Phylogenetic placement of *Ceratophyllum submersum* based on a complete plastome sequence derived from nanopore long read sequencing data

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Abstract

Objective

Eutrophication poses a mounting concern in today's world. *Ceratophyllum submersum* is a plant capable of living in eutrophic conditions, therefore it could play a critical role in addressing the problem of eutrophication. This study aimed to take a look at the genetic basis underlying *C. submersum*.

Results

Sequencing of gDNA from *C. submersum* yielded enough reads to assemble a plastome. Subsequent annotation and phylogenetic analysis validated existing information regarding angiosperm relationships and the positioning of Ceratophylalles in a wider phylogenetic context.

Keywords: ONT, angiosperms, Ceratophyllaceae, Ceratophyllales plastome, plastomics, chloroplast-genome phylogenomics, phylogenetic pipeline, supermatrix tree

Introduction

Ceratophyllum submersum, commonly known as soft hornwort, is a subaquatic plant whose genus is the only extant member in the order of Ceratophyllales, placed as a sister clade to the eudicots [1,2]. It is native to Europe, Africa and Asia and grows in stagnant freshwater bodies [3]. Morphological features include long, branching stems that can reach up to several metres in length and leaves that are forked into narrow, filament-like segments that grow in multiple whorls around the stem (Figure 1) [4]. The plant is often green, but can vary in colour from brownish to reddish depending on environmental conditions. Anthocyanins contribute to the colouration of many plant species, and metabolic analyses have detected several

derivatives in *C. submersum* [5–7]. It thrives in eutrophic conditions, characterised by low light intensity and high nutrient levels [8,9]. Eutrophication of aquatic environments is indicated by the accumulation of nutrients albeit other parameters and multiple classification systems exist [10]. Due to anthropogenic effects the occurrence of eutrophic environments is rising which poses a problem e.g. for greenhouse gas emissions [11]. In aquatic systems, eutrophication is inducing harmful algal blooms (HABs), which are responsible for environmental hazards like the Oder ecological disaster in 2022 [12]. *C. submersum* has the ability to enhance the water quality by absorbing nutrients (especially nitrogen and phosphor) and is therefore popular in amateur aquacultures. It can also prevent the formation of HABs, even though it suffers from them [7].

Figure 1: *Ceratophyllum submersum* and its habitat. A: The pond (Braunschweig, 52.28062 N / 10.54896 E) where *C. submersum* was collected, **B**: Whole *C. submersum* plant, **C**: Close up view of *C. submersum*.

Further research of *C. submersum* can help to develop ecosystem engineering solutions to combat human-induced eutrophication and HABs in freshwaters. Here, we used the potential of nanopore long read sequencing to unravel the plastome sequence of *C. submersum* and provide a comprehensive annotation.

Main Text

Results and Discussion

A total of 1,544 long reads representing the plastome were extracted from the raw read sequencing data (Additional file 1, PRJEB62706). From that, 752 reads were passed to the Flye assembler by ptGAUL to reach a maximum coverage depth of 50x. The plastome assembly of *C. submersum* resulted in a 155,767 bp long sequence with a GC content of 38.26%. In total, 75 unique protein coding genes were annotated (Additional file 2). The *C. submersum* plastome sequence is 485 bp shorter than the reference plastome sequence of *C. demersum* that has a length of 156,252 bp [13]. While the GC content is similar (*C. demersum*: 38.22, *C. submersum*: 38.26) the amount of unique annotated genes in *C. submersum* is smaller than in *C. demersum* (75 against 79). After the annotation some predicted coding sequences were flawed (e.g. containing multiple stop codons). Manual evaluation revealed four homopolymeric regions in which a frameshift would correct the annotation. Since homopolymers are a frequent error type in ONT reads, manual correction in those regions is appropriate [14]. The comparison of those regions to all plastome reads suggested a correction of two of these regions, namely the adenine at position 3,094 and the thymines at position 3,143, at position 86,261, and at position 86,262 were inserted.

To validate the plastome assembly from *C. submersum*, a phylogenetic tree was calculated (Figure 2).

