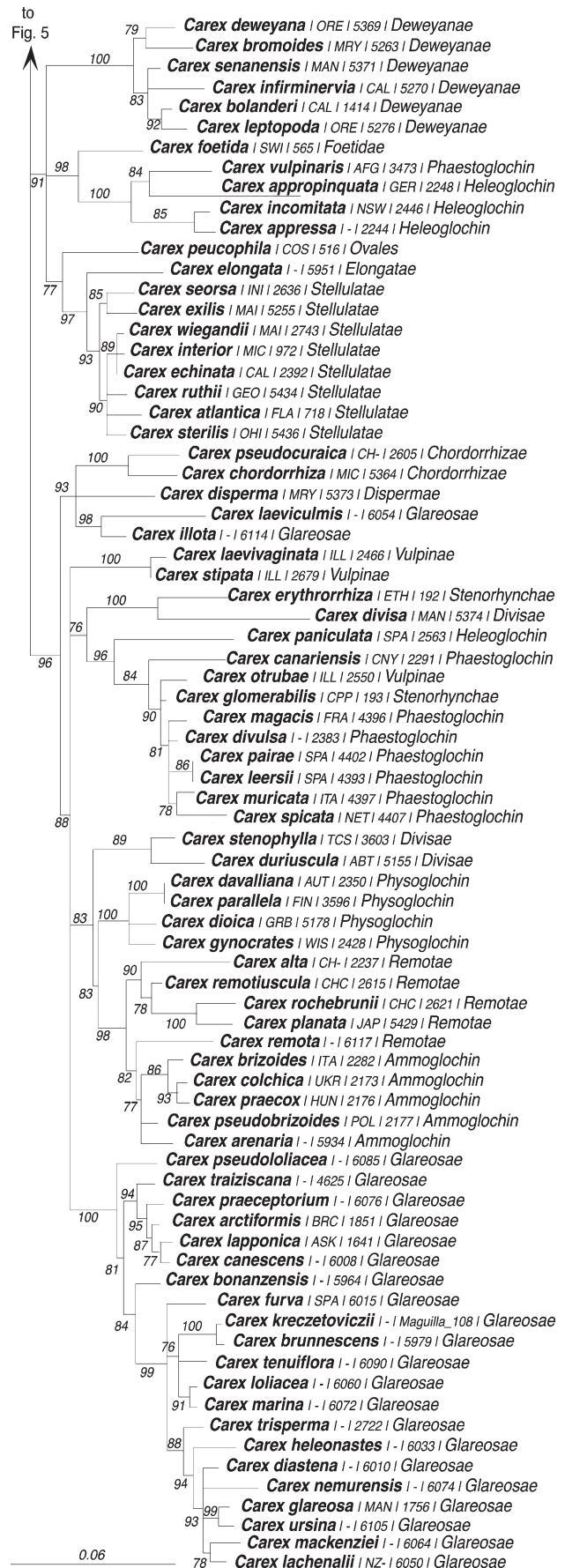
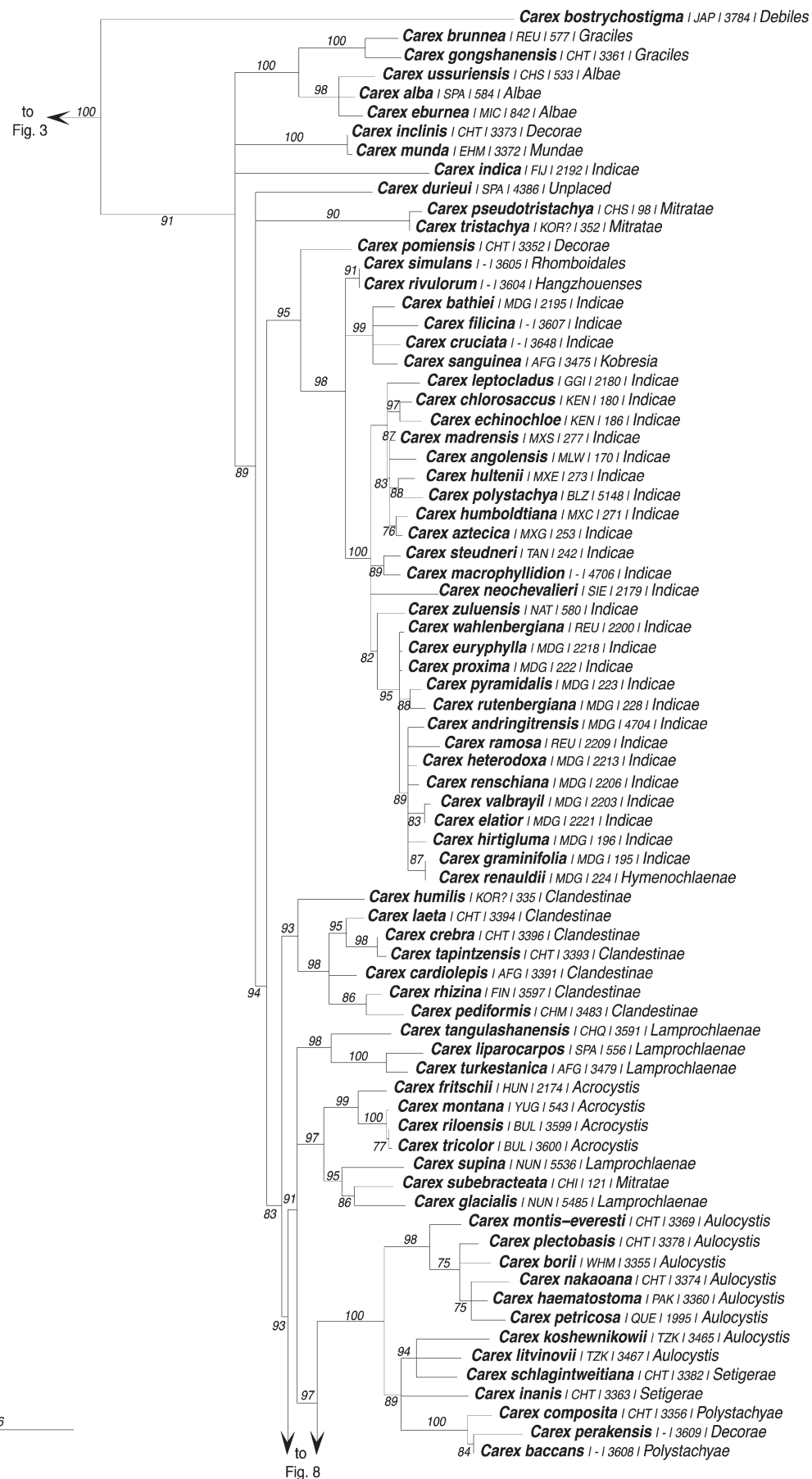


