Plastome Phylogenomics and Historical Biogeography of Aquatic Plant Genus *Hydrocharis* (Hydrocharitaceae)

Zhi-Zhong Li  
Chinese Academy of Sciences

Samuli Lehtonen  
Herbarium, University of Turku

Andrew W. Gichira  
Chinese Academy of Sciences

Karina Martins  
Universidade Federal de São Carlos

Efremov Andrey  
State Pedagogical University

Qing-Feng Wang  
Chinese Academy of Sciences

Jin-Ming Chen (jmchen@wbgcas.cn)  
Chinese Academy of Sciences

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**Research Article**

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Abstract

Background

*Hydrocharis* L. and *Limnobium* Rich. are small aquatic genera, including three and two species, respectively. The taxonomic status, phylogenetic relationships and biogeographical history of these genera have remained unclear, owing to the lack of Central African endemic *H. chevalieri* from all previous studies. We sequenced and assembled plastomes of all three *Hydrocharis* species and *Limnobium laevigatum* to explore the phylogenetic and biogeographical history of these aquatic plants.

Results

All four newly generated plastomes were conserved in genome structure, gene content, and gene order. However, they differed in size, the number of repeat sequences, and inverted repeat borders. Our phylogenomic analyses recovered non-monophyletic *Hydrocharis*. African species *H. chevalieri* was fully supported as sister to the rest of the species, and *L. laevigatum* was nested in *Hydrocharis* as a sister to *H. dubia*. *Hydrocharis-Limnobium* initially diverged from the remaining genera at ca. 53.3 Ma, then began to diversify at ca. 30.9 Ma. The biogeographic analysis suggested that *Hydrocharis* probably originated in Europe and Central Africa.

Conclusion

Based on the phylogenetic results, morphological similarity and small size of the genera, the most reasonable taxonomic solution to the non-monophyly of *Hydrocharis* is to treat *Limnobium* as its synonym. The African endemic *H. chevalieri* is fully supported as a sister to the remaining species. *Hydrocharis* mainly diversified in the Miocene, during which rapid climate change may have contributed to the speciation and extinctions. The American species of former *Limnobium* probably dispersed to America through the Bering Land Bridge during the Miocene.

Background

*Hydrocharis* L., an aquatic monocot genus, has broad native distribution in tropical and temperate regions of the Old World [1]. As traditionally circumscribed, the genus comprises only three allopatric species: *Hydrocharis chevalieri* (De Wild.) Dandy, *H. morsus-ranae* L. and *H. dubia* (Blume) Backer. Among them, *H. chevalieri* is only found in small sedge swamps or wetlands in central Africa [1, 2]. It is easily distinguished from the other two species by unique morphological features, e.g., long and erect petioles and the number of primary veins (>4) in leaf lamina [1]. In comparison, *H. morsus-ranae* and *H. dubia* are more widely distributed. The native range of the former covers West and North Eurasia, whereas the latter is natively distributed in Southeast Asia [1, 3]. Beyond their natural range, these two species have invaded Northeast America and Australia, respectively [3], and cause serious damage to the local...
environment [2]. However, due to the deterioration of the aquatic habitats resulting from human activities, they have been considered threatened in part of their native distributions [3]. *Hydrocharis* and the closely related *Limnobium* are unusual in Hydrocharitaceae by having aerial leaves only, and therefore are significant for understanding the evolution of aquatic adaptations in the family [1, 4]. Additionally, *H. morsus-ranae* has been proved to have the ability to accumulate heavy metal elements making it a possible bioindicator of trace element pollution in freshwaters [5].

