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Uncovering ancient allopolyploidy through genomic evidence: A case study in *Carex* subgenus *Uncinia*

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ABSTRACT

The signature of allopolyploidy becomes increasingly blurred through time, hindering evaluation of its evolutionary role, and ancient hybridization remains difficult to detect even with large-scale genomic data. Understanding these processes is critical for reconstructing plant diversification at deep time scales. We focus on the enigmatic *Carex* subg. *Uncinia* (Cyperaceae), which shows unusual genomic and morphological traits and whose phylogenetic placement has long been controversial. To address this, we combined phylogenomic reconstruction with genome size estimates, allele divergence and locus heterozygosity analyses, and ploidy-level inference, together with phylogenetic network approaches. Our framework explicitly integrates allele-aware analyses that are often overlooked in plant phylogenomics. We find that genome size in *Uncinia* is approximately threefold larger than in other *Carex* lineages, consistent with an ancient polyploidy event. Phylogenetic networks further suggest that subg. *Uncinia* was originated through a hybridization event between subgenera *Euthyceras* and *Vignea* during the Early Miocene. This signal is reinforced by allele phasing and paralog analyses, with *Uncinia* gene copies clustering with different parental clades and phased alleles frequently segregating into opposite lineages. These complementary approaches reveal that *Uncinia* originated through ancient hybridization and allopolyploidy, and that its conflicting placement in previous studies reflects a reticulate evolutionary history. More broadly, our study highlights the power of combining genome size, network analyses, and allele phasing to uncover hidden reticulate processes in plants and emphasizes the importance of explicitly accounting for such processes when investigating diversification in complex evolutionary lineages.

1. Introduction

Polyploidy —the increase in nuclear genome number within cells, resulting in the presence of more than two complete sets of chromosomes in an organism— is a pervasive and evolutionarily significant feature of plant genomes. It has long been recognized as a major driver of speciation and diversification in angiosperms, with recent genomic analyses revealing that all extant flowering plants bear the

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imprint of recurrent whole-genome duplication (WGD) events (Wendel, 2015; Soltis et al., 2015). These events, followed by genome reshuffling, gene loss, and diploidization —the loss of duplicated genes and return to a diploid genome— have shaped the architecture and function of modern plant genomes (Conant et al., 2014; Wendel, 2015; Soltis et al., 2015; Qiao et al., 2019). Polyploidy often remains cryptic, with its signatures masked by subsequent genomic remodelling that can obscure the evolutionary signals accessible through conventional molecular or cytogenetic techniques. This genomic complexity challenges our ability to reconstruct evolutionary relationships and functional gene dynamics, and underscores the need for integrative approaches to uncover the hidden layers of polyploid history in plant lineages (Wendel, 2015).

Recent comparative studies using phylogenetic methods support the hypothesis that polyploid lineages diversify at higher rates than their diploid relatives (Landis et al., 2018; Han et al., 2020), challenging earlier perspectives and reinforcing the view of polyploidy as a key driver of plant diversity. Evidence linking polyploidy with diversification has been reported in several plant families such as Orchidaceae, Brassicaceae, Poaceae, Asteraceae, Liliaceae *s.l.*, and ferns (Amich et al., 2007; Marques et al., 2017; Qiao et al., 2019; Mandáková & Lysak, 2018; Heslop-Harrison et al., 2023), and related to rapid radiation in certain lineages (e.g. *Dianthus* (Caryophyllaceae), Balao et al., 2010; *Allium* (Amaryllidaceae), Han et al., 2020). However, polyploidy has also been considered to lead to evolutionary "dead-ends", lineages that fail to diversify and instead exhibit higher extinction rates compared to their diploid relatives (Mayrose et al., 2011). The so-called "dead-end hypothesis" proposes that while polyploidy may provide short-term advantages (e.g., ecological novelty or reproductive assurance), many polyploid lineages do not persist or generate diverse descendant clades. This contrasting idea is supported by comparative phylogenetic studies showing reduced diversification rates in polyploid lineages (Mayrose et al., 2011; Arrigo & Barker, 2012; Soltis et al., 2014) and remains a subject of ongoing debate (Mayrose et al., 2011; Soltis et al., 2014; Mayrose et al., 2015; Baduel et al., 2018; Hagen & Beaulieu, 2024).

Over the past century, methods for estimating the ploidy level in plants have evolved substantially, from microscopy-based Feulgen densitometry techniques to high-throughput sequencing (HTS) approaches (Doležel et al., 2007; Weiß et al., 2018; Soraggi et al., 2022; Sun et al., 2023). The advent of flow cytometry in the 1980s revolutionized the field, enabling rapid and accurate DNA quantification across numerous samples (Doležel et al., 1989, 1992, 2003). It has since become a standard in genome size estimation, greatly enhancing our understanding of genome size variation and its evolutionary significance in plants regarding polyploidization (Pellicer & Leitch, 2020, Pellicer et al., 2021). However, a characterization of both genome size and chromosome number is needed to properly identify polyploidy events (Pellicer et al. 2021). More recently, advances in HTS have allowed genome size inference through read depth and k-mer analysis (Straub et al., 2012, Vurture et al., 2017; Hesse, 2023; Natarajan et al., 2025), while genome skimming has become a powerful tool for capturing repetitive and organellar sequences in large-scale phylogenetic studies (Dodsworth, 2015).

Hybridization and polyploidy have long been recognized as major evolutionary processes in the order Poales, contributing to both diversification and taxonomic complexity (Hilu, 2004; Paterson et al., 2004; Soltis et al., 2009; McKain et al. 2016). Phylogenomic data now reveal multiple WGD events across the order, shedding light on its intricate evolutionary history (Wang et al., 2024). Complementary evidence from genome size analyses indicates a wide range of values—from very small to substantially larger genomes—often linked to variation in ploidy level (Winterfeld et al., 2025). Together, these genomic perspectives provide a more nuanced understanding of the evolutionary dynamics that have shaped Poales.

Cyperaceae is a large family within Poales (c. 5700 species; Larridon et al. 2021; Jiménez-Mejías et al. 2025) characterized by remarkable karyotypic and genomic diversity (Márquez-Corro et al., 2019; Johnen et al., 2020; Elliott et al., 2022). A defining feature of the family is the presence of holocentric chromosomes, which lack a localized centromere and instead exhibit diffuse kinetochore activity along their entire length (Hipp et al., 2009; Márquez-Corro et al., 2021; Márquez-Corro et al., 2023). This cytological peculiarity facilitates chromosomal rearrangements such as dysploidy (including chromosome fission and fusion), translocations and duplication, while allowing for a correct chromosome segregation. As a result, holocentricity enables rapid karyotypic evolution and may promote lineage diversification (Tena-Flores et al., 2014; Márquez-Corro et al., 2019; Johnen et al., 2020; Tribble et al., 2025). Some studies have documented frequent dysploidy and polyploidy in several genera within this family, often co-occurring as in *Schoenoplectus* (Tena-Flores et al., 2014) and *Rhynchospora* (Mata-Sucre et al., 2024). Allopolyploidy cases have been also documented in *Eleocharis* (Johnen et al., 2020). Altogether, these patterns highlight the role of hybridization, polyploidy, and holocentricity in Cyperaceae chromosomal evolution (Nowak et al., 2020).

Carex includes over 2000 species (Roalson et al., 2021; Jiménez-Mejías et al., 2025), which makes it among the five largest plant genera. Extensive chromosomal variation is known in the genus, though polyploidy appears to be infrequent with confirmed cases representing only a small fraction of its diversity (see Márquez-Corro et al., 2021). The best-studied polyploid group is found in subgenus *Siderosticta*, where *C. siderosticta* exhibits diploid ($2n = 12$), triploid ($2n = 18$), and tetraploid ($2n = 24$) cytotypes, the latter being the most frequent (Tanaka, 1940; Chung et al., 2013; Yano et al., 2014; Chung, 2024). Additional potential polyploid cases have been identified in a few small groups and isolated species by their increased chromosome number (see results in Márquez-Corro et al., 2021). Previous genome size estimates for *Carex* (Nishikawa et al., 1984; Bai et al., 2012; Chung et al., 2012; Lipnerová et al., 2013; Zonneveld et al., 2019; Elliott et al., 2022; Takahashi et al., 2023), have mostly reported a relatively small genome size (average 2C value of 0.88 ± 0.32 pg (Leitch et al., 2019). Exceptions to this include species such as the polyploids *C. siderosticta* (Nishikawa et al., 1984) and *C. flacca* (as *C. cuspidata*; Lipnerová et al., 2013). Our recent report (García-Moro et al., 2025) confirmed these patterns and, at the same time, revealed that certain species and groups have unexpectedly large genome sizes within other subgenera.

Carex subg. *Uncinia* is one of the six recognized subgenera (Villaverde et al., 2020; Jiménez-Mejías et al., 2025). Some of its members were previously treated as a separate genus, the former genus *Uncinia,* characterized by unique morphological traits that distinguished them from the remaining *Carex* species, while also forming a monophyletic group nested within the genus *Carex* (García-Moro et al., 2022). This lineage is known to have a problematic phylogenetic placement, varying between Sanger vs HTS approaches, and also between nuclear and plastid data (Jiménez-Mejías et al., 2016; Villaverde et al., 2020). Although these results could point to a hybrid origin of the lineage, this possibility has not been previously tested. The few available data on chromosome numbers for subg. *Uncinia* are known to fall within the larger end of the range observed for *Carex* (Márquez-Corro et al., 2021). Moore (1968) first reported for *Carex delacosta* $2n = 38$ (as *Uncinia brevicaulis*), but without citing any material or showing any illustrative plate. Remarkably, from an early report of New Zealand *Uncinia* karyotypes, 31 out 33 species have been reported to uniformly show $2n = 88$ chromosomes, except for *C. parvispica* (=*U. sinclairii*) and *C. subtilis* (=*U. elegans*) which had $2n = 94$–96 (Beuzenberg, 1970). In addition, based on its chromosome number, a tentative dodecaploid species was later reported (*C. perplexa*, $2n = 132$; Heenan and de Lange, 2001; de Lange and Murray, 2002). Regarding genome size, only four estimates are known for *Uncinia*: *C. meridensis* (2C = 2.7 pg; Bennett, 1982) *C. dikei* (2C = 2.89 pg; Chau et al., 2020) *C. uncinata* (2C = 2.83 pg; Elliott et al., 2022), and *C. fraseriana* (2C = 2.92 pg; Elliott et al., 2022), which stand as abnormally high for *Carex*. Genome size sampling for *Uncinia* has been considerably expanded (43 additional species) by the recent report by García-Moro et al. (2025), resulting in an average size of 2C = 3 pg for

the entire subgenus, therefore confirming notably higher values than the genus average (as previously mentioned).

Considering these unusual cytological features, together with the genome size and the ambiguous phylogenetic position of *Carex* subg. *Uncinia*, this lineage provides an ideal framework to investigate its evolutionary origin. To this end, we first conduct a comprehensive macroevolutionary survey of genome size variation across *Carex*. We then apply multiple cutting-edge, HTS-based approaches to infer ploidy levels, explore phylogenetic placement and potential reticulation events, and examine patterns of gene paralogy and allele distribution. By integrating evidence from these complementary analyses, we aim to clarify the evolutionary processes underlying the distinctive genomic and cytological features of subg. *Uncinia*.

2. Material and methods

Our analyses have a primary focus on testing whether subg. *Uncinia* has a hybrid origin involving subgenera *Euthyceras* and *Vignea*. Hereafter, for the sake of simplicity, when referring to these subgenera they will be mentioned as *Euthyceras*, *Uncinia* or *Vignea*. Fig. 1 summarizes the employed analytical pipeline detailing the main inputs, the analytical tools applied, and the outputs obtained at each step.

2.1. Genome size data across Carex phylogeny

We compiled more than 900 available genome size estimates for *Carex* species from recent literature and curated databases (e.g., Lipnerová et al., 2013; Leitch et al., 2019; Pellicer & Leitch, 2020; Elliott et al., 2022; Takahashi et al., 2023; García-Moro et al., 2025; see Suppl. Table 1). Species were grouped into subgenera according to the most recent accepted systematic classification (Villaverde et al., 2020; Roalson et al., 2021; Jiménez-Mejías et al., 2025). To explore how genome sizes are distributed across subgenera, we used the genomic-constrained Sanger phylogeny of *Carex* from Martín-Bravo et al. (2019) —the most complete to date— and pruned it to retain only the terminals for which genome size data were available. Genome size values were then mapped onto this pruned tree to visualize their variation across the subgeneric framework (Fig. 2).

2.2. Genomic sequencing

We compiled a genomic dataset of 50 species (49 from the genus *Carex* and one *Schoenoplectus* as outgroup), encompassing the phylogenetic breadth of the genus according to the megaphylogeny in Martín-Bravo et al. (2019). We made a particular emphasis on *Uncinia* (see Suppl. Table 2), for which we included at least one species from each currently recognized section (García-Moro et al., 2022). Two putative hybrids of subg. *Carex* and one of subg. *Vignea* were included as these could be relevant to interpret the results of allele analyses (see below; indicated in Suppl. Table 2). This sampling strategy was designed to maximize phylogenetic breadth while keeping the number of taxa within computational limits of downstream analyses (see Phylogenomic reconstruction below).

Most of our data derive from Hyb-Seq, combining previously published sequences with newly generated ones (Suppl. Table 2). For subg. *Siderosticta*, where sampling was limited, we complemented our dataset with Whole Genome Sequencing data obtained from GenBank. Full details, including BioProjects and sample information, are provided in Suppl. Table 2.

2.3. Ploidy level estimation for subgenus Uncinia species

To estimate ploidy levels on the genomic sequences we performed the bioinformatic workflow described in Viruel et al. (2023), which

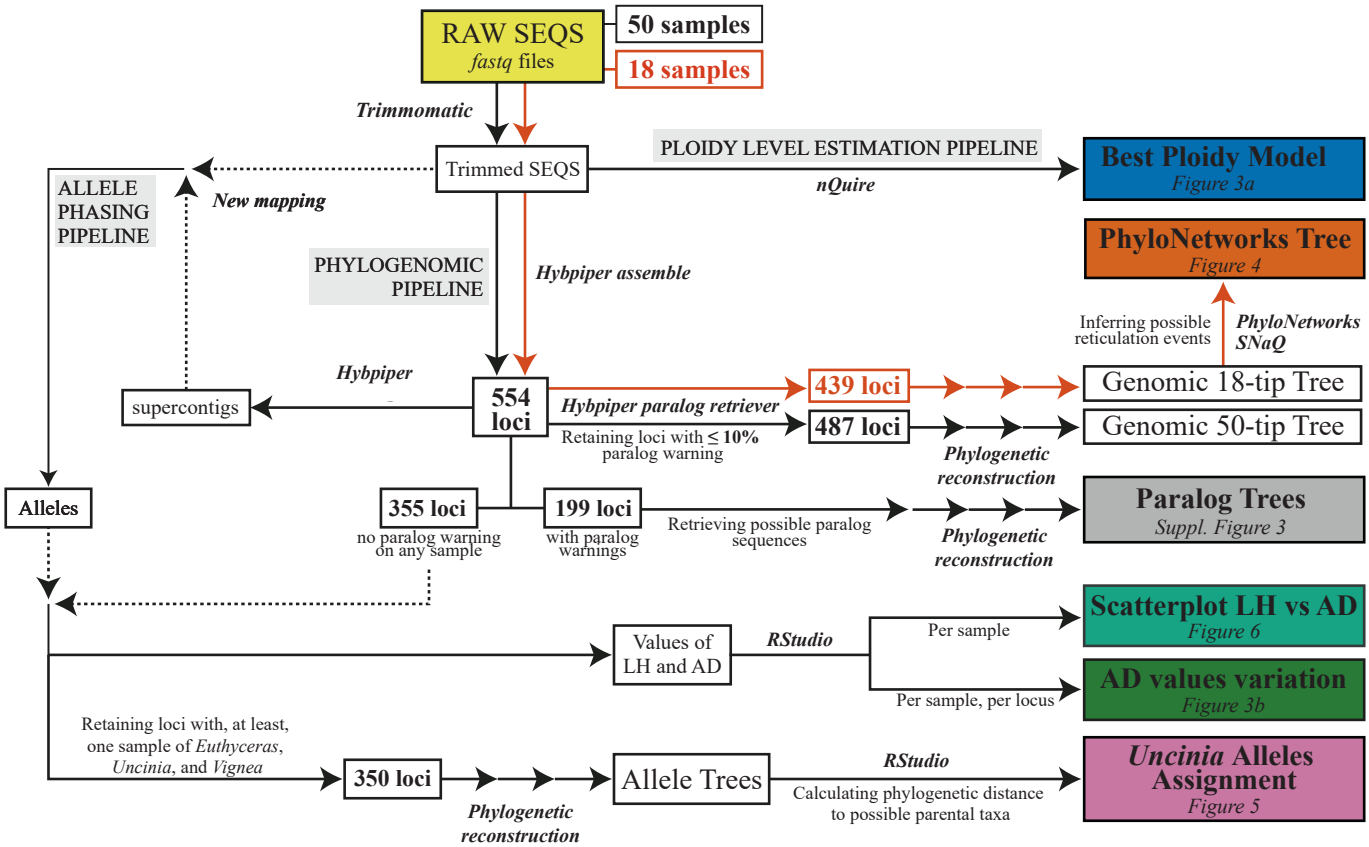

Fig. 1. Workflow of the genomic data and analysis used in the present study.

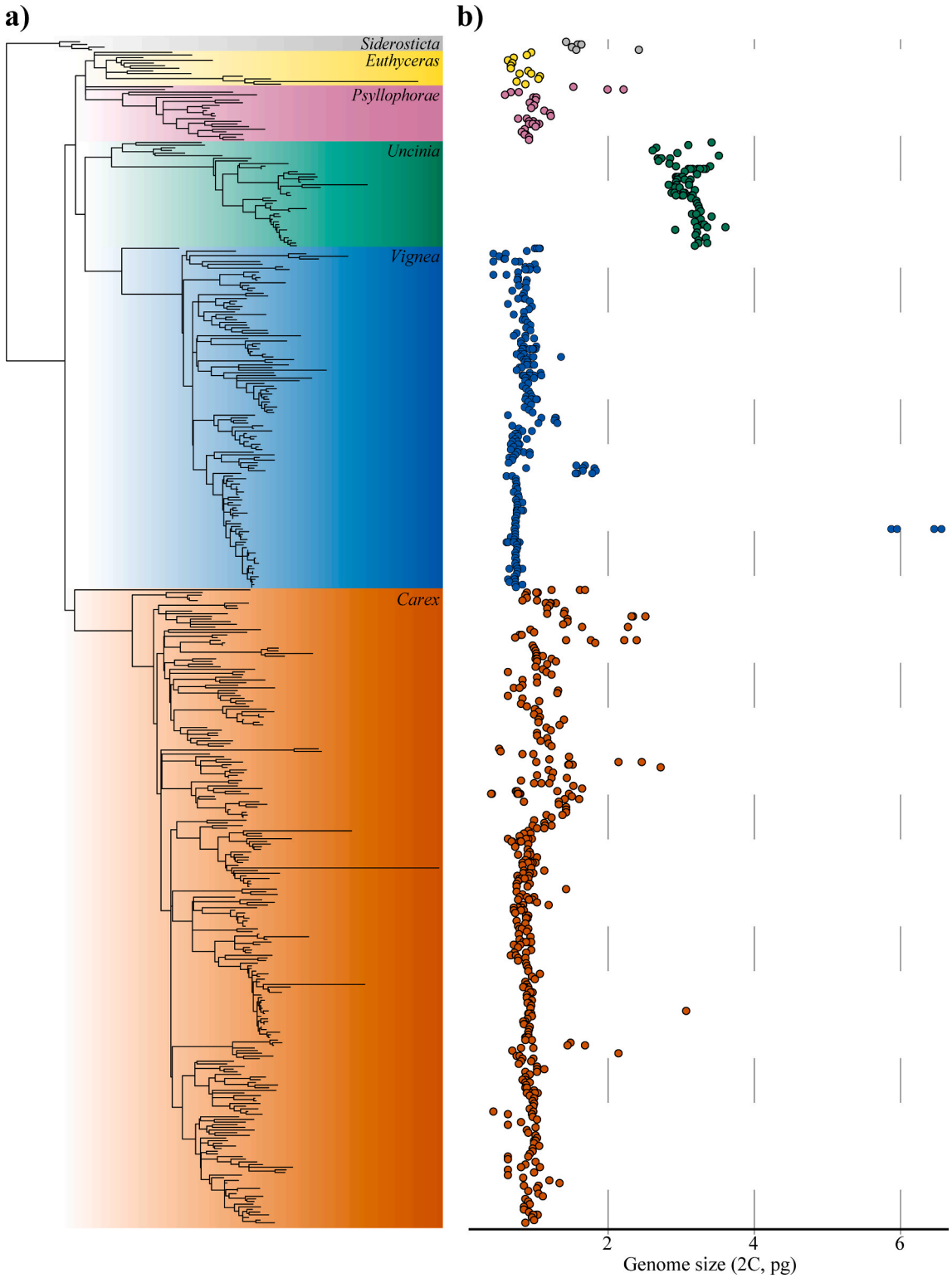

Fig. 2. Genome size distribution in the genus *Carex*. a) Most recent phylogenetic tree based on Sanger sequencing using ITS, ETS and *matK* (Martín-Bravo et al., 2019) pruned to the tips that have genome size data. b) Genome size for each tip in picograms (2C). Data for each subgenus is coloured accordingly.

estimates ploidy based on allele frequency distributions derived from target capture data. Raw paired-end reads were quality-checked and trimmed using **Trimmomatic v0.39** (Bolger et al., 2014) to remove adapters and low-quality regions. The custom bait set with target nuclear loci for Cyperaceae, designed by Villaverde et al. (2020), was first indexed using **BWA v0.7.17** (Li & Durbin, 2009), and we subsequently used this reference to map the cleaned reads against it. The resulting alignments were processed with **SAMtools v1.18** (Li et al., 2009) to generate sorted BAM files, which were then analysed with **nQuire v.201805** (Weiß et al., 2018). This software fits Gaussian mixture models to the distribution of biallelic SNP frequencies in order to distinguish between diploid, triploid, and tetraploid configurations. To reduce noise from sequencing errors, we evaluated multiple minimum allele frequency thresholds (0.05, 0.1, and 0.2), retaining the latter for

being the most stringent (Viruel et al., 2019; 2023). The best-fitting ploidy model for each sample was selected based on the highest coefficient of determination (R^2). These outputs were subsequently processed in **R v4.4.0** (R Core Team, 2025) to calculate allelic ratios, including the median, mean, and proportion of SNPs with ratios below 2, following the criteria outlined in Viruel et al. (2019; 2023).

2.4. Phylogenomic reconstruction and potential reticulation events for the subgenus Uncinia

We reconstructed the evolutionary framework of our focal group using the **HybPiper v2.1.6** pipeline (Johnson et al., 2016). We included the same 50 sequences presented above. Starting from the trimmed paired-end reads generated in the previous step, we mapped target nuclear loci using the HybPiper *assemble* function and the previously mentioned custom Cyperaceae baits (Villaverde et al., 2020). This function coordinates several tools to recover coding regions: **BBMap v39.01** was used for the initial mapping of reads to the target references; **SPAdes v3.15.4** performed the *de novo* assembly of mapped reads into contigs; and **Exonerate v2.4.0** aligned these contigs to the reference sequences to accurately recover the target regions. We ultimately recovered a total of 554 exon-derived loci through this pipeline.

To optimize phylogenetic inference, we manually screened all loci for evidence of paralogy and discarded any locus showing a paralog signal in more than 10% of the samples. This filtering step resulted in a curated dataset of 487 matrices retained for downstream analyses. Multiple sequence alignments were generated using **MAFFT v7.505** (*−auto*) (Katoh and Standley, 2013) and subsequently refined with **TrimAl v1.4.1** (Capella-Gutierrez et al., 2009) using the *−automated1* function, a heuristic approach based on similarity statistics. We inferred a gene tree for each locus using **RAxML v8.2.13** (Stamatakis, 2014) under a *GTR* model and 300 bootstrap replicates (Villaverde et al., 2020; Larridon et al., 2020). We subsequently used these gene trees to infer a coalescent species tree using **ASTRAL-III v5.7.8** (Zhang et al., 2018).

We evaluated potential reticulation events at deeper levels of the phylogeny, focusing on *Uncinia*. We ran ten independent network inferences using the *SNaQ* function, implemented in the PhyloNetworks package (Solís-Lemus et al., 2017), running in **Julia v1.10**. Given the computational limits of the function, that decreases its efficiency when there are more than 20 terminals (Hejase & Liu, 2016), we selected 18 taxa representing all *Carex* subgenera and all *Uncinia* sectional lineages (Roalson et al. 2021; García-Moro et al., 2022) and followed the same workflow explained above to obtain the corresponding coalescence and gene trees for the selected samples. *SNaQ* estimates networks under a pseudolikelihood framework by evaluating alternative topologies that incorporate up to a given number of hybridization events (*h*). We evaluated values of *h* ranging from 0 (strictly bifurcating tree) to 10 and selected the best-supported network based on changes in log pseudolikelihood and topological stability. After obtaining the best tree with reticulation events, we subsequently applied function *bootnet* to estimate bootstrap support for the branches of our network.

Divergence times were estimated using **treePL v1.0** (Smith & O'Meara, 2012) under a penalized likelihood framework. Analyses followed the approach of Martín-Bravo et al. (2019), with the optimal smoothing parameter selected by cross-validation, and fossil calibrations compiled in Jiménez-Mejías et al. (2016). Dating analyses were applied to the same phylogeny used to infer hybridization events. The crown node of the genus *Carex* was calibrated using the fossil *Carex colwellensis*, with minimum and maximum bounds of 33.9–38.0 Ma. The crown node of subgenus *Vignea* was constrained based on the fossil *Carex marchica*, with an age range of 16.0–23.0 Ma. The crown node of subgenus *Euthyceras* was calibrated using a secondary calibration fixed at 23.06 Ma, following Martín-Bravo et al. (2019). No calibration was applied to the crown node of *Uncinia*, allowing its divergence time to be inferred solely from the calibrated nodes elsewhere in the tree.

2.5. Analysis of paralogous and potential orthologous loci

To investigate potential paralogy for the sequenced *Uncinia* species and the phylogenetic affinity of the orthologous copies, we employed the *paralog retriever* function implemented in HybPiper (Johnson et al., 2016). This tool allowed us to systematically identify which samples and loci exhibited evidence of multiple gene copies. Briefly, the sequences were aligned with **MAFFT** and trimmed with **TrimAl** to remove poorly aligned regions, and Maximum Likelihood gene trees were inferred using **IQ-TREE v2.3.4** (Minh et al., 2020) with *−m TEST, −bb 1000,* and *−alrt 1000* options on each alignment. All resulting gene trees were individually examined to assess their topologies and detect potential paralogy. We discarded 40 loci that exhibited a complete duplication with each subset of copies yielding mirror phylogenetic relationships, which were considered true paralogs. Of the remaining loci, we focused on analyzing the phylogenetic distribution of *Uncinia* samples in those gene trees where subg. *Euthyceras* and *Vignea* formed monophyletic clades.

2.6. Allele phasing and assessment

For all the sequenced *Carex* samples, we explored the phylogenetic affinity of the potential alleles co-existing within a single locus. First, we retrieved *supercontigs* with HybPiper *retrieve sequence* function (Johnson et al., 2016). We subsequently assembled a new, sample-specific, reference set. These references were then used to map the raw sequencing reads and detect variants representing differences between a sample's DNA sequence and the new reference set.

Reference indexing and alignment were performed with BWA, while SAMtools was used for converting, sorting, and indexing the resulting BAM files. Duplicate reads were marked and read group information was managed using **Picard v2.25.0** (Broad Institute, 2019), generating cleaned BAM files suitable for variant calling. We called variants using **GATK v4.5.0.0** (McKenna et al., 2010): with the *HaplotypeCaller* function we generated individual genomic VCF files, followed by joint genotyping and variant selection with *SelectVariants* to retain high-quality SNPs. The resulting multi-sample VCF file was phased with **WhatsHap v2.6** (Patterson et al., 2015; Martin et al., 2016), which implements read-backed phasing to resolve phase blocks by connecting heterozygous variants supported by the same sequencing reads (e.g., Kates et al., 2018). We then ran *haplonerate.py* (Kates et al., 2018) to identify the longest phase block per sample and extract the corresponding allelic sequences. At this stage, either one sequence (indicating homozygosity) or two sequences (two alleles, indicating heterozygosity) could be recovered for each sample and locus. All sequences belonging to the same gene were merged into gene-specific FASTA files for downstream phylogenetic analyses. Phased VCFs were converted into haplotype-specific FASTA sequences using the *consensus* function in **BCFtools v1.18** (Li, 2011). The resulting files—containing one or two sequences per sample depending on the zygosity—were manually inspected to verify which samples were successfully recovered at each locus.

Following Kates et al. (2018), we aligned the exonic regions using **MACSE v2.07** (Ranwez et al., 2011). To maintain methodological consistency, we restricted this workflow to exon data, which is the type of nuclear DNA used throughout our phylogenetic tree reconstructions. Alignments were then trimmed with trimAl to remove low-quality regions and columns with excessive gaps. At this point, we applied a two-step filtering process: (1) we removed all loci that triggered a paralog warning in any sample, to ensure we retained only single-copy loci and maximize the likelihood of recovering true alleles (199 loci), and (2) we excluded loci whose alignments lacked representation from any of the three subgenera *Euthyceras*, *Vignea*, and *Uncinia* (5 loci). The result of these filterings was a final set of 350 loci alignments. Maximum likelihood phylogenies were inferred for each of these alignments using **IQ-TREE**, generating gene trees with branch length estimates. These trees were imported into R, and pairwise phylogenetic distances were

calculated using the *cophenetic* function from the *ape* package (Paradis & Schliep, 2019). In addition, to quantify whether *Uncinia* alleles were potentially more closely related to other subgenera representatives than to each other (see Results), for each *Uncinia* allele we determined the minimum patristic distance to the nearest terminal belonging to *Euthyceras* or *Vignea*. This distance-based approach provided an objective criterion for assigning alleles to one of the two subgenera regardless of branch support.

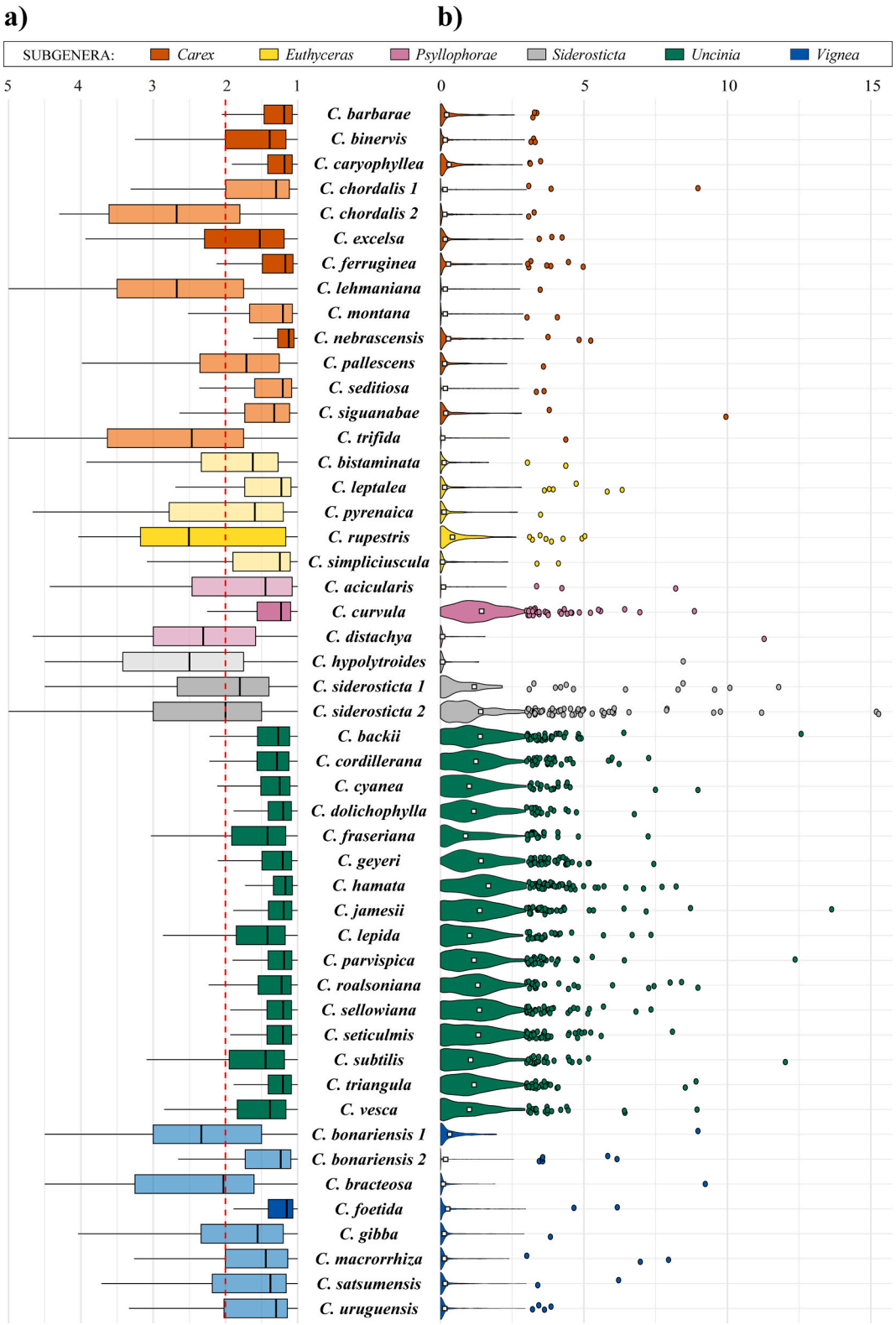

Fig. 3. a) Boxplots of the allelic frequency ratios obtained from nQuire. The red line indicates a ratio of 2, depicting the diploid threshold. Boxplots shown in full color represent samples with >2000 SNPs, whereas those with reduced color intensity indicate samples with fewer SNPs. b) Violin plots indicating the variation of Allele Divergence (AD) values across all sampled loci.

We developed a Python custom script to automatically score zygosity at each locus and calculate both the total length of the locus and the number of SNPs between alleles. These metrics were then used to calculate allele divergence values for each sample. We plotted locus heterozygosity (LH) against total allele divergence (AD), as these metrics may provide insights into potential polyploidy and hybridization (Hendricks et al., 2023). We also plotted density and violin plots for each sample to represent the variation of AD across individual loci.

3. Results

3.1. Genome size estimation and character distribution

In this study, we have significantly expanded the understanding of genome size across the evolution of the genus *Carex*. Specifically, we plotted 447 taxa, representing ca. 20% of the genus, all six subgenera (according to Roalson et al. 2021; Jiménez-Mejías et al. 2025) (Fig. 2). Notably, most *Carex* lineages demonstrate remarkable uniformity, typically slightly deviating from a median of 0.90 pg of 2C DNA content. However, all species belonging to *Uncinia* exhibit distinctly higher genome sizes, consistently falling within a range of 2C = 2.60–3.50 pg (Fig. 2). Furthermore, we observed within certain taxonomic groups, such as the Flacca Clade (subg. *Carex*), in which higher values around 2C = 2 pg are frequent, section *Inversae* (subg. *Vignea*), where particular species exhibited notable increases in DNA content compared to closely related taxa, or even single species in section *Cyperoideae* with extremely large genomes (i.e., *Carex longii* with 2C = ca. 6 pg). These findings highlight intriguing genomic variability within the genus and remark the subgenus *Uncinia* as particularly exceptional in its genomic composition.

3.2. Genomic sequencing

For our 49 *Carex* samples, obtained using the Hyb-Seq sequencing technique, we recovered more than 74% of target loci for all but four samples: two belonging to subgenus *Siderosticta* (6% and 7%) and two to subg. *Vignea* (8% and 35%). The number of sequenced reads in this dataset ranged from 728,908 to 79,421,024. One of our most remarkable findings was the number of paralog warnings recovered for *Uncinia* samples. The mean of "ParalogWarningsLong" in *Uncinia* (45.0) compared to the mean in the rest of the subgenera (12.1) indicates that values in *Uncinia* are approximately 3.7 times higher. A similar pattern is observed for "ParalogWarningsDepth", with a mean of 218.6 in *Uncinia* and 28.3 in the remaining subgenera, i.e. 7.7 times higher.

3.3. Ploidy level estimations with emphasis in subgenus Uncinia

The ploidy levels estimation pipeline inferred several instances of polyploid behaviour based on allele frequency for our samples, including species in the subgenera *Carex*, *Euthyceras*, *Psyllophorae*, *Siderosticta,* and *Vignea*. Despite polyploidy being regarded as rare in *Carex*, we found several instances in different subgenera, especially within *Siderosticta* (Fig. 3a).

The log-likelihood differences (ΔlogL) obtained from the nQuire analysis further reinforced the diploid assignment for all *Uncinia* samples. For each sample, the log-likelihood of the unconstrained (free) model was compared against those of the constrained diploid, triploid, and tetraploid models. In all cases, the diploid model yielded the smallest ΔlogL values (mean = 1,081.6; range = 269.4–4,212.5), while those for the triploid (mean = 23,625.6; range = 7,972.2–38,153.9) and tetraploid (mean = 16,198.4; range = 6,678.9–22,681.8) models were substantially higher (Suppl. Table 2). On average, ΔlogL values for the diploid model were approximately 22-fold lower than those for the triploid model and 15-fold lower than for the tetraploid model. This consistent pattern across all *Uncinia* samples confirms that the allele frequency distributions closely matched the expectations under diploidy and deviated markedly from those predicted under triploid or tetraploid scenarios. The log-likelihood comparison thus provides a robust statistical criterion to rule out any evidence of recent polyploidization within these individuals.

We also obtained low relative heterozygosity (0.99–2.47%) and median allele ratios around 1.2–1.5 (Fig. 3a) further supported this conclusion, matching the expected values for diploids as validated previously by Viruel et al. (2019).

3.4. Phylogenomic placement for the subgenus Uncinia and its ancient hybrid origin

The 50-tip species tree inferred under the multi-species coalescent model, based on 487 gene trees generated through Hyb-Seq data, revealed a well-resolved phylogenetic structure within *Carex* (Suppl. Fig. 1). According to this topology, subg. *Siderosticta* represents the earliest-diverging lineage and is recovered as sister to the rest of the genus. The remaining subgenera exhibit a sequential pattern of divergence: subgenus *Carex* is the next lineage to split off and appears as sister to the clade containing the other four subgenera. Within this clade, subg. *Psyllophorae* diverges next and is sister to a group comprising subg. *Vignea*, *Euthyceras*, and *Uncinia*. Finally, *Euthyceras* branches off as sister to the clade formed by both *Vignea* and *Uncinia*. All nodes corresponding to the origin of each subgenus are strongly supported, with posterior probabilities of 1.

In our 18-tip species tree used for the PhyloNetworks analysis the position of subgenera *Vignea* and *Euthyceras* appear swapped, with *Euthyceras* recovered as the sister clade to *Uncinia*. After obtaining all networks returned by the *SNaQ* function, we visualized each resulting topology individually in order to examine the placement and consistency of reticulation events across networks. Among these, the best-supported model was the one incorporating a single reticulation event. Plotting the negative log pseudolikelihood values against the number of allowed reticulations (*hmax*) revealed a pronounced decrease in slope from $h = 0$ to $h = 1$, followed by a near-flat curve, indicating that additional reticulations did not substantially improve model fit. The inferred inheritance parameter ($\gamma \approx 0.5$) suggests that an ancestral lineage of subgenus *Vignea* contributed approximately half of its genetic material to an ancestral lineage within subgenus *Euthyceras*. This hybridization likely occurred along the branch that subsequently gave rise to *Uncinia*, supporting its origin as the result of an ancient hybridization event. In addition, the crown node age of subgenus *Uncinia* was estimated at 21.60 Ma using treePL, placing its origin in the Early Miocene (Suppl. Fig. 2).

The different phylogenomic topologies obtained from the 50-tip tree and the 18-tip tree used in the PhyloNetworks analyses are presented in the Supplementary Discussion section.

3.5. Assessment of paralogous and potential orthologous loci

After running the *paralog retriever* function from HybPiper, we examined all 199 genes showing potential signs of paralogy in any of the samples (Fig. 1). Remarkably, within *Uncinia* we recovered 3.7 times more paralogs than in the rest of *Carex* subgenera (Suppl. Table 2). We discarded 40 loci considered true paralogs (showing complete duplication; Suppl. Fig. 3a). A total of 159 loci were retained after this filtering. Among those, these *Uncinia* samples with a single retrieved copy were consistently nested either within *Euthyceras* or *Vignea* clades. However, when two copies were recovered for the same *Uncinia* sample, each copy was placed in either of those two clades and never recovered as sister sequences. These results indicate that each copy may have originated from a different subgeneric lineage (*Vignea* and *Euthyceras*), although most loci yield only a single sequence per sample.

3.6. Allele phasing and assessment

The 350 gene trees obtained showed highly variable topologies regarding the placement of *Uncinia* samples. In a subset of genes, both alleles of a given *Uncinia* sample formed monophyletic clades with minimal divergence, indicating that intraspecific variability was lower than interspecific divergence within the broader phylogeny. In contrast, other gene trees revealed a pattern where *Uncinia* alleles were phylogenetically dispersed, suggesting that intraspecific divergence surpassed interspecific differentiation. In these cases, the involved alleles were potentially more closely related to representatives of other subgenera and not sister to one another. For the *Uncinia* samples, both alleles were assigned to *Euthyceras* in approximately 53–67% of gene trees, both to *Vignea* in 21–29% of loci (Fig. 5). Remarkably, in 5–24% of the trees, the two alleles from the same sample were assigned to different subgenera—one to *Euthyceras* and the other to *Vignea*—indicating substantial intraindividual phylogenetic discordance.

The plot of LH vs total AD values (Fig. 6) revealed the structuring of the samples in three clusters: one first cluster A with high LH and AD, another intermediate cluster B with high values of LH and low AD, and a last cluster C with low LH and AD. The first cluster included all *Uncinia* samples together with *C. curvula*, *C. rupestris*, and *C. siderosticta* (89.7–99.7% LH; 0.87–1.67% AD). The second cluster included a set of taxonomically heterogeneous taxa together with the samples of putative hybrid origin (57.1–92.2% LH; 0.11–0.46% AD). The last cluster contained the rest of the samples (22–51.5% LH; 0.04–0.19 AD), including the samples of *C. bracteosa*, *C. distachya and C. lehmanniana*, inferred as possible polyploids. This distribution of the *Uncinia* samples in the first cluster suggests that, proportionally to the other subgenera, they are more heterozygotic and their alleles are more divergent by accumulating more SNPs. The variation of AD across loci (Fig. 3b) shows that the samples of the first cluster including all *Uncinia* species display a relative high number of loci with high scores when compared with the other two clusters.

4. Discussion

4.1. The hybrid origin of subg. Uncinia based on phylogenetic conflicts

The placement of subg. *Uncinia* is problematic across different reconstructions, with phylogenetic incongruence affecting subgeneric relationships at deep nodes, depending on the genome (nuclear vs. plastid), sequencing technique (Sanger vs. Hyb-Seq), and sampling size (Suppl. Figs. 1, 4). *Uncinia* has been previously recovered as sister to subg. *Vignea* (Villaverde et al., 2020), sister to subg. *Euthyceras* (García-Moro et al., 2022), or nested within it (Starr et al., 2004; 2008; Jiménez-Mejías et al., 2016; Villaverde et al., 2020). Interestingly, the HTS evidence presented by Villaverde et al. (2020) retrieved subg. *Uncinia* as sister to subg. *Vignea* using nuclear loci but deeply nested within subg. *Euthyceras* when using plastid data. Our coalescence species trees using nuclear loci (Suppl. Figs. 1, 4) placed *Uncinia* either as sister to *Euthyceras* or to *Vignea* —both relationships supported with maximum support (local posterior probabilities = 1)— depending on sampling size (see also Supplementary Discussion). All these incongruences can be interpreted as a signal of hybridization (Fehrer et al., 2007; Pokorny et al., 2024).

Our phylogenetic networks analysis (Fig. 4) further supports a reticulate evolutionary origin for subg. *Uncinia*, with the most likely scenario involving an ancient hybridization event. The nearly equal genomic contribution of the two putative parental subgeneric lineages (*Vignea* 49%, *Euthyceras* 51%) suggests a punctual hybridization rather than introgression (Solís-Lemus et al., 2017; Valdés-Florido et al., 2024). The hybridization appears to involve an ancestral, perhaps stem lineage of subg. *Vignea*, and a second lineage nested within subg. *Euthyceras* (probably a member of the Rara Clade according to Villaverde et al. (2020, plastid tree). This finding is consistent with results by Jung et al. (unpublished), who used the HyDE framework to identify historical hybridization events across *Carex*. Remarkably, the ages and ancestral areas previously inferred for all the putative parental and descending lineages involved in the hybridization event (Martín-Bravo et al. 2019; García-Moro et al. 2022) are consistent with an ancient hybridization event that occurred during the Early Miocene in the Northern Hemisphere, probably in regions surrounding the Pacific Ocean. The crown

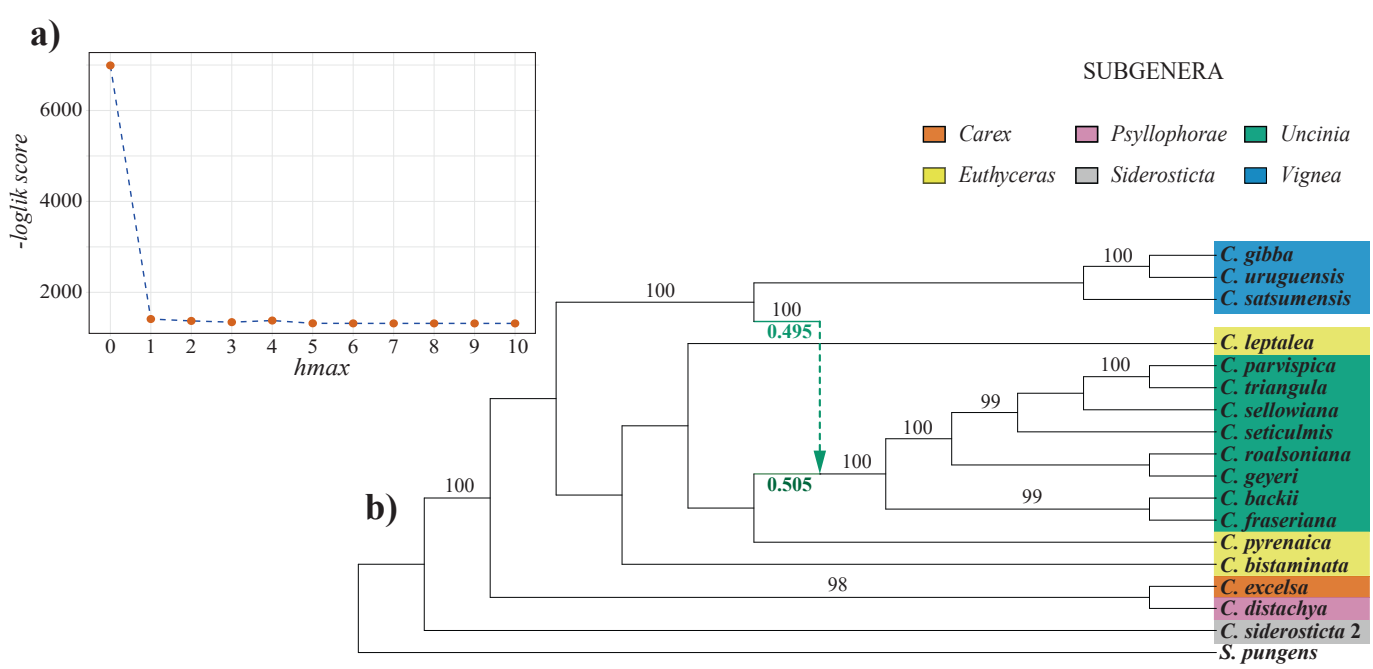

Fig. 4. a) Comparison of the number of reticulation events (*hmax*) against the log pseudolikelihood (*-loglik*) scores, used to identify the optimal number of reticulations that best fit our data. b) Phylogenetic network inferred with *SNaQ* (PhyloNetworks), showing a reticulation event from a basal lineage of subgenus *Vignea* towards an internal branch of subgenus *Euthyceras*. Branches with high bootstrap support are indicated (> 90%).

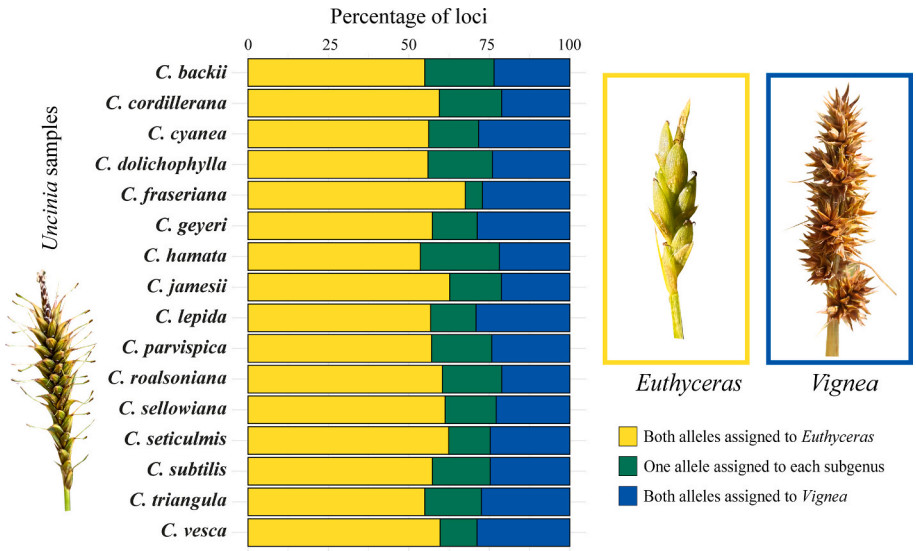

Fig. 5. Percentage of loci of *Carex* subg. *Uncinia* according to their allele's assignment to subgenera *Euthyceras* or *Vignea* based on minimum phylogenetic distance.

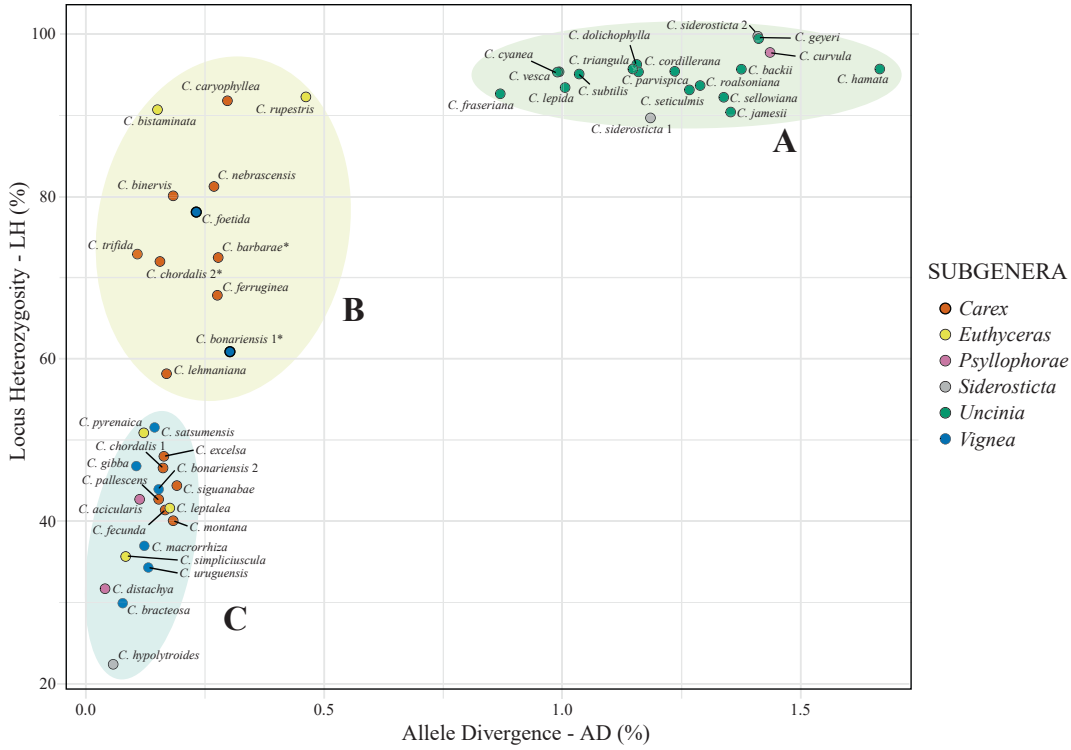

Fig. 6. Scatterplot displaying Locus Heterozygosity (LH) vs Allele Divergence (AD). Coloured ovals indicate the three clusters in which our samples are organised: Cluster A, with high LH and high AD; Cluster B, with high LH and low AD; Cluster C, with low LH and low AD. Putative hybrid samples indicated with an asterisk (*).

age inferred for *Uncinia* in this study is consistent with previous dated phylogenies of the group, including those for the whole genus *Carex* (Martín-Bravo et al., 2019) and for subg. *Uncinia* (García-Moro et al., 2022), despite differences in taxon sampling and molecular datasets (Sanger-based loci versus genomic data).

4.2. Ancestral polyploidy and subsequent diploidization involved in the origin of subgenus Uncinia

One of the most remarkable results of our comprehensive genome size compilation in *Carex* are the consistently high values observed in species of *Uncinia* (average 2C = 3.03 pg), which are approximately three times larger than the average of the remaining *Carex* (2C = 0.96 pg; Fig. 1; Supplementary Table 1). We initially hypothesized that these species could be functional polyploids, with genome size increasing proportionally to chromosome set multiplication, as observed in other plant groups (Leitch et al., 2008; Peng et al., 2022; Hutang et al., 2023; Kobrlová et al., 2024). A relevant example within *Carex* is subg. *Siderosticta*, where the larger genome sizes we recovered are consistent with its known karyotypic variation in ploidy levels (Tanaka, 1940; Chung et al., 2013; Yano et al., 2014; Chung, 2024).

However, the polyploid origin of *Uncinia* was challenged after ploidy levels estimates using allele frequency distribution (nQuire), which consistently supported a diploid model for all the analysed subgenus

species (Fig. 3a). As in other studies (Viruel et al., 2019), the allele ratio distributions in *Uncinia* and most species fell below a ratio of two, supporting diploidy. Polyploid nature was however confirmed in several samples of other subgenera by a majority of their allele ratios being larger than two (Fig. 3a). This included several *Siderosticta* accessions. Our results challenge the claim that polyploidy is rare in *Carex* as previously inferred from chromosome number evolution (Márquez-Corro et al. 2019) and point to the need of use of HTS-based tools in future analyses evaluating polyploidy.

Therefore, while genome size seems to indicate a polyploidization event involved in the origin of *Uncinia*, allele frequencies suggest its genome has subsequently undergone diploidization. Indeed, the abnormally high number of paralogs across most *Uncinia* samples as retrieved by HybPiper may be a residual sign of polyploidy (see next section). This residual polyploidy is also suggested by the comparatively higher number of loci with high AD in *Uncinia* (Fig. 3b). Under this hypothesis of polyploidization with subsequent diploidization, a large genome size may have been retained, despite the return to a diploid-like allelic state for some loci. A lack of direct connection between genome size and ploidy level has been documented in other groups, such as *Nicotiana* (Leitch et al., 2008), where some polyploid species experienced genome downsizing, while others retained or increased their genome size relative to their diploid progenitors. Therefore, although ploidy level estimation favours a diploid model for *Uncinia*, it does not rule out an ancient polyploid origin, particularly if diploidization has progressed to the point where allele frequency-based ploidy estimates no longer reflect the genome's evolutionary history. Diploidization may be achieved through different genomic processes including gene loss, genome downsizing and chromosomal fusions (reviewed in Benítez-Benítez et al., 2025). In the case of *Uncinia*, the persistence of large genome sizes and relatively high and constant chromosome numbers (Roalson et al. 2008; Márquez-Corro et al. 2021) alongside the overall diploid allele frequencies could reflect a lineage that has undergone substantial diploidization through gene loss but without prevalent chromosome fusion and genome downsizing. This highlights how polyploid lineages may follow contrasting genomic trajectories. In *Uncinia* this is further illustrated by the heterogeneous retention of copies, depending on the locus, that are exclusively allied to one of the two putative parental lineages (*Vignea* or *Euthyceras*; Fig. 5). Future studies incorporating chromosome-level whole-genome sequencing and synteny analyses will be essential to assess the extent and genomic consequences of this putative diploidization process.

4.3. An allopolyploid scenario supported by the signal of potential alleles and paralogs

The combined evidence of hybrid origin, genome size and ploidy level seem to point to an historical hybridization event linked to polyploidization (ancient allopolyploid scenario; Wendel, 2015) and later diploidization. This is further supported by the combined data from heterozygosity and allele distribution.

Our AD vs. LH values plot (Fig. 6) displays a clustering pattern similar to that retrieved by Hendriks et al. (2023). According to that theoretical framework, high AD and LH are indicative of polyploid origin, while hybrids tend to be recovered with high LH but lower AD. Species in *Uncinia* exhibit the highest AD and LH values among our sampling, suggesting a polyploid origin (Hendricks et al. 2023) despite being characterized as current diploids (Fig. 3a, Suppl. Table 2). Moreover, *Uncinia* samples clustered with the two samples of *Carex siderosticta,* which has been documented as a polyploid (Tanaka, 1940; Hoshino, 1981; Yano et al., 2014). In contrast, the putative recent hybrids included in our analyses are placed in a different cluster of samples, with high LH but lower AD than the polyploids. The fact that all samples of *Uncinia* display similar values also argues in favour of a single ancient event at the origin of the group −likely occurred in the early Miocene, when the subgenus originated (Martín-Bravo et al., 2019; García-Moro et al. 2022) rather than multiple independent recent hybridizations involved in all *Uncinia* sampled taxa.

Additional support for the allopolyploid scenario comes from heterozygosity estimates based on SNPs (extracted from the ploidy level estimation pipeline) and from the counts of loci marked as paralogs by HybPiper (Suppl. Table 2). SNP counts in *Uncinia* (average = 18,973) are markedly higher than *Siderosticta* (2,403) and also exceed those of the other *Carex* lineages (2,292). Mean heterozygosity in *Uncinia* (1.97) is comparable to that of *Siderosticta* (2.15), which is well known to have polyploid species, and both are markedly higher than in the remaining *Carex* lineages (0.28). Regarding loci marked as paralogous, we recovered in *Uncinia* an abnormally high number of putative paralogs (average of 45 vs 12.1 in all the other non *Uncinia* subgenera). In several of these cases, two distinct sequences of the same gene were retrieved from *Uncinia* samples, with one copy consistently clustered with *Vignea* and the other with *Euthyceras* (Suppl. Fig. 3b). These patterns are consistent with the retention of homoeologous gene copies, a hallmark of allopolyploidy, where gene copies from both parental genomes persist after hybridization (Kyriakidou et al., 2018). The presence of divergent homeologs with such contrasting phylogenetic affinities in all *Uncinia* samples may support them having been retained from the ancestral hybridization between *Vignea* and *Euthyceras* and a residual signal of ancient polyploidy.

Aufiero et al. (2024) highlighted that homeologs retention in allopolyploid plants can provide evolutionary flexibility, allowing duplicated genes to diverge and acquire novel functions or enable ecological adaptation. The extremely divergent morphologies displayed by several lineages of *Uncinia* when compared with most *Carex* lineages, such as the insect-pollinated *C. fraseriana* or the exclusive hooked utricles of sect. *Uncinia* may have been facilitated by the allopolyploid origin of the subgenus.

5. Conclusions

Carex subg. *Uncinia* originated through an ancient allopolyploid event, during the Early Miocene, involving ancestral lineages of *Euthyceras* and *Vignea*. This allopolyploidization was followed by partial diploidization, as shown by balanced allele frequencies and the ploidy level estimated as diploid. Independent sources of evidence, including its comparatively large genome size relative to the rest of the genus, the high number of putative paralogous copies, elevated allele divergence and locus heterozygosity, and the inferred hybridization event at the stem node, consistently support this scenario. These combined results indicate that *Uncinia*'s complex evolutionary history reflects an ancient allopolyploid origin.

Genomic sequencing approaches and analytical pipelines may still have limitations in distinguishing between alleles of the same ortholog, different homoeologous copies, and paralogs (Ning et al., 2024), since this distinction depends on read depth per locus. Nevertheless, the congruence among independent sources of evidence lends robustness to these conclusions. Overall, this study illustrates how advanced genomic tools can unmask obscure, complex polyploidization events and motivates further investigation into *Carex*, the Cyperaceae family, and other plant groups to uncover ancient polyploidization processes that may have remained overlooked.

6. Supplementary discussion

The topology recovered in our 18-tip species tree (Suppl. Fig. 4) used in the PhyloNetworks analysis was largely congruent with that of our 50-tip species tree (Suppl. Fig. 1), with two notable exceptions.

First, in our 18-tip species tree subg. *Psyllophorae* appeared as sister to subg. *Carex*, and together they formed a clade sister to subgenera *Euthyceras*, *Vignea*, and *Uncinia*. Interestingly, in the 50-tip species tree this placement changes, and subg. *Psyllophorae* is the sister group to the clade containing *Vignea*, *Euthyceras* and *Uncinia*. This placement is

congruent with the HTS-based topologies presented by Martín-Bravo et al. (2019) and Villaverde et al. (2020), as well as with previous Sanger-based phylogenies (Jiménez-Mejías et al., 2016).

Second, in our 18-tip species tree, *Euthyceras* and *Uncinia* form a monophyletic group sister to *Vignea*, while in the 50-tip species tree, *Euthyceras* and *Vignea* appeared interchanged, being *Vignea* the sister clade to *Uncinia*. The first placement is consistent with other reconstructions based on Sanger data (García-Moro et al., 2022) and HTS analyses for the plastid (Villaverde et al., 2020, plastid phylogenies), while the second agrees with previous nuclear HTS analyses (Villaverde et al., 2020, Fig. 2).

These changes in the topological relationships after using different sampling sizes highligths that the limited sampling included in this study may affect the phylogenetic reconstructions. Such limitation may be behind both topological discrepancies with previous studies. Assuming the hybrid-origin of the subg. *Uncinia* lineage, our smaller sampling may condition the retrieving of the clade as sister of *Euthyceras* instead of *Vignea*, the two parents involved in the origin of the subgenus. After discarding methodological artifacts, it seems that that the phylogenetic position of subg. *Uncinia* cannot be determined solely based on the best-supported genomic topology, in light of the results presented in this study.

CRediT authorship contribution statement

Pablo García-Moro: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Se-Eun Jung:** Resources, Investigation, Data curation, Conceptualization. **Ana Valdés-Florido:** Writing – review & editing, Resources. **Jérémie Morel:** Resources. **María Sanz-Arnal:** Resources. **Tamara Villaverde:** Writing – review & editing, Methodology. **Robert F.C. Naczi:** Writing – review & editing, Resources. **Kerry Ford:** Writing – review & editing, Resources. **Sangtae Kim:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Santiago Martín-Bravo:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Pedro Jiménez-Mejías:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Juan Viruel:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **José Ignacio Márquez-Corro:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of use of AI tools

We used ChatGPT (GPT-4, accessed during May and June 2025, OpenAI) to assist in identifying literature related to allopolyploidy for the Introduction section. All references suggested by the tool were subsequently verified by the authors before being cited in the text. ChatGPT was also used for grammatical correction of the manuscript, prior to the careful revision by two English-speaking coauthors who ensured the accuracy of the grammar, and for debugging our Python script employed to extract LH and AD values from phased sequences (mentioned above). All scientific content, data analyses, and interpretations remain the sole responsibility of the authors. Also, we used GPT-5 (accessed September 2025) to unify the format of all cited literature in the References section. All scientific content, data analyses, interpretations, and figure preparation are the sole responsibility of the coauthors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2026.108603.

Data availability

The Python script described in the Materials and Methods to extract AD and LH information is available at GitHub (https://github.com/pgarciamoro/heterozygositysummary). The script *haploneraty.py*, required for the allele phasing, is available at a separate GitHub repository (https://github.com/mossmatters/phyloscripts/tree/master/alleles_workflow). All newly generated genomic sequences will be available in GenBank in due course.

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