FIG. 6. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

FIG. 7. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

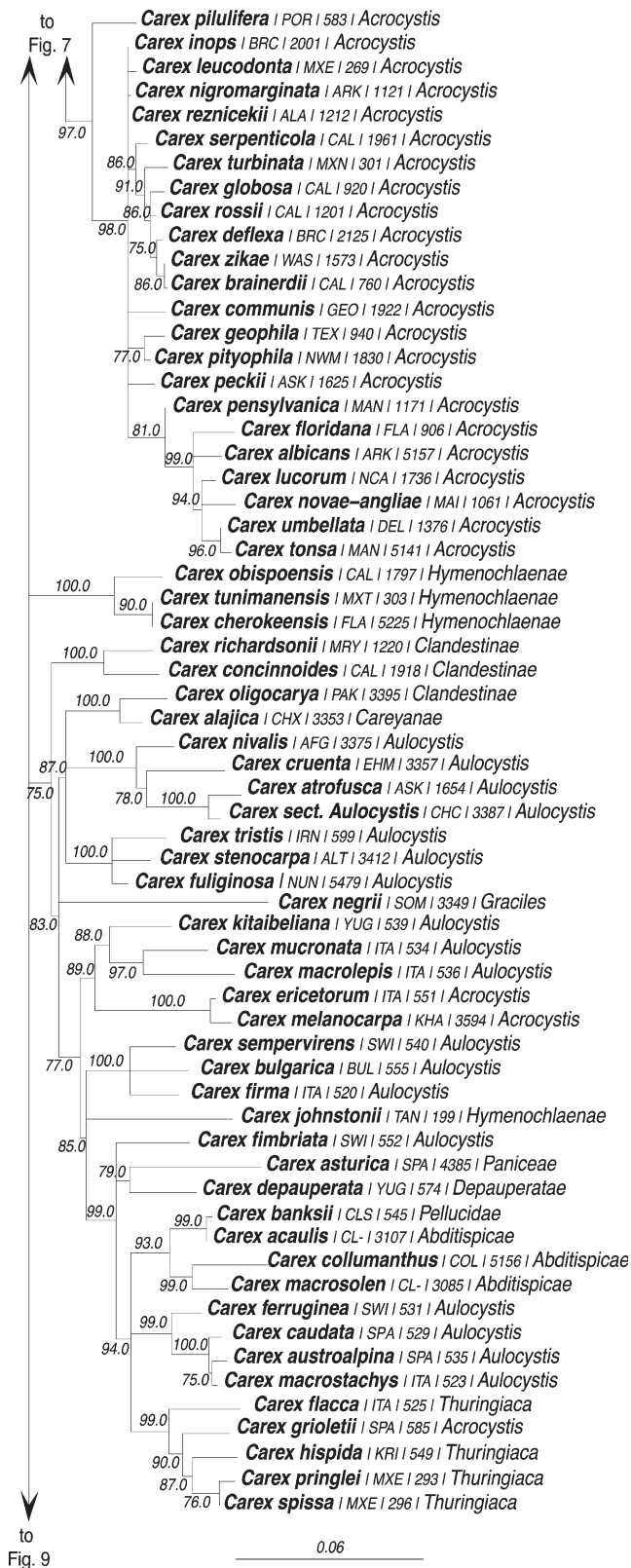


FIG. 8. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

Thus, isolated placement of particular species needs to be taken as tentative, especially if it is recovered among apparently unrelated taxa (e.g. *C. foetida*, Fig. 6; *C. iljinii*, Fig. 5; *C. ivanoviae*, Fig. 12).

**The Conflicting *Carex* Sectional Classification**—It is apparent that significant changes will be required to revise the sectional classification of *Carex* based on molecular data, just as such changes have been necessary in other plant groups (e.g. Larridon et al. 2011, 2013, *Cyperus*, Cyperaceae; Downie et al. 2010, Apiaceae; Harbaugh et al. 2010, Caryophyllaceae; Patchell et al. 2014, Cleomaceae; among others). Over the past several decades a general consensus has emerged that classifications based on common ancestry (i.e. phylogenetic classifications) should be followed, rather than alternative classifications that try to account for plesiomorphies and so accept paraphyletic taxa (i.e. phyletic classifications; e.g. Brummit 2014). Such systematic rearrangements have already significantly reorganized the traditional Angiosperm classification (Angiosperm Phylogeny Group 2009), and this has promoted similar changes at lower taxonomic ranks. In *Carex*, the first step toward a more natural classification has been performed by merging all the previously recognized genera in Cariceae into a more broadly circumscribed *Carex* (Global *Carex* Group 2015). However, as our knowledge about the phylogenetic arrangement of the genus *Carex* has broadened, the taxonomic scenario has become progressively more complicated. The dramatically increased sampling presented here reveals extensive polyphyly of sections as traditionally conceived. This is not only an elaboration of the already known cases of rampant polyphyly, such as sections in subgenus *Vignea* (Ford et al. 2006, 2012; Molina et al. 2015), the subgenera *Psyllophora* (Starr et al. 1999, 2004; Starr and Ford 2009) and *Vigneastra* (Starr et al. 2015), or the former genus *Kobresia* (Starr et al. 2004; note also the recovery of *C. sanguinea* Boott, formerly *Kobresia sanguinea* (Boott) Raymond according to Govaerts and Simpson 2007, within core *Carex* in this study; Fig. 7), but also sections that were thought to be morphologically well-defined are placed by our analysis into more than one clade (e.g. *Lamprochlaenae* (Drejer) L. H. Bailey, Figs. 7, 12, and *Mitratae*, Figs. 7, 9). A few large groups with morphological integrity remain largely monophyletic (e.g. sections *Glareosae*, Fig. 6, *Spirostachyae*, Fig. 11, former genus *Uncinia*, Fig. 3), but even in some of these, species from other sections are unexpectedly nested within them (e.g. section *Echinochlaenae* Holm nested within section *Spirostachyae*, Fig. 11). Polyphyly was also found, as expected, in the case of sections that have largely been considered taxonomic dumping grounds for “orphan” species (e.g. *Aulocystis* Dumort., Figs. 7–9, 11; *Clandestinae* G. Don, Figs. 7–10, 12; *Hymenochlaenae* (Drejer) L. H. Bailey, Figs. 7–8, 10, 13; and *Phaestoglochyn*, Figs. 4, 6), when monotypic or very narrowly defined small sections ended up nested within other larger groups (e.g. sect. *Granulares* (O. Lang) Mack. within sect. *Griseae* (L. H. Bailey) Kük., Fig. 9; sect. *Lupulinae* Tuck. ex J. Carey within sects. *Vesicariae* (Heuffel) J. Carey and *Phacocystis* Dumort. s. l., Figs. 12, 14), or after controversial treatments (e.g. the recent rearrangement of section *Careyanae* Tuck. ex Kük., Figs. 8–9, see Dai et al. 2010).

Problems with the current sectional arrangement are largely due to the homoplasy of characters that have been selected a priori as “relevant” in *Carex* taxonomy (Naczi 2009). Many examples of homoplasy can be found in different phylogenetic studies on *Carex* (Roalson et al. 2001; Ford et al. 2006; Hipp et al. 2006; Ford et al. 2012; Jiménez-Mejías et al. 2012b; Gebauer et al. 2015; Maguilla et al. 2015; Molina et al. 2015), some of which represent clear cases of reversions (e.g. three stigmata in section *Macrocephalae*, nested



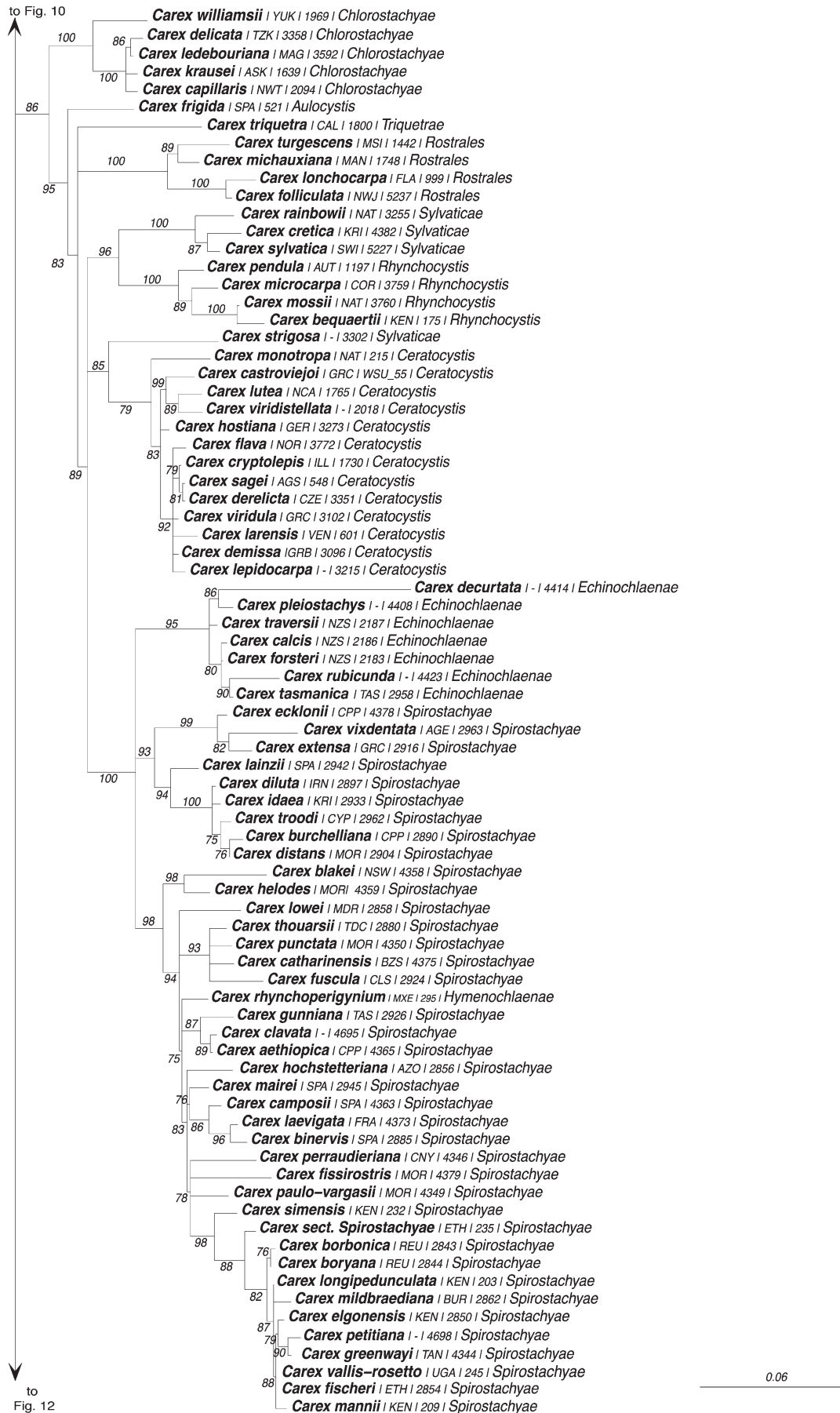
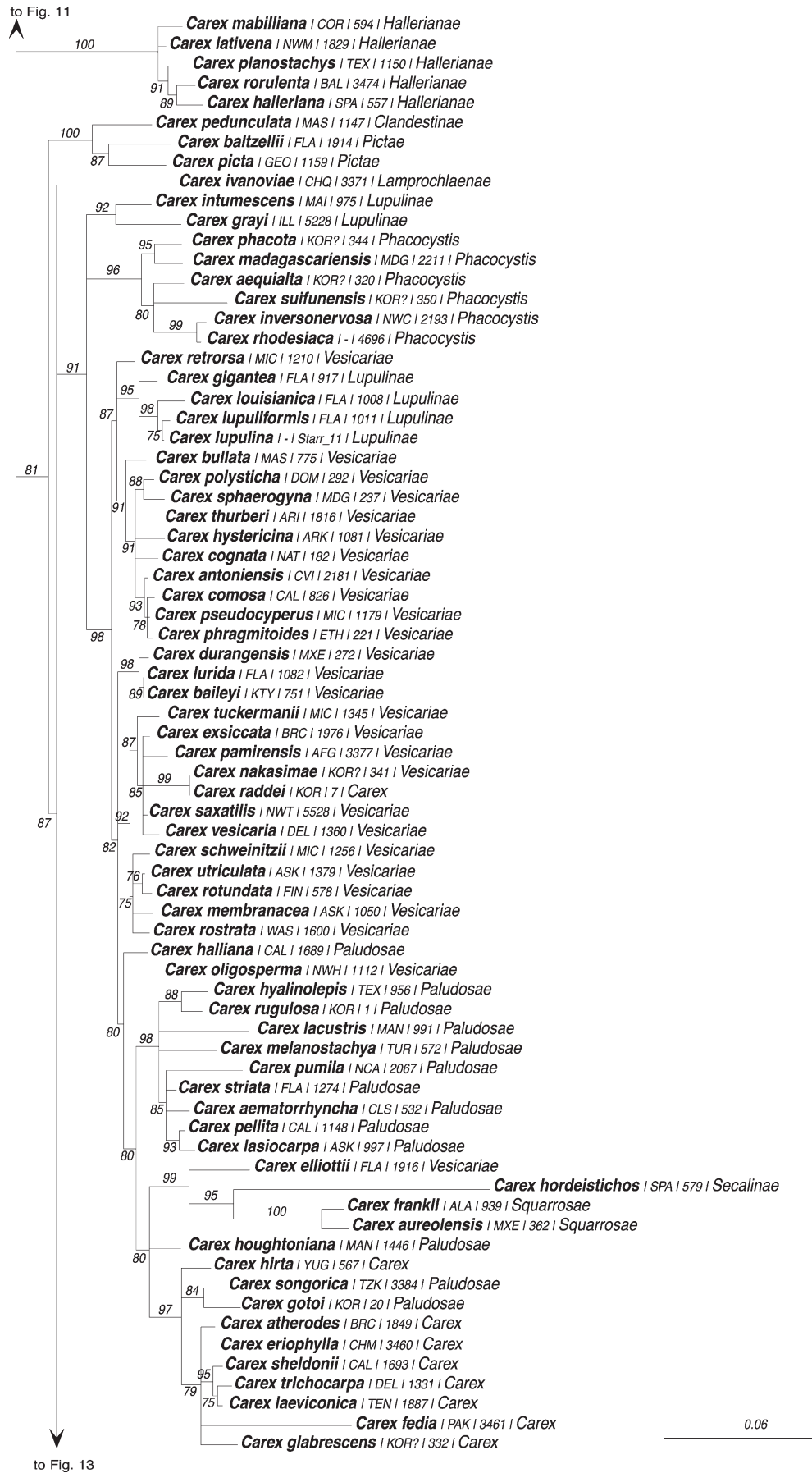


FIG. 11. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

FIG. 12. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

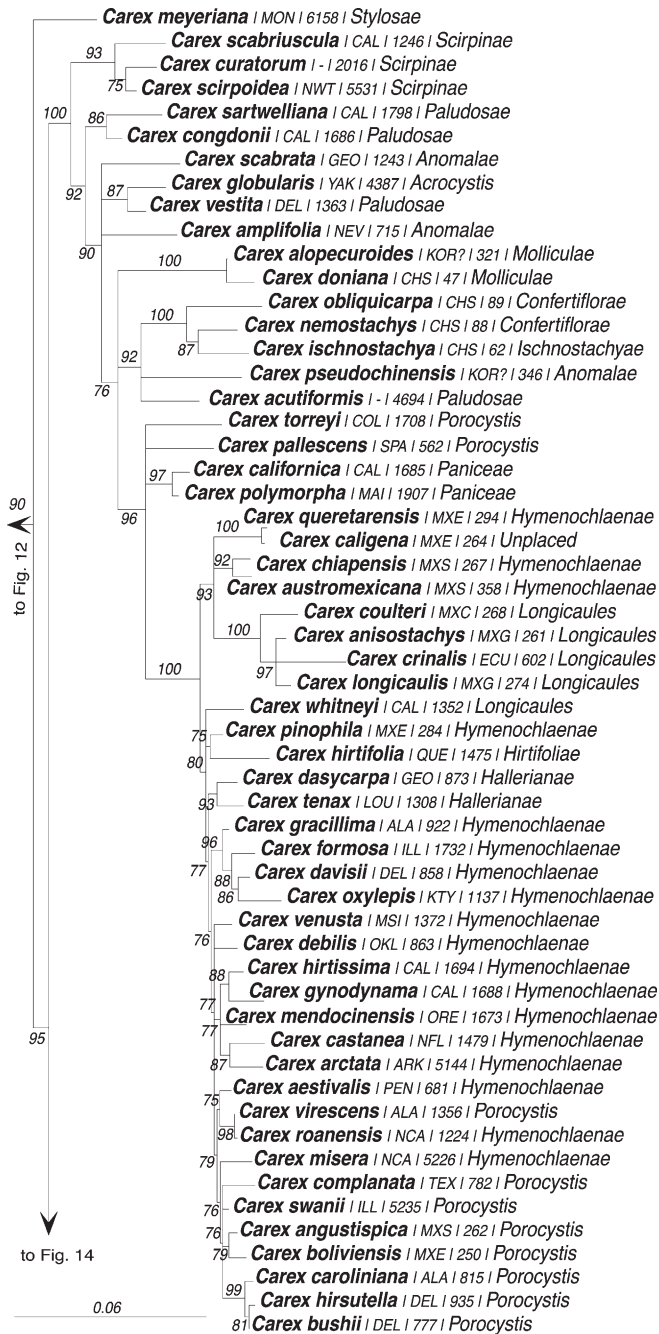


FIG. 13. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.

within the mostly two-stigmatic subgenus *Vignea*; Starr and Ford 2009), and affecting those characters believed to be highly conserved and key for higher ranks (Starr et al. 2004; Molina et al. 2012). This is especially evident within those sections that display the highest TDI values (Supplemental Data 3). Those are indeed formed by species whose morphology is quite characteristic but whose members have been split among some of the major phylogenetic partitions of the *Carex* tree (e.g. large paniculate inflorescences with bisexual spikes and utriculiform cladophylls in section *Indicae*; small reduced bisexual unispicate inflorescences with narrowly oblong, patent or deflexed utricles in section *Leucoglochin* Heuff.). The resemblance of the species within

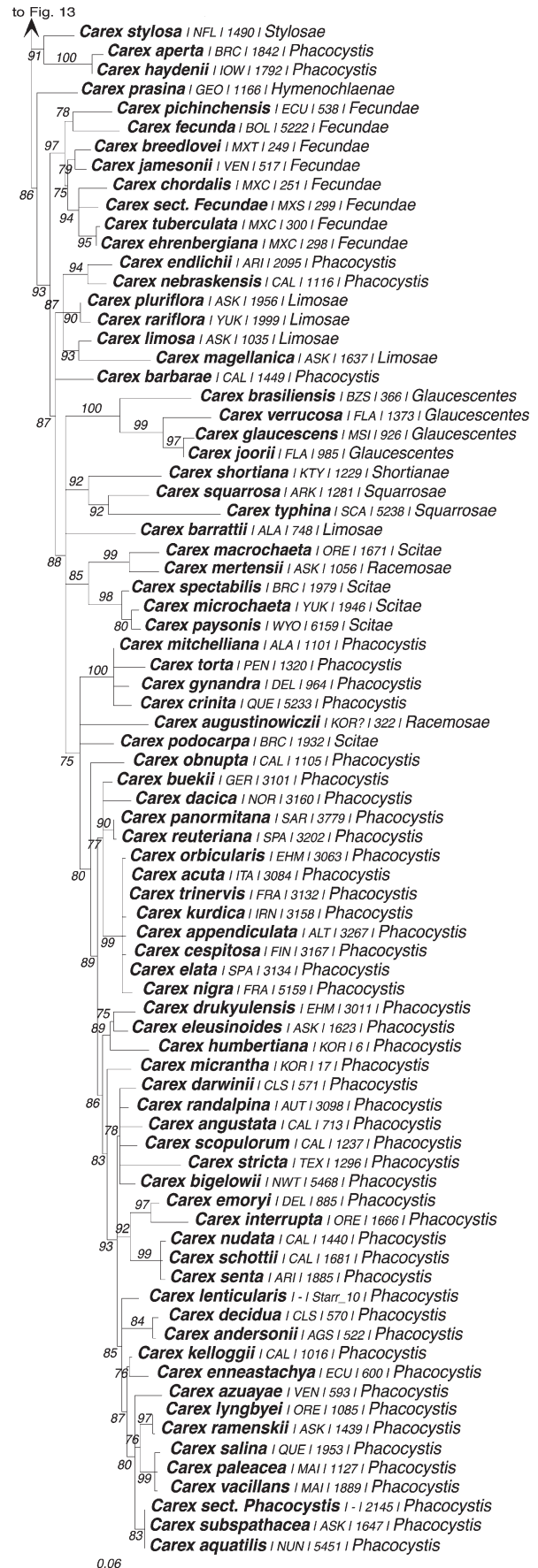


FIG. 14. Continuation of maximum likelihood phylogenetic hypothesis of *Carex*; see legend to Fig. 2 for details.



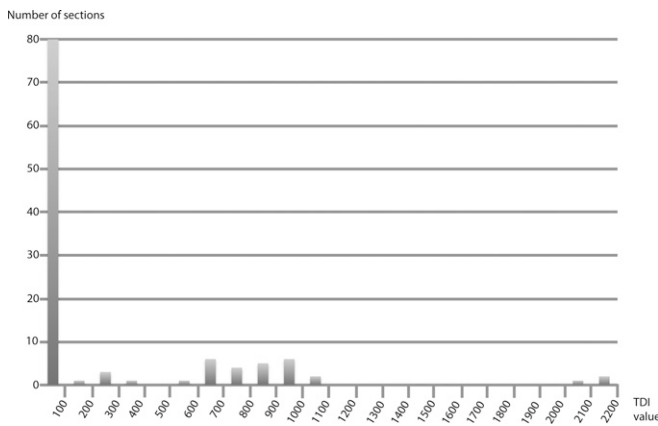


FIG. 15. Distribution of TDI (taxonomic disparity index) for the considered *Carex* sections sampled in this study.

these polyphyletic assemblages is so striking that some instances have been considered to be cases of almost perfect convergence (Escudero et al. 2010).

Homoplasy in *Carex* classification (and in taxonomy in general) should not be simply understood as the inappropriate selection of section-defining characters from a random set of morphological features. Polyphyletic classifications also result from an incorrect understanding of how the characters that vary have evolved within the genus. Examples of studies at the species level in *Carex* have shown that wider variation than expected blurs species limits and inflates the account of hybrids due to apparent intermediacy (Smith and Waterway 2008; Jiménez-Mejías et al. 2011, 2014; Řepka et al. 2014) as well as the number of species by the description of extreme variants as different species (e.g., *C. rivulorum* Dunn and *C. simulans* C. B. Clarke, see Global *Carex* Group 2015;

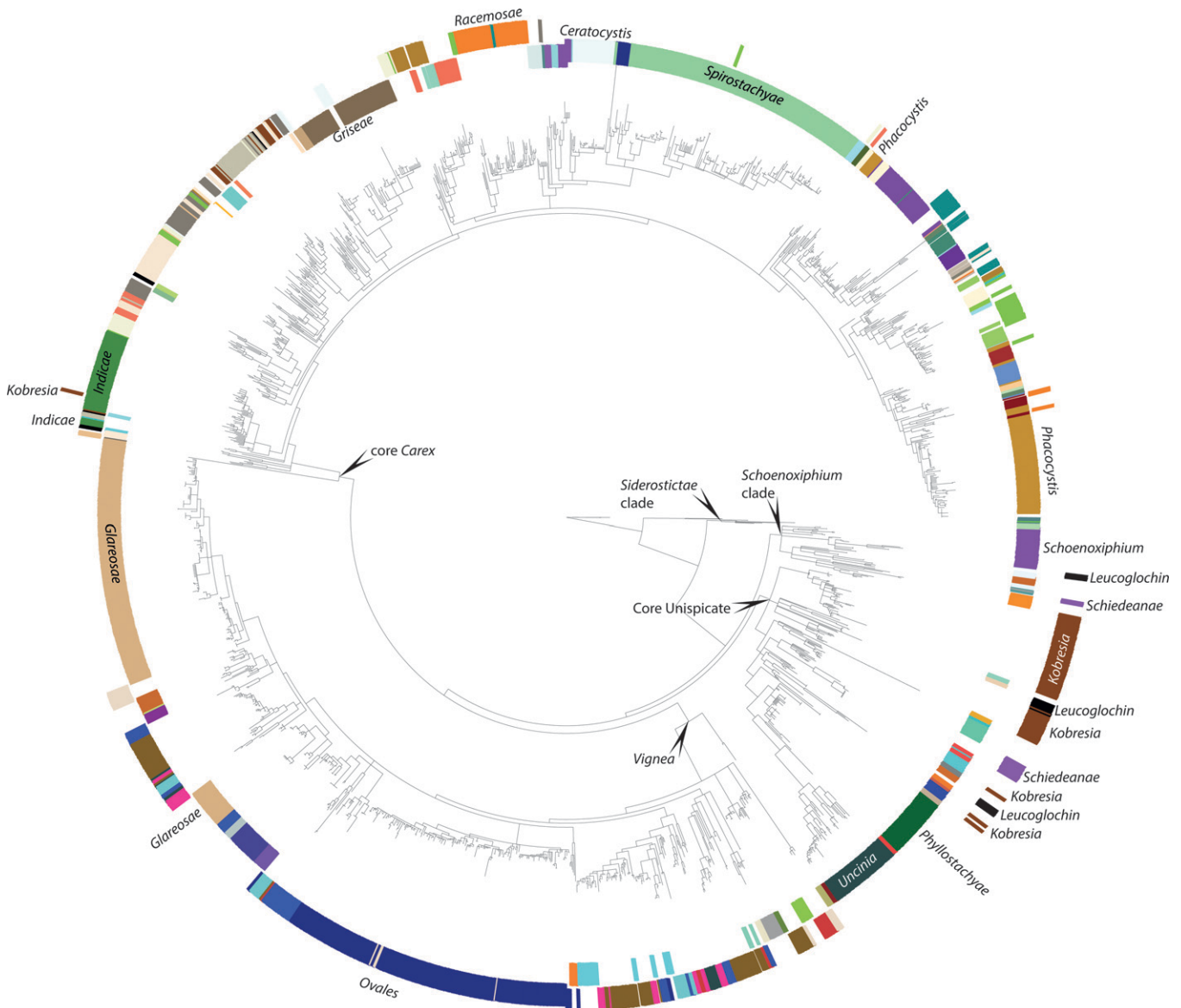


FIG. 16. Placement of sections on the query tree. The four formerly recognized genera of the tribe Cariceae (*Cymophyllus*, *Kobresia*, *Uncinia*, and *Schoenoxiphium*) are also displayed. Major groups and groups with the highest TDI values are labeled on the tree. The three concentric rings match the three clusters of sections following TDI values (Fig. 15), the outermost ring being the highest scores, and the innermost the lowest ones.

*C. nigra* (L.) Reichard, *C. juncella* (Fr.) Th. Fr. and allies, see Košnar et al. 2012; Jiménez-Mejías et al. 2012a, 2015). However, there are also many examples where the blurring of species limits is a consequence of an incomplete understanding of character state variation resulting in an underestimation of taxonomic diversity as was found in sects. *Griseae*, *Ovales*, and *Phyllostachys* (see Ball and Reznicek 2002). Misconception about which characters are variable and which are not is often the real source of the problem with morphologically-based classifications and the phylogenetic relationships revealed by DNA sequencing. A clear example in *Carex* is presented by the first inferences about perigynium evolution based on DNA phylogenetics. The character state “closed” for the perigynia of *Carex* was traditionally considered to be derived, whereas the state “open” (observed in the former genus *Kobresia*, and resembling in some cases a glume) was considered plesiomorphic (see Reznicek 1990). It was indeed the basis for considering *Kobresia* a distinct, allegedly more primitive genus than *Carex* (Kükenthal 1909; Egorova 1999). It was surprising for the *Carex* systematics community when the phylogenetic data revealed that the open perigynia of *Kobresia* evolved several times from the ancestral closed utricle (Starr and Ford 2009).

Several factors contribute to the difficulty of choosing characters that reflect phylogenetic relationships and are thus appropriate for defining sections. Definition of taxonomic groups based on local or regional diversity and the bias created by this non-global perspective are significant contributing factors. Taxonomists with an exhaustive knowledge of a local flora have tended to make taxonomic groups based on such geographically-biased sampling without a comprehensive prior knowledge of how their scheme fits into the diversity found in the rest of the world (see examples below). Remarkably, this geographical bias has had two radically different effects on the classification of *Carex*. In some cases, poorly known species were included within better-known groups, even if they did not quite fit, for lack of a better place to classify them, creating “wastebasket” sections. This has affected numerous classifications, including the large-scale revisions of Kükenthal (1909; see sections *Maximae* (Asch.) Kük. [= *Rhynchocystis* Dumort.] and *Spirostachyae*) and Egorova (1999; see sections *Sylvaticae* and *Microrhynchae* (Drejer) L. H. Bailey [= *Racemosae*]). On the other hand, quite divergent taxa have sometimes been accommodated within otherwise monotypic or small and narrowly defined sections if no close relatives are obvious (e.g. sections *Leptocephalae* L. H. Bailey or *Obtusatae* Tuck.).

To date, classification of *Carex* has relied largely on morphological characters, particularly those related to reproductive structures, with special emphasis on qualitative rather than quantitative characters (Gebauer et al. 2015). Based on the new relationships suggested by molecular phylogenetic hypotheses, this emphasis on reproductive characters appears inappropriate for the delimitation of natural groups of species. Studies prior to 2000 presented hypotheses of polarity, homoplasy and plasticity of characters in *Carex* that remain largely unsupported by phylogenetic hypotheses based on DNA sequence data (e.g. Reznicek 1990; Egorova 1999). Despite this lack of congruence between previously proposed classifications based on morphology and molecular phylogenetic hypotheses, it is probable that a new examination of morphological variation in light of the phylogenetic hypotheses may help to identify morphological characters

that can be used to define infrageneric taxa that are congruent with phylogenetic lineages, and thus natural groups. Many studies suggest the use of combinations of characters to define sections in a new classification (Hipp et al. 2006; Gebauer et al. 2015; Molina et al. 2015) as a probable way to circumvent the confounding effects of homoplasy when a few characters are considered independently (e.g. Maguilla et al. 2015). Also, the consideration of new non-traditional characters as sources of variation, e.g. anatomical and micro-morphological (Roalson et al. 2001; Naczi 2009; Proctor and Bradshaw 2014, 2015), carpological (Jiménez-Mejías and Martinetto 2013; Martinetto et al. 2014; Jiménez-Mejías et al. 2016b) and vegetative (Gebauer et al. 2015), seems promising to help develop a new sectional scheme, as it has already been proven to be useful in other large and taxonomically complex large groups (e.g. Larridon et al. 2011; Zamora et al. 2014). Character congruence as a goal of natural classifications has been criticized since the first molecular studies initiated the current stage of classification revision. Even today there is a defeatist trend in the consideration of morphologically-based taxonomy as a dying science in favor of using only DNA-based tools (e.g. Figueiredo and Smith 2015). However, there are more and more examples of morphologically-based revised classifications at different ranks which suggest that, rather than being impossible, the new delimitation of groups just requires careful study. Being able to circumscribe groups using morphology is a valuable tool that fosters a broader understanding of classification and makes identification possible using simple technology such as a magnification lens, thus making it accessible in the field and more understandable for the general public. This accessibility is critical for inclusion and outreach to ecologists, conservation biologists, biodiversity scientists, amateurs, and the general public, which is critical for the long-term synthesis of global biodiversity knowledge.

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