Since the taxonomic revision of the genus *Hydrocharis* by Cook and Lüönd [1], only a few studies have focused on the systematics and taxonomy of the genus. Chen et al. [4] reappraised the generic relationships of Hydrocharitaceae using eight genes representing all three genomes. The genus *Hydrocharis* was resolved as a monophyletic and highly supported sister of *Limnobium*, a genus with two currently accepted species [6]. In that study, both of the two *Limnobium* species were sampled, but one of the *Hydrocharis* species remained unsampled. Similarly, Ross et al. [7] resolved *Hydrocharis* and *Limnobium* as sisters in a phylogenomic analysis of 83 plastid genes, but they only sampled one species of each two genera. Bernardini and Lucchese [8] further studied the phylogeny of Hydrocharitaceae using a dataset of ITS and five plastid genes. In their study, the taxon sampling and results concerning *Limnobium* and *Hydrocharis* were comparable with Chen et al. [4]. They suggested that these two genera could be merged into a single genus. This has also been supported by morphologic evidence [6, 9, 10] (Cook and Urmi-Konig, 1983; Shaffer-Fehler, 1991; Les et al., 2006) and recently Plant Gateway [11] concluded – without further justification – that *Limnobium* is part of *Hydrocharis*. However, the systematic relationships of *Hydrocharis* and *Limnobium* remain unclear due to the lack of morphologically intermediate Central African endemic *H. chevalieri* from all previous studies.

Previous biogeographic analyses suggested that the most recent common ancestor (MRCA) of *Hydrocharis-Limnobium* clade originated in the Oriental region approximately 15.9 Ma, and long distance dispersal (LDD) might contribute to its current distributions [4]. However, numerous fossils, including more than 10 *Hydrocharis* species, which mainly occurred during the Oligocene and Miocene epochs, were discovered in Europe and the Far East [3, 12–15]. No fossils have been found in Africa. The biogeographic history of African species *H. chevalieri* has been unsolved, limiting a comprehensive understanding of the biogeographic history of the genus.

As the plastome has become more easily retrievable, it has dramatically enhanced the resolution of phylogenetic relationships at various taxonomic levels [7, 16–18]. Here, we sequenced and assembled plastomes of all three *Hydrocharis* species and *Limnobium laevigatum*. The objectives of this study were to 1) investigate the plastome evolution in this group; 2) clarify the phylogenetic relationships of *Hydrocharis* and *Limnobium*; and 3) infer the historical biogeography of the group.

## Results

### Plastome feature
After de novo assembly, we obtained the complete plastome of three *Hydrocharis* species and *L. laevigatum* from genome skimming data. The coverage of each plastome ranged from ~ 807× to ~ 878× (Table 1). The size of newly assembled plastomes ranged from 153,373 bp to 159,698 bp and exhibited the typical quadripartite structure (Fig. 1), including a pair of IRs (22,292 bp – 30,605 bp) separated by the Large Single Copy (LSC, 85,578 – 89,518 bp) and Small Single Copy (SSC, 11,278 – 21,476 bp) regions (Table 1). Except for the *rps16*, lost from the plastome of *H. chevalieri*, the other plastomes encoded 113 unique genes, which is similar to most of the plastomes in angiosperm. Unique genes comprised 79 PCGs, 30 tRNA genes, and four rRNA genes. Both the average gene density (~0.8) and the overall GC content (~37%) of plastomes were conserved across the species. Furthermore, a small inversion of the *trnN-GUU* gene was detected in *H. dubia* plastome (Fig. 1).
Table 1
Detailed information of the newly assembled plastomes

| Name of organism                      | Hydrocharis dubia | Hydrocharis chevalieri | Hydrocharis morsus-ranae | Limnobium laevigatum |
|---------------------------------------|-------------------|------------------------|--------------------------|----------------------|
| Voucher No.                           | HIB-LZZ-SB12      | HIB-LZZ-Cam19          | 14513                    | HIB-CJM-Bra50        |
| Collection Sites                      | China; Wuhan      | Cameroon; Mangueme     | -                        | Brazil; Rio de Janeiro |
| GenBank accession number              | OK326868          | OK326871               | OK326869                 | OK326870             |
| SRA accession number                  | SRR16469680       | SRR16469681            | SRR16469679              | SRR16469678          |
| Raw data (G)                          | 8.5               | 7.7                    | 8.5                      | 7.4                  |
| Clean data (G)                        | 8.2               | 7.5                    | 8.1                      | 7.2                  |
| Mean coverage                         | 828×              | 807×                   | 872×                     | 878×                 |
| Genome size (bp)                      | 159,698           | 158,066                | 153,881                  | 153,373              |
| LSC length (bp)                       | 89,518            | 85,578                 | 88,098                   | 87,313               |
| SSC length (bp)                       | 18,211            | 11,278                 | 20,695                   | 21,476               |
| IR length (bp)                        | 25,953            | 30,605                 | 22,544                   | 22,292               |
| Number of genes                       | 113               | 112                    | 113                      | 113                  |
| Number of protein-coding genes (duplicated in IR) | 79 (4) | 78 (6) | 79 (3) | 79 (3) |
| Number of tRNA genes (duplicated in IR) | 30 (7) | 30 (7) | 30 (7) | 30 (7) |
| Number of rRNA genes (duplicated in IR) | 4 (4) | 4 (4) | 4 (4) | 4 (4) |
| Number of genes with one intron (two introns) | 16 (2) | 15 (2) | 16 (2) | 16 (2) |
| Proportion of coding to non-coding regions | 0.73 | 0.74 | 0.72 | 0.73 |
| Average gene density (genes/kb)       | 0.8               | 0.82                   | 0.83                     | 0.83                 |
| GC content (%)                        | 37.2              | 37                     | 37.2                     | 37                   |

Comparison of border regions and sequence identity

The IR/LSC and IR/SSC junctions were compared to assess the IR expansion or contraction of the plastome. Here, the LSC/IRa (JLA) and LSC/IRb (JLB) junctions were identical for all four species; IR
expanded into the *rpl23* gene at the JLB, and the JLA were located at the *tmI-trnH* intergenic spacer (Fig. 2). Substantial divergence was detected at the SSC/IRa (JSA) and SSC/IRb (JSB) junctions. In *H. dubia* IR expanded into the *ycf1* gene at the JSA, whereas in *H. chevalieri* the *ndhA* gene overlapped with the JSA and in *H. morsus-ranae* and *L. laevigatum* JSA was placed in *ycf1-trnN* intergenic spacer. In comparison, at the JSB the IR regions expanded into genes in *H. dubia* (*ycf1*), *H. morsus-ranae* (*ndhF*) and *L. laevigatum* (*ndhF*). The JSB of *H. chevalieri* lay in the *ndhH-ndhF* intergenic spacer.

Both the sequence identity analysis and Pi values revealed a high differentiation among the newly assembled plastomes. The divergent regions were mostly derived from IGS regions (Fig S1,S2), e.g., *ycf1-trnN*-GUU, *rps16-trnQ-UUG*, *tmI-CAU-ycf2*, *ycf2-trnI-CAU*, *tmK-UUU-rps16*. Additionally, the top five PCGs, *ycf1*, *rps12*, *rps18*, *rps3*, *accD*, showed relatively high nucleotide diversity and had the potential to be developed into molecular markers for further phylogenetic research.

**Sequence Repeat and SSR analysis**

The SSR analysis revealed a similar number of SSRs (Table S1), ranged from 64 (*H. morsus-ranae*) to 69 (*H. dubia*), within *Hydrocharis* species. In contrast, a clearly higher number of SSRs was detected in *L. laevigatum* (85). Moreover, species of *Hydrocharis* had the largest number of tri-, tetra-, and hexa-nucleotide repeats, whereas *L. laevigatum* was rich in mono-, and di- nucleotide repeats were more common in *L. laevigatum* (Table S1). Four types of repeat elements, forward, palindromic, reverse, and complementary, were identified for *Hydrocharis* and *L. laevigatum* plastomes. The total number varied from 383 (*H. morsus-ranae*) to 689 (*H. dubia*) repeats of 30-317 bp length for the newly generated plastomes (Fig S3). Forward repeats were the most common and complementary repeats were the rarest repeat type. Based on the repeat size, we further divided all repeats into the following categories: 35-45 bp, 45-60 bp, 60-75 bp, 75-90 bp, and ≥ 90 bp (Fig. 3). At least half of the repeats belonged to the size class of 35-45 bp, and the majority of long repeats (≥ 90 bp) were located in the plastome of *H. dubia* (107).

**Phylogenetic analysis**

Both ML and BI methods, regardless of partitioning strategy, revealed completely identical topologies and robust support for most of the nodes (Fig. 3). Two major clades were identified with high support (BS = 99/100, PP = 1/1) and the genus *Stratiotes* was fully resolved as the sister to these two clades. *Hydrocharis* and *Limnobium* were clustered with full support (BS = 100/100, PP = 1/1) and resolved as the sister group of the remaining genera within Clade I. However, *Hydrocharis* did not form a monophyletic group, but *L. laevigatum* was nested within it as a fully supported sister to *H. dubia*. Additionally, African species *H. chevalieri* was fully supported as sister to the rest of the species within (*Hydrocharis* + *Limnobium*) group, and *H. morsus-ranae* shared a common ancestor with (*L. laevigatum* + *H. dubia*).

**Divergence time estimation and biogeographical reconstruction**
The MRCA of Hydrocharitaceae was inferred at ca. 61.7 Ma [Fig. 3, 95% highest posterior densities (HPD): 54.6–71.8 Ma] and the Clade I and Clade II split from each other at 57.6 Ma (95% HPD: 49.5–67.4 Ma). In Clade I, Hydrocharis-Limnobium initially diverged from the remaining genera at ca. 53.3 Ma (95% HPD: 43.7–63.1 Ma), and then began to diversify at ca. 30.9 Ma (HPD: 13.8–49.9 Ma). The MRCA of H. dubia and L. laevigatum was dated at ca. 12.84 Ma (95% HPD, 3.8–24.1 Ma) and diverged of H. morsus-ranae and the remaining species was estimated at ca. 17.5 Ma (95% HPD: 6.6–30.5).

Biogeographical reconstruction was implemented using RASP [39] with DIVALIKE model supported as the best-fit model (LnL = -8.23, AICc = 24.46, AICc_wt = 0.56; Table 2) from BioGeoBEARS analysis. However, the ancestral area remained uncertain. The scenario with the highest probability (40%) suggested that the family originated in Europe and Central Africa, followed by diversification due to three vicariance and two dispersal events (Fig. 3).

| Model         | LnL  | num params | d     | e     | j    | AICc | AICc_wt |
|---------------|------|------------|-------|-------|------|------|---------|
| DEC           | -9.26| 2          | 0.018 | 0.027 | 0    | 26.52| 0.2     |
| DEC+J         | -6.02| 3          | 1.00E-12 | 1.00E-12 | 0.19 | 30.03| 0.035   |
| DIVALIKE      | -8.23| 2          | 0.008 | 3.60E-05 | 0    | 24.46| 0.56    |
| DIVALIKE+J    | -5.28| 3          | 1.00E-12 | 1.00E-12 | 0.15 | 28.56| 0.072   |
| BAYAREALIKE   | -9.82| 2          | 0.027 | 0.047 | 0    | 27.65| 0.11    |
| BAYAREALIKE+J | -6.66| 3          | 1.00E-07 | 1.00E-07 | 0.2  | 31.32| 0.018   |

### Discussion

#### Plastome evolution

Similar to the most reported plastomes of angiosperms [17, 40], all the plastomes in our study exhibited relatively conservative genome structure, gene content, and gene order [41, 42]. However, when compared to H. morsus-ranae and L. laevigatum, H. dubia (159,698 bp) and H. chevalieri (158,066 bp) had larger plastomes. One reason for plastome size variation is the expansion or contraction of IRs [43, 44]. Indeed, H. dubia and H. chevalieri had one (ycf1) and three more genes (ycf1, rps15, and ndhH) in IRa, respectively, than the other two species. In addition, typical patterns of the plastome evolution associated with the fluctuation of plastome length include the gain/loss of genes, pseudogenization, and variations in intergenic regions [44–46]. Our results revealed the loss of gene rps16 in African endemic H. chevalieri. This gene has been reported missing from plastome in many plant groups, e.g., Oxalidaceae [47, 48], Podostemaceae [49], and Violaceae [45]. It seems that rps16 has been frequently transferred to the nuclear genome [40, 50]. Additionally, a small inversion of the trnN-GUU gene near the IR boundary was
detected in *H. dubia*. The inversion was likely related to the shift in the IR boundary (Fig. 2), as identified and demonstrated by Zhu et al.[51].

Previous studies [4, 8] have applied only five plastid genes (*rbcL, matK, rpoB, rpoC1, trnK*) to resolve the phylogeny of the genus *Hydrocharis*. However, there was only low nucleotide diversity (*Pi* < 0.06) within these five genes, which might have affected the accuracy and resolution of phylogenetic reconstruction[52]. In our study, a number of regions (Fig S2), including five plastid genes (*ycf1, rps12, rps18, rps3, accD*) and five intergenic spacers (*ycf1-trnN-GUU, rps16-trnQ-UUG, trnL-CAU-ycf2, ycf2-trnl-CAU, trnK-UUU-rps16*), were detected as hotpots and may be helpful even in population genetic studies in the genus.

The high frequency of repeat sequences has been demonstrated to be one of the leading causes of plastome rearrangement and divergence [53]. Our analysis identified many repetitions (> 350) with more than 30 bp length in all newly assembled plastomes. Among these, short repeat sequences (30-45 bp) were dominant, similar to other plant plastomes that have not undergone large-scale structural variation. SSRs are widely distributed in plant plastomes and exhibit relatively high polymorphism, which can be used in population genetics [54]. The majority of SSRs were mono- and di- nucleotides in four newly assembled plastomes, which have been reported in other angiosperm plastomes, e.g., *Primula* [55], *Dendrosenecio* [18], and *Oxalis* [48]. The cpSSRs reported here could be used as genetic markers for future studies into the genetic diversity of *Hydrocharis*.

### Phylogeny and biogeographical reconstruction

Prior to this study, no molecular information was available for the African endemic *H. chevalieri*. Here we have clarified the relationships within *Hydrocharis* by assembling and reporting the plastomes of all three *Hydrocharis* species and *Limnobium laevigatum*. Unlike previous studies [4, 8], we recovered non-monophyletic *Hydrocharis*. The genus *Limnobium* represented here by *L. laevigatum* was nested in *Hydrocharis* as sister to *H. dubia* with robust support (Fig. 3, BS = 100/100; PP = 1/1). This contradicting result might be because previous studies did not include *H. chevalieri* and used only a limited number of molecular markers [4, 8]. Furthermore, a series of morphological features support the current phylogenomic relationships. Vegetatively, *Hydrocharis* and *Limnobium* are indistinguishable, but *H. chevalieri* has stout, erect petioles, and laminas with a large number of primary veins, some of which originate from the lower half of the midrib [6]. The remaining species have slender petioles and primary veins originating from the point of petiolar attachment [6]. *Limnobium* differs from *Hydrocharis* by its rudimentary petals [6]. Given the morphological similarity and small size of the genera, only two species in *Limnobium* and three species in *Hydrocharis*, the most reasonable taxonomic solution to the non-monophyly of *Hydrocharis* is to treat *Limnobium* as the synonym of *Hydrocharis*. Nomenclatural combinations already exist, i.e., *Hydrocharis spongia* Bosc and *Hydrocharis laevigata* (Willd.) Byng & Christenh.

Our time-calibrated tree indicated stem node age of about 53 Ma for *Hydrocharis* (Fig. 3), which is similar to the previously reported median age of 54.7 Ma [4] and in agreement with the oldest known *Hydrocharis*
fossils from the Eocene [56]. The crown age of *Hydrocharis* was estimated to be approximately 31 Ma, much older than the 15.9 Ma reported in Chen et al. [4] based on analysis that lacked *H. chevalieri*, the sister species of the remaining genus. Based on our results, *Hydrocharis* mainly diversified in the Miocene, which is consistent with the time inferred for many other extant plant species [57, 58]. Rapid climate change during the Miocene may have contributed to the speciation of *Hydrocharis* as well as extinctions, given that the fossil diversity is high in comparison to the limited extant diversity [59, 60]. The recent analysis of the distribution patterns of *Hydrocharis* indicated that the mean annual temperature is the main factor to impact the distribution in this genus [3]. Furthermore, paleoclimatic changes may have induced such changes in water bodies that some groups were driven to extinction [61]. This might explain why there are only three extant species, and a majority of fossil groups only occurred in Europe during the Miocene [3].

The area of origin remained uncertain for the genus because several alternative areas with low probabilities were recovered for the deeper nodes (Fig. 3). This may, of course, also reflect a widespread ancestral distribution. However, our model with the highest probabilities for the ancestor of the genus in Europe, Central Asia, and Central African regions (Fig. 3) contrasted the biogeographical model of Chen et al. [4], which suggested origination in the Oriental area. Additionally, our results indicated that at least three vicariance and two dispersal events had shaped the current distribution of the genus (Fig. 3). The Bering Land Bridge (BLB) has been proved to play an important role in the dispersal of different plant lineages between Asia and America [62–64]. The former genus *Limnobium*, only recorded in America, may have dispersed to America through the BLB during the Miocene [64, 65].

**Methods**

**Taxon Sampling and DNA extraction**

All the samples used in this study were listed in Table 1. The fresh leaves of two *Hydrocharis* species, *H. chevalieri* and *H. dubia*, were collected in 2019 from Cameroon and China, respectively. *Limnobium laevigatum* was sampled from a small swamp in Brazil. The field collection followed the ethics and legality of the local government and was permitted by the government. Total genomic DNA of these three species was extracted from silica-dried leaves using the MagicMag Genomic DNA Micro Kit (Sangon Biotech, Shanghai, China) following the manufacturer’s protocol. The genomic DNA of *H. morsus-ranae* was retrieved from the Kew DNA bank (ID: 14513) and used for the subsequent analysis.

**Genome skimming, Plastome assembly, and Annotation**

The construction of all sequencing libraries and genome skimming was carried out following Li et al. [17]. Approximately 8.5G paired-end reads (150 bp) were yielded for each sample. After trimming and filtering using Fastp v. 0.20.0 [19] with default parameters, at least 7G clean reads were obtained. The complete plastomes were assembled de novo using GetOrganelle v. 1.7.5 with the recommended settings [20]. Web applications Geseq [21] and PGA [22] were applied to annotate genes in the newly generated plastomes using default settings, with manual adjustment of the start/stop codons were manually adjusted through
comparison with a reference plastome (Ottelia alismoides; NC_057494). Circular maps of plastome were created using OGDraw v1.3.1 [23]. All newly generated sequencing data and annotated plastomes were submitted to the GenBank (Table 1).

**Comparative analysis and Divergence hotspot identification**

The web-based software mVISTA [24] was used to identify the sequence and structural variations in Hydrocharis and Limnobium, using the plastome of H. dubia as reference. The comparison of expansions/contraction of IR in Hydrocharis and Limnobium was conducted using Geneious v 5.6.3 [25].

All the protein coding genes (PCGs) and intergenic spacers (IGSs) were extracted separately, and aligned in MAFFT v. 7.221 [26] with default settings. The program DnaSP v.6 [27] was used to assess the nucleotide diversity (Pi) for all PCGs and IGS of the studied plastomes. Additionally, the online application MISA [28] was employed to predict the simple sequence repeats (SSRs) for each plastome. The minimum number of repetitions was set to 10, 5, 4, 3, 3, and 3, for mono-, di-, tri-, tetra-, penta-, and hexanucleotides, respectively. Furthermore, we explored the forward, reverse, palindromic, and complementary repeats for each plastome using REPuter web-based tools [29], with a minimum repeat size of 30 bp and Hamming distance equal to 3.

**Phylogenetic analysis**

Six plastomes of Hydrocharitaceae (Elodea canadensis NC_018541; Enhalus acoroides NC_048519; Halophila beccarii NC_051970; Najas flexilis NC_021936; Ottelia cordata NC_046891; Thalassia hemprichii NC_043774) were downloaded from GenBank. The PCGs were extracted from each of the plastomes and used in the phylogenetic analysis. A total of 78 PCGs in 15 Hydrocharitaceae species, three Alismataceae species (Alisma triviale, Baldellia ranunculoides, and Hydrocleys nymphoides), and Butomus umbellatus representing Butomaceae were retrieved from Ross et al. [7]. Finally, we compiled all 78 PCGs from 29 samples for our phylogenetic analysis (Table S2). Three Alismataceae species and B. umbellatus were used as outgroups.

All PCGs were aligned using MAFFT v. 7.221 [26] and ambiguously aligned regions were removed using trimAl v. 1.2 [30]. Unpartitioned and partitioned strategies of both Maximum likelihood (ML) and Bayesian inference (BI) methods were utilized for phylogenetic inference. For the unpartitioned strategy, we concatenated all 78 PCGs sequences as a supermatrix to infer the phylogenetic relationship with the best-fit model of nucleotide substitution estimated by ModelFinder [31]. For partitioned strategy, PartitionFinder 2 [32] was used to select the best-fit partitioning schemes and models for 78 PCGs via the rcluster algorithm. All phylogenetic analyses were performed with ML using IQ-Tree v. 1.6.12 [33] and BI using MrBayes v. 3.2.7 [34]. An ultrafast bootstrap approximation was used to estimate ML branch support values with 5,000 replicates. BI was run using two independent runs of eight million generations, and four Markov chains were set for each run, sampling trees every 5,000 generations. After verifying that an average standard deviation of split frequencies was less than 0.001, the initial 25% of the trees were discarded as burn-in. A consensus tree with Bayesian posterior probabilities (PP) was constructed from the remaining trees.
Molecular dating and ancestral area reconstruction

Three credible fossils from Hydrocharitaceae with Lognormal prior distributions were employed to calibrate the divergence times of the lineages within Hydrocharitaceae, following previous studies [4, 17, 35]. The first calibration point was assigned between *Stratiotes* and the rest of the Hydrocharitaceae based on the fossil seeds of *Stratiotes* from the Paleocene-Eocene boundary [35]. This node was constrained to a minimum of 54.5 Ma (offset = 54.5, mean = 1.0, SD = 1.0). The fossil *Thalassites* was applied to constrain the stem node of *Enhalus + Halophila + Thalassia* to the Middle Eocene. Thus, the split between (*Enhalus + Halophila + Thalassia*) and (*Nechamandra + Vallisneria*) was constrained to a minimum of 38.0 Ma (offset = 38.0, mean = 0.8, SD = 0.9). The oldest fossil from *Ottelia* recorded in the Upper Eocene was used to constrain the split between *Blyxa* and *Ottelia* with a minimum age of 33.7 Ma (offset = 33.7, mean = 1.1, SD = 1.2). An uncorrelated Lognormal relaxed clock model with Yule tree prior was applied for molecular dating implemented in BEAST v.1.10.4 [36]. We ran 8.0 × 10^8 iterations of Markov chain Monte Carlo (MCMC) and sampled every 2,000 iterations. The program Tracer v. 1.7.1 [37] was used to check the effective sample size (ESS) for the convergence of each parameter discarding the initial 25% generations as burn-in.

According to the existing distribution patterns of *Hydrocharis* [1, 3], four major areas were identified for biogeographic analysis: A) East and Southeast Asia; B) Europe and Central Asia; C) Central Africa; D) America. The ancestral states were reconstructed using the BioGeoBEARS package [38] implemented in RASP v. 4.0 [39]. The best fit biogeographic model was selected by BioGeoBEARS [38] based on the Akaike Information Criterion cumulative weight (AICc_wt). The condensed tree and 100,000 sampled trees from BEAST analysis were used as input.

Conclusion

In this study, we firstly assembled plastomes of three *Hydrocharis* species and *Limnobium laevigatum*. Our phylogenomic analysis recovered non-monophyletic *Hydrocharis* and fully supported *H. chevalieri* as a sister to the rest of the species within the (*Hydrocharis + Limnobium*) group. Based on the morphological and phylogenomic evidence, we suggested treating *Limnobium* as the synonym of *Hydrocharis*. Moreover, we reappraised the divergence time and historical biogeography of *Hydrocharis*. *Hydrocharis* mainly diversified in the Miocene, rapid climate change during the Miocene may have contributed to the speciation of *Hydrocharis* and extinctions. The American members of the genus, i.e. former *Limnobium*, probably have dispersed to America through the Bering Land Bridge during the Miocene. In summary, our study provides new insights into the plastome evolution, phylogeny, and biogeography of the genus *Hydrocharis*.

Abbreviations

AICc_wt: Akaike Information Criterion cumulative weight; BI: Bayesian inference; bp: Base pair; BS: Branch support; ESS: Effective sample size; IGSs: