Genome size and GC content in the genus *Carex*

Introduction
The sedge genus *Carex* with around 2000 species is one of the most specious angiosperm genera (Reznicek 1990). Sedges exhibit some remarkable karyological characters unusual in other angiosperms: (*i*) diffuse centromeres (holocentric or holokinetic chromosomes), (*ii*) pseudomonad origin of pollen grain, and (*iii*) post-reductional meiosis (reviewed in Hipp *et al.* 2009). The first mentioned character, holocentric chromosome nature, have important consequences in the evolution of genus.

x. Agmatoploidy and symploidy in *Carex*
Chromosomal fission (agmatoploidy) in the most angiosperms results usually into deletion of generated acentric fragments, but in *Carex* due to its holocentric chromosome nature such deleterious effect of chromosomal rearrangement is not present. Although the viability is lower in structural heterozygotes (possessing unbroken chromosome together with its homolog fragments) (Dernburg 2001), new fragmented (or fused) karyotype could be easily homologized via selfing or backcrossing which fixation could result to establishment of new cytotypes or chromosomal races followed by forming of reproductive barriers resulting to new species origin (Hipp 2007). Based on these assumptions, the higher frequency of chromosomal fusion or fission in *Carex* and their key role in karyotype evolution of the genus is supposed by Heilborn (1924), Luceño and Castroviejo (1991), Escudero *et al.* (2008), Hipp (2007) and Hipp *et al.* (2007, 2009). The probabilistic models of karyotype evolution in *Carex* favorites gain or loss of individual chromosome as the most probable step in karyotype evolution in sedges suggesting that fission an fusion plays much important role than polyploidy (Mayrose *et al.* 2010) or even that exclusively fission and fusion proceed the karyotype evolution in this genus (Hipp *et al.* 2009).

The presence of symploidy and agmatoploidy in karyotype evolution in the genus is indirectly suggested also (*i*) by the wide range of chromosome number variation in the genus as a whole varying from *n*=6 to 66 (Tanaka 1949, Roalson 2008, Hipp *et al.* 2009, Rotreklová *et al.* in press); (*ii*) by the presence of meiotic trivalents frequently observed in natural populations (Wahl 1940, Faulkner 1972, Hoshino 1971, Luceño and Castroviejo 1991, Luceño 1994, Hoshino and Waterway 1994, Escudero *et al.* 2008), (*iii*) by occasionlay detected intraplant variation in chromosome number, observed for example in *Carex disticha* by Luceño (1992), (*iv*) by the presence of more than one cytotype within the species, as was documented in more than 100 *Carex* taxa (Roalson 2008, Hipp *et al.* 2009)

xx. Polyploidy in *Carex*
In *Carex*, polyploidy seems to be rarer compared other *Cyperaceae* (Hipp 2007, Hipp *et al.* 2007, 2009, Roalson 2008). The best documented example of intraspecific ploidy variation is *Carex siderosticta* where autotetraploidy was confirmed by the presence of tetravalents in meiosis by Tanaka (1940, 1949). The other examples of proven polyploidy in *Carex dolichostachya*, *C. jackiana*, *C. roraimensis* are reviewed in detail by Hipp *et al.* (2009).

xxx. Phylogenetic pattern in karotype variation in *Carex*
In the genus as a whole, the species with the smallest numbers of the largest chromosomes (*C. siderosticta*, *C. pachygyna*, and *C. ciliato-marginata*) belonging to the section *Siderosticae* form the basal clade of the whole genus (Nishikawa *et al.* 1984, Waterway *et al.* 2009). The evolutionary trend from lower to higher chromosome numbers in *Carex* was assumed already by Heilborn (1924). While at the broad evolutionary scale this Heilborn hypothesis seems to

be true, in more fine scale also reverted process was found to be probable at least at some sections as document recent probabilistic models of chromosome number evolution in *Carex* sect. *Ovales* (Hipp 2007, Mayrose *et al*. 2010) but as they suggested both descending and ascending karyotype orthoselection could be present in particular phylogenetic lineages resulting to. In another holokinetic genus from the same family, *Eleocharis*, species with the largest chromosomes of the type section seem to be more evolved while numerous and very small chromosomes are typical for species of basal phylogenetic lineages of subgenera *Limnochloa* and *Zinserlingia* (Yano *et al*. 2004, da Silva *et al*. 2010, Zedek *et al*. 2010), similar pattern might be present also in *Schoenus* (Bhatti *et al*. 2007). Actually the largest chromosomes of type section *Eleocharis* enlarged via massive proliferation of Ty1-*copia* LTR transposons (namely particular specific recently recognized family Helos1, Zedek *et al*. 2010).

xx. Genome size and GC content as an aditional phylogenetic parameters
The genome size diverzification is one of the most important evolutionary processes in plants, and number of recent studies have documented significant differences in genome size associated with evolution of taxa at different levels, groups of different ecological specialization or geographical origin (e.g., Leitch *et al.* 2005, Bancheva and Greilhuber 2006, Šmarda *et al.* 2008).
Informations about genome size in the genus *Carex* and family *Cyperaceae* are scarce relatively to the high species count. In Angiosperm C-value Database (Bennet and Leitch 2005), there are 49 species of *Cyperaceae* present with 36 entries for the genus *Carex* (approximately 2% of its species). Most of the sedge values come from a single study (Nishikawa *et al*. 1984) not directly concerning estimating of genome size, rest of *Cyperaceae* come from several studies involving just one or two species of this family. Values from the database suggest that sedges have relatively small genome size, they are around modal value of angiosperm genome sizes (0.56 pg/1C, Temsch and Greilhuber 2010). There are no published data for GC content in the genus *Carex* or family *Cyperaceae*, but the comparision with grasses (*Poaceae*), another monocot family, is possible. *Poaceae* exhibit extremely high portion of GC in their genomes (Barow and Meister 2001, Meister and Barow 2007, Šmarda *et al*. 2008). Although sedge family (*Cyperaceae*) is included in *Poales* clade (APG III, Stevens 2001 onwards), its holokinetic nature of chromosomes and generally lower genome sizes distinguishes it markedly from *Poaceae*. This genomic parameter, GC content, should be examined to find out whether it is or not on the levels observed in *Poaceae*.
Thus the knowledge of genome size and GC content can shed unique light on the understanding of evolution processes in this genus and its connection to well-studied chromosomal variability of the sedges.

xx. Aims
In the present study we would like to answer following questions: (i) What is phylogenetic pattern of genome size and genomic base composition within the genus? (ii) Is there general pattern in relation between genome size and chromosome number within the genus? (iii) Are they lineary related at least at some phylogenetic lineages suggesting presence of polyploidy or aneuploidy?

Materials and methods
Samples were collected in the field (Euroasian species) or in the Botanical Garden and Arboretum of Mendel University and in the Botanical Garden of Masaryk University, Brno (North American species) from 2006 to 2009. Voucher specimens of all of the plants were deposited in the herbarium at the Department of Botany and Zoology of Masaryk University,

Brno (BRNU). All of the samples were determined by R. Řepka and the species collected in Russia by A. E. Kozhevnikov (Institute of Biology and Soil Science of the Russian Academy of Science, Vladivostok, Russia). One plant was usually sampled per species. The nomenclature follows Egorova (1999) for Eurasian species and Ball and Reznicek (2002) for North American species.

xx. Genome size and GC content estimation

The term „genome size" refers to 1C value in whole study. The genome size and GC content were measured by flow cytometry using propidium iodide (PI) and the DAPI fluorochromes. Measurements were performed by flow cytometers CyFlow (Partec GmbH, Germany) equipped by 100 mW Cobolt Samba green laser and ploidy analyzer PA-1 (Partec GmbH, Germany). A two step procedure (Otto 1990) following Šmarda and Bureš (2006) was used. As a primary standard we used *Oryza sativa* subsp. *japonica* 'Nipponbare' (International Rice Genome Sequencing Project 2005), for the cases where rice was not appropriate we used internal standards *Lycopersicon esculentum* and *Carex acutiformis*.