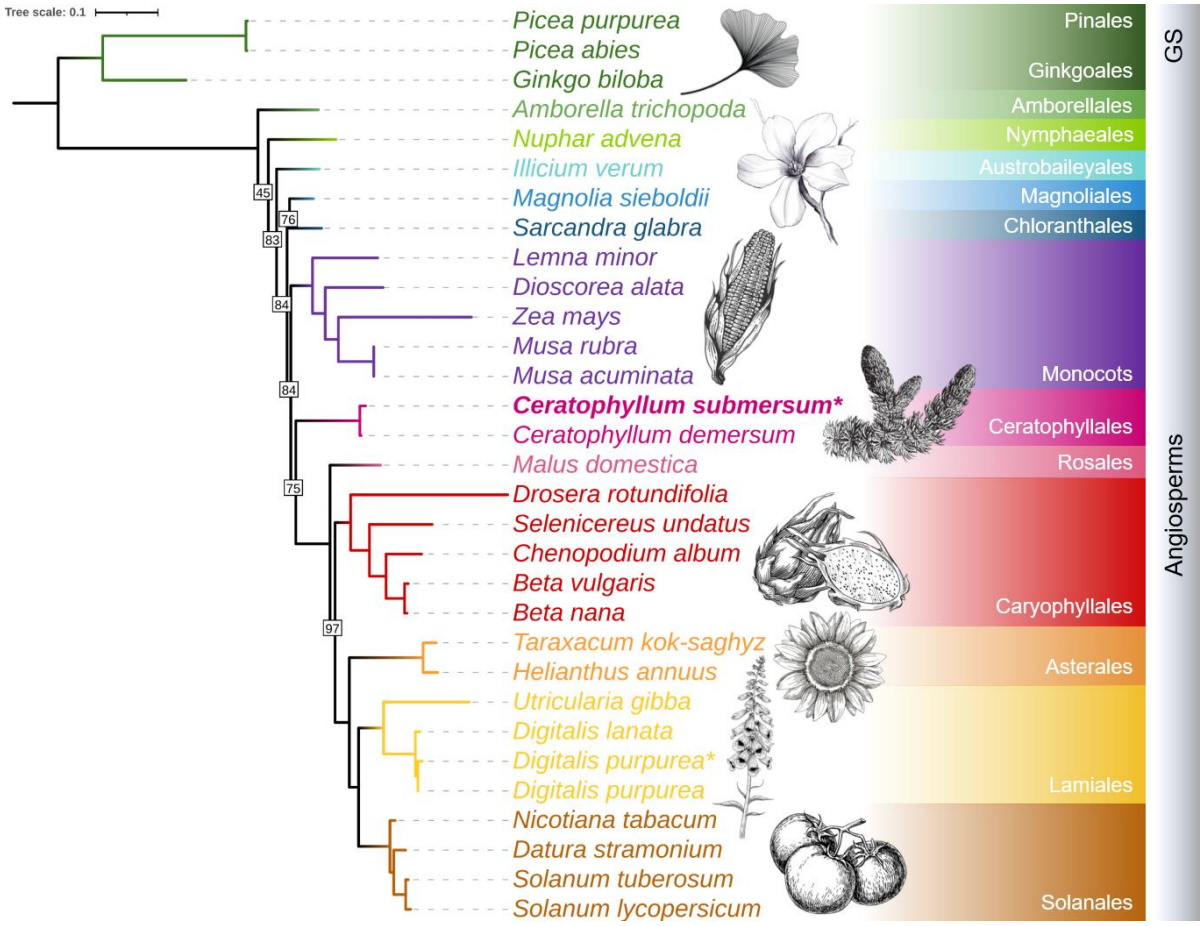

Figure 2: Phylogenetic tree of selected spermatophytes based on plastome protein sequences. The asterisks (*) indicate plastome assemblies generated in this study. Within the angiosperms, the orders are differently coloured. The eudicotyledons are the only exception as they are represented by several orders. All nodes received full bootstrapping support (100%), except those displaying the actual value. Please see the material and methods section for further description of the tree calculation. Visualisation was done in iTOL 6.7.6 [15]. The full tree including the outgroup *Chlamydomonas reinhardtii* can be found in the additional files (Additional file 4). GS = gymnosperms. The sketches are licensed by adobe and depositphotos (Standard License: https://wwwimages2.adobe.com/content/dam/cc/en/legal/servicetou/Stock-Additional-Terms_en_US_20221205.pdf, https://depositphotos.com/license.html).

Multiple plastome reference sequences were chosen to represent the angiosperm clade as well as *Chlamydomonas reinhardtii* as outgroup (for full list and references see Additional file

3). Additionally, our own plastome assembly of *Digitalis purpurea* was included as a control, because gDNA from this plant was also sequenced on the same flow cell as the gDNA from *C. submersum* (*D. purpurea* data: PRJEB62706). The phylogenetic tree (figure 2, for full tree see Additional file 4) classifies *C. submersum* close to their reference *C. demersum* and our *D. purpurea* plastome assembly close to the RefSeq *D. purpurea* plastome from Zhao *et al*., 2023 [16]. The angiosperm clade is represented in accordance with the current APG IV classification except for the exact separation/placement of the Magnoliids and the Chloranthales, which is still controversial [2]. This underlines the significance of plastome sequences for modern plant phylogenetics [17].

The plastome assembly and phylogenetic analysis presented in this study provide initial insights into the genetic basis of *Ceratophyllum submersum*. Further research is needed to unravel its nuclear genome and to explore potential human applications of this plant, such as its use as a valuable resource or as an agent to mitigate environmental hazards.

Material and Methods

Plant Material, gDNA extraction and sequencing

Ceratophyllum submersum was collected from a small pond in Braunschweig (52.28062 N / 10.54896 E) and kept in cultivation. The artificial pond needs occasional water replenishment. Foliage from surrounding shrubs and dead aquatic plants lead to a high eutrophy (see figure 1A). *C. submersum* shoot tips (figure 1C) were harvested for gDNA extraction conducted with a CTAB method [18–20]. Short DNA fragments were depleted with the Short Read Eliminator kit (Pacific Biosciences). Library preparation for ONT sequencing was started with 1µg of DNA that was first repaired with the NEBNext® Companion Module and then processed according to the SQK-LSK109 protocol (Oxford Nanopore Technologies). For ONT sequencing, a R9.4.1 flow cell was used with a MinION. Prior to *C. submersum* sequencing, the flow cell was already utilised for *Digitalis purpurea* gDNA sequencing. Processing of raw data was performed with

guppy v6.4.6+ae70e8f (https://community.nanoporetech.com) and minimap2 v2.24-r1122 [21] on a graphical processor unit (GPU) in the de.NBI cloud to generate FASTQ files.

Plastome Assembly and Annotation

The FASTQ files were subjected to a plastome assembly with ptGAUL using standard parameters [21–24]. As reference the *C. demersum* plastome from the NCBI RefSeq database (release 216) was used [13,25]. Since the ptGAUL results consist of two assemblies (one per path) the OGDRAW plastome maps were compared between the reference and the assemblies to decide which one is closer to the reference. The *C. submersum* assembly needed to be reverse complemented and the sequence start was adjusted to the *C. demersum* sequence. GC contents of the assembly were calculated using the contig_stats.py script [26]. For annotation GeSeq was applied (see Additional file 5 for parameters) [27–36]. Protein coding genes were extracted from the resulting GBSON.json file using our own script JSON_2_peptide_fasta_v1.0.py [37]. Manual evaluation of wrong annotated CDSs was conducted with the help of EMBOSS Sixpack to have an overview of all reading frames which could then be compared to the results from NCBI blastx to find possible wrong frameshifts [38,39]. Further evaluation of these frameshifts was conducted with the help of a read mapping. All plastome reads were mapped against the plastome sequence with minimap2 v2.24-r1122 [21]. Subfurther generation of a bam file and its indexing with samtools 1.10 [40] was conducted to be able to analyse the mapping in the integrative genomics viewer (IGV) 2.16.1 [41]. Assembly corrections were only then conducted, if a significant number of reads support it.

Data preparation prior to submission was performed by extracting all the read IDs from the ‘new_filter_gt3000.fa’, generated by ptGAUL, which contains all the identified plastome reads. The plastome reads were extracted from the FAST5 and FASTQ raw read datasets via the ont_fast5_api tool and a custom script [37,42].

Phylogenetic analysis

Reference data retrieval and phylogenetic supermatrix tree construction was performed by our newly developed Python pipeline PAPAplastomes (Pipeline for the Automatic Phylogenetic Analysis of plastomes) which integrates established external tools for complex steps (Additional file 6) [43]. Reference species, closely related species of interest, and outgroup species can be specified via a config file. First, NCBI RefSeq plastome data is downloaded and the reference plastome peptide sequences are extracted from this collection. These peptide sequences are further combined with the peptide sequences derived from the assemblies representing plastomes of interest. The pre-OrthoFinder trimming step removes potential paralogs with the exact same sequence, sequences which contain asterisks and sequences that are shorter than 10 amino acids (this threshold value is adjustable). Next, OrthoFinder v2.5.4 [44–47] is applied. Post-Orthofinder processing includes four steps: (1) removal of outlier sequences, (2) deletion of orthogroups missing the species of interest or their references, (3) removal of orthogroups harbouring fewer species than the pre-defined outgroup species, (4) paralog cleaning. (1) Since the OrthoFinder results include phylogenetic trees of each orthogroup, outlier identification was conducted with the help of the Python module Dendropy v4.5.2 [48]. Per orthogroup the edge length for each taxon is accessed except for outgroup species. Based on these lengths, outliers are identified by the 1.5*IQR method (inter quartile range) i.e. sequences with a distance larger than 1.5 times the variation are excluded. (2) The species of an orthogroup are listed and if pre-defined species are all either present or absent the orthogroup will be kept. Otherwise, the orthogroup will be discarded. This is suggested for closely related species i.e. from the same genus and can be specified by the user in the config file. (3) Orthogroups consisting solely of the outgroup species are exempt from this criterion. (4) From remaining paralogs within one species, only the longest sequence is kept.

The cleaned orthogroups are then aligned with MAFFT v7.453 (--maxiterate 1000 --localpair) [49,50] and alignments are concatenated. Phylogenetic supermatrix tree calculation is

performed by IQ-TREE (multicore version 1.6.12 for Linux 64-bit built Aug 15 2019; with the '-nt AUTO -bb 1000' options, seed: 291752) based on the concatenated alignment of all orthogroups [51–53].

Limitations

Before sequencing *C. submersum*, the flow cell had already been utilised for sequencing gDNA of *D. purpurea*. Insufficient DNA availability hindered the complete sequencing and assembly of *C. submersum*'s nuclear genome. Future optimisations in DNA extraction methods dedicated to small aquatic plants could overcome this limitation.

Abbreviations

CDS - coding sequence

gDNA - genomic DNA

GPU - graphical processing unit

GS - gymnosperm

HAB - harmful algal bloom

RefSeq - NCBI Reference Sequence Database

IQR - interquartile range

ONT - Oxford Nanopore Technologies

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and analysed during the current study are available under PRJEB62706. Scripts developed for the data analysis are available from Codeberg (https://codeberg.org/snmeckoni/scripts, https://codeberg.org/snmeckoni/PAPAplastomes) and GitHub (https://github.com/bpucker/GenomeAssembly).

Competing interests

The authors declare that they have no conflict of interest.

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Author's contributions

All authors contributed to the concept and design of the studies, wet and dry lab work and manuscript writing.

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Legends

Additional file 1: FASTQ stats of the *Ceratophyllum submersum* plastome reads. The stats were calculated with the help of the script FASTQ_stats3.py (https://github.com/bpucker/GenomeAssembly).

Additional file 2: *Ceratophyllum submersum* plastome map derived from OGDRAW.

Additional file 3: List of references of the reference plastomes. The data was extracted by PAPAplastomes and originates from the RefSeq database.

Additional file 4: Full phylogenetic tree with outgroup species *Chlamydomonas reinhardtii*.

Additional file 5: Exact parameters for the GeSeq run. Some parameters from the online tool GeSeq consist of a checkbox that has to be checked. Therefore, the value "check" is stated.

Additional file 6: PAPAplastome workflow chart.