xx. Chromosome counts

Chromosome numbers were estimated for 96 species of Carex (Rotreklová *et al*. in press), other counts were adopted from Roalson (2008).

xx. Phylogenetic tree

There is no phylogenetic study involving sufficient number of our target species, so we choosed to make phylogenetic tree with as much of our species as possible (108 species) from previously published ITS sequences from several studies (Hendrichs *et al*. 2004a,b, Starr *et al*. 2004, Ford *et al*. 2006, Starr and Ford 2009) from the NCBI Gen Bank database (Benson *et al*. 2010; for acession numbers see Fig. XXX strom). We choosed species *Scirpus radicans* and *Eriophorum vaginatum* as an outgrup according to phylogeny of the family *Cyperaceae* proposed by Muasya *et al*. (2009). Preliminary alignment was done in ClustalX (Thompson *et al*. 1997) with program's default settings for slow-accurate alignment. Obtained alignement was adjusted manually using method proposed by Starr *et al*. (2004) in program MEGA4 (Tamura *et al*. 2007). To choose appropriate model for phylogenetic analysis we used Modeltest (Posada and Crandall 1998) via online accesible FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). Bayesian analysis was performed in MrBayes 3.1 (Ronquist and Huelsenbeck 2003) using Markov Chain Monte Carlo algorithm run of 4 000 000 generations with tree sampled every 100th generation. A general-time-reversible model incorporating gamma-shaped rate variation with a proportion of invariable sites was used. Final tree topology was summarized using trees from all partitions after discarding trees from first 1 000 000 generations.

xx. Phylogenetic analysis

We used a method of independent contrasts (Felsenstein 1985) incorporated in Phylocom (Webb *et al*. 2008) modul AOTF (Ackerly 2006) to test correlations/relationship in phylogenetic frame. Genome size and GC-ratio weren't transformed, chromosome number was transformed to 1/2n. Obtained trees and results of phylogenetic analyses were displayed in program TreeGraph 2 (Stöver and Müller 2010). Statistical analyses with exception of analyses of independent contrasts were carried in STATISTICA 9.1 software (Stat Soft Inc. 2010). Spearman rank order correlations were run for the genus *Carex*, for two subgenera of genus *Carex* (subg. *Carex* and subg. *Vignea* as delimited in morphological systems (Reznicek 1990, Egorova 1999) and for the dataset of species used for phylogenetic analyses. Final phylogenetic tree correspond with trees published in previous studies (see above). We adopted

names for two main clades in phylogenetic tree from Waterway and Starr (2007) as a clade Vignea (species from the subgenus *Vignea* and both dioecious species of *Carex*) and clade core Carex (species of subgenus *Carex*).

Results

We measured 159 species of genus *Carex* (Tab. 1 lists minimum, maximum and mean 1C and GC content values for the whole genus and both main subgenera). Overall the genome size within the genus *Carex* ranges from 0.24 pg in halophytic *C. secalina* to 1.64 pg in italian subspecie *C. flacca* subsp. *serrulata*, GC content values ranged from 34.8 % in *C. secalina* to 40.6 % in alpine *C. firma.*

There are substantial differences in genome size between subgenera *Carex* and *Vignea* ($CV_{subg.Carex}$ = 41 %, $CV_{subg.Vignea}$ = 20.4 %), as the former exhibits wider range of genome sizes than the latter (Mann-Whitney U test, Z=5.576, p=0.000; Fig. 1 - boxploty). The variation in GC content also differ between these two subgenera ($CV_{subg.Carex}$ =2.5 %, $CV_{subg.Vignea}$=1.8 %), but not significantly (M-W U test, Z=1.315, p=0.189; Fig. 1). This is mirrored in phylogenetic tree, where the highest contribution index, showing which nodes on tree were important for changes in genome size and GC content, are the same for both characters. This highest index is associated with a node dividing clade Vignea and clade core Carex as there are relatively average genome sizes in clade Vignea and more divergent genome sizes in clade core Carex which contains species with the largest observed genomes of the sedges themself. Node with second highest contribution indixes is also common for genome size and GC content values, other contribution indices are lower of order of magnitude (Tab. 2).

Several sections defined in morphology-based systems exhibit good similarity in the genomic characters, like sect. *Phaestoglochin* (subg. *Vignea*) also known as *C. muricata* agg., sect. *Ammoglochin* (subg. *Vignea*) also know as *C. praecox* agg. or species from sect. *Ceratocystis* (subg. *Carex*) also known as *C. flava* agg. (Fig. 2ABC). There are also sections showing differnt pattern. It is case of sect. *Phacocystis* (Fig. 2D), where the genome size is quite stable but GC content differs in nearly 2 % (from 35.9 % to 37.8 %), or the most diverse sect. *Aulocystis* (Fig. 2E) with one of the smallest and the highest genome size and GC content values (0.38-1.15 pg, 35.4-40.6 %). Nevertheless, these sections form well supported monophyletic clades in phylogenetic analysis with exception of *C. frigida* belonging to sect. *Aulocystis* but with weakly supported different position on the phylogenetic tree along with *C. capillaris* (sect. *Chlorostachyae*) and *C. sylvatica* (sect. *Sylvaticae*). On the other hand, the sect. *Acrocystis* (Fig. 2F) splits to two groups per 3 species differing in genome size and GC content and members of this section are scattered across the clade core Carex.

We found strong positive correlation between genome size and GC content supported by analysis of phylogenetic contrasts (Tab 3a). This relation was significant in whole genus and both main subgenera. The relation between genome size and chromosome number was also found across the whole genus *Carex* and in both its main subgenera, but it wasnt proved by the analysis of phylogenetic contrasts (Tab. 3b).

Our data suggest possibility of polyploid origin of some species in sect. *Digitatae*, where doubled chromosome count in *C. callitrichos*, *C. lanceolata*, and *C. pediformis* subsp. *rhizina* is connected with twofold DNA content than in *C. humilis* and *C. pediformis* subsp. *macroura* (Tab. 4a; Rotreklová *et al*. in press). Another circumstantial evidence of polyploidy can be found in sect. *Microrhynchae*, where *C. aterrima*, *C. parviflora*, *C. atrata* and *C. norvegica* have almost doubled genome size and chromosome count in comparision with *C. buxbaumii* and *C. adelostoma* (Tab. 4b).

Discussion

Estimates of genome size obtained in this study do not fit well with those reported in Angiosperm C-value Database (Bennet and Leitch 2005). The differences may be due to unclear and probably not correct method of obtaing genome size values from the study of Nishikawa *et al*. (1984) in which just relative DNA contents are published. Our estimates suggest that genus *Carex* have genomes near the below margin of variation of genome size in angiosperm as a whole. Our results cannot prove the smallest sedge genome size (0.15 pg in Angiosperm C-value Database vs. our 0.24 pg) and we found the new highest genome size for sedges in *Carex flacca* subsp. *serrulata*. In more detailed comparision sedges have genome sizes like other members of the family *Cyperaceae* except the genus *Eleocharis* and somewhat comparable with values of sister family *Juncaceae*.

Comparin to grasses genus *Carex* has lower GC content (mean 43.7 % in *Poaceae* vs. 36.7 % in *Carex*), it is comparable to levels found in monocotyledonous *Liliaceae* or dicotyledonous *Urticaceae* (Meister and Barrow 2007). It is a question whether this lowering of GC content is result of decrease of before increased levels or it indicates that the increase of GC content in *Poaceae* came after split of lineages leading to *Cyperaceae* and *Poaceae*.

xx. Pattern of genome size and GC content in the genus

Found differences in genome size and GC content between subg. *Carex* and subg. *Vignea* are in agreement with general considerations of the state of these subgenera. Subgenus *Vignea* is considered to be a homogenous natural group, on the contrary the monophyly of subg. *Carex* as a large and highly variable group is object of discussions based on evidence from morphological (e.g., Koyama 1962, Reznicek 1990), phylogenetical (e.g., Yen and Olmsted 2000, Starr *et al*. 2004) or smut host-parasite studies (Nannfeldt 1977), reviewed in Starr and Ford (2009). The considerable difference between variation among these two subgenera also could indicate their difference in evolutionary age or rate of evolution. Relativelly uniform subg. *Vignea* could be somewhat recently diverged group of species in contrast to older and thus more diverged subg. *Carex*. Second possibility is that the subg. *Vignea* could be group of relatively old species with low rate of evolution and the subg. *Carex* could comprise species with higher rate of evolution.

The dicsrepancies in genomic characters on lower taxonomical levels (e.g., sections) can indicate irregularities in genome constitution (polyploidy, agmatoploidy, amplification of transposable elements etc.) or the polyphyletic state of target group of species. The latter could be case of sect. *Acrocystis* as mentioned in result. It is in agreement with several recent phylogentic studies, where the position of Eurasian species in this section was in doubt (Hendrichs *et al*. 2004, reviewed in Roalson and Friar 2004). In general it can be said that closely related sedges have and probably would have suchlike values of genome size and GC content, so that the evolution of genome size and GC content follow evolutionary trends in the genus.

xx. Relation of genomic parameters to chromosome numbers

Lack of phylogeneticaly independent relation between genome size and chromosome numbers suggest that chromosomal evolution, on the contrary of genome size evolution, is not directly connected with evolution of genome sizes and evolution of sedges at least for the analysed species. It indeed does not negate potential role of chromosomal changes in speciation of sedges as proposed by several authors (see Introduction). There is no general pattern in relationship between these two characters. Nevertheless, there seems to be several patterns in lineages inside the genus suggesting both polyploidy and fussions or fissions as a *mode* of chromosomal evolution in sedges.

We found indirect proofs of polyploid origin of some species in sect. *Digitatae* and sect. *Microrhynchae*. These polyploid events occur in one clade containing species from both

sections as can be seen from phylegentic tree (Fig XXX). Thus the polyploidy seems to play an important role in chromosomal evolution of this lineage of sedges.

In sect. *Aulocystis* different pattern is observed. Chromosome changes are not connected with changes in genome size as the increase in genome size is accompanied with decrease in chromosome number (Fig. XXX grafiky). The GC content increases with increasing genome size. This indicate possibility of enlarging of chromosomes via massive proliferation of GC rich transposable elements like in the genus *Eleocharis* (Zedek *et al*. 2010). Explanation involving trend of decreasing chromosome size and GC content with stable chromosome number would take in massive decrease of genome size via unequal homologous or illegitimate recombination deleting preferentialy GC rich regions at very high rates.

We found evidence of agmatoploidy, namely fission events, as a prevalent mode of chromosomal evolution in sect. *Phacocystis* in contrary to hypothesis of quantitative aneuploidy as an mode of evolution in this section as proposed by Shmid (1982) and Hipp *et al*. (2009). Roalson *et al*. (2006) suggest that the increase in number of chromosomes (aka fission events) could be connected with continual losses of genome sizes mediated by some losses of broken ends of DNA before healing of the new chromosome ends. In sect. *Phacocystis* such pattern is seen as number of chromosomes increase and genome size decrease with phylogenesis. Simply said, younger species have more chromosomes and lower genome size (Fig. XXX – bud na stromu nebo grafiky).

The decrease of genome size and increase of number of chromosomes in sect. *Phacocystis* is strikingly accompanied with decrease in GC content. There is no known mechanism explaining such situation, but some possible explanations could be found. One possible explanation arise from studies of role of chromosomal rearrangments in creating reproductive barriers in genus Carex (Hipp 2007, reviewed in Hipp *et al*. 2010) showing that the rearrengments should be somewhat larger than just few rearrangment events. Such larger genomic rearrangement would be connected with shift in GC content as complementary way to enlarging potential to create speciation barrier. Analogous situation was suggested by Feldman *et al*. (1997) in allopolyploid wheat, where ensuring of proper chromosome pairing after polyploid event seems connected with differentation of homeologous chromosome arms via several chromosome-specific regions. The differentiation in sequencies of these specific regions could be certainly associated with shift in their GC content. Neutral explanation cannot be left out as observed relation could be just efect of two independent processes, first one association of chromosome number increase with genome size decrease in way hypothesized by Roalson *et al*. (2006) and the second one independent amplifying of transposable elements with AT rich sequences.

Fussion events werent detected in this study. Nevertheless, with broader phylogenetic background a deeper insight into occurence of symploidy and agmatoploidy would be possible and thus it would facilitate stating if there is one prevalent way of chromosomal evolution in the genus *Carex*.

Conclusions

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Table 1. Minimum (min), maximum (max) and mean 1C DNA amounts and GC content for measured *Carex* species

1C	N	min (pg)	max (pg)	mean (pg)
genus *Carex*	159	0.24	1.64	0.46
subg. *Carex*	108	0.24	1.64	0.50
subg. *Vignea*	40	0.24	0.75	0.37

| GC content	N	min (%)	max (%)	mean (%)
genus *Carex*	159	34.75	40.61	36.70
subg. *Carex*	108	34.75	40.61	36.73
subg. *Vignea*	40	35.22	38.08	36.52

Fig. 1. Boxplots of genome size (1C) and GC content in two subgenera of genus *Carex*. The differences in genome size are statistically significant.
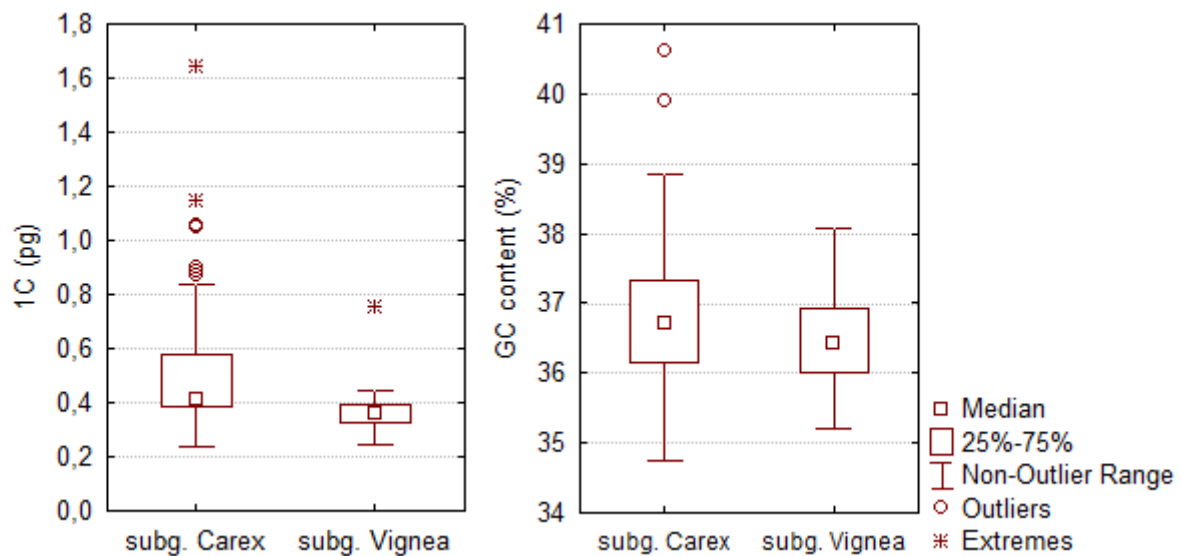
Tab 2. The top five divergencies making contributions to today 1C and GC content variation in genus *Carex*.

	1C DNA contribution				GC content contribution		
1C rank	score	P-value	Divergence description		GC rank	score	P-value
1	0.209		Divergence at base of clade Vignea and clade core Carex		2	0.120	
2	0.197		Divergence at base of clade core Carex		1	0.268	
3	0.111		*C. baldensis* and *C. pauciflora* vs. rest of sedge species		22	0.021	
4	0.101		Divergence between basal species and two main clades		11	0.038	
5	0.086		Divergence of *C. atrofusca* and rest of core Carex clade		7	0.051	

Fig. 2. Relation of genome size (1C) and GC content in selected setions of the genus *Carex*. Related groups of species usually exhibit similar values of these genomic parameters (**A** sect. *Phaestoglochin*, **B** sect. *Ammoglochin*, **C** sect. *Ceratocystis*), variability could indicate polyphyly (**D** sect. *Acrocystis*), proliferation of transposable elements (**E** sect. *Aulocystis*) or symploidy (**F** sect. *Phacocystis*). See text for further details.
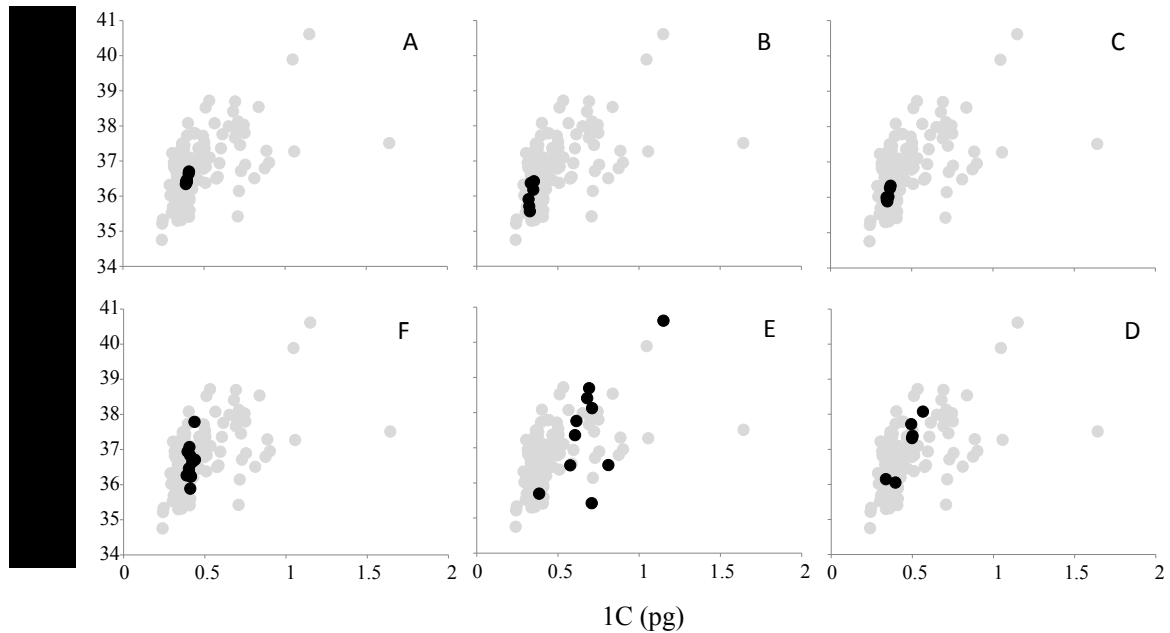
Tab. 3. Results of nonparametric correlations and analysis of phylogenetic contrasts of relation between genome size and GC content and chromosome number.

a) genome size versus GC content							
Spearman rank correlation	N	R	P-value	Independent contrast	N	R	P-value
genus *Carex*	157	0.588	0.000	1C vs. GC content	108	0.396	0.000
subg. *Carex*	108	0.660	0.000				
subg. *Vignea*	40	0.675	0.000				
b) genome size vs. chromosome number							
Spearman rank correlation	N	R	P-value	Independent contrast	N	R	P-value
genus *Carex*	150	-0.231	0.004	1C vs. 2n	108	-0.068	0.487
subg. *Carex*	102	-0.270	0.006				
subg. *Vignea*	39	-0.147	0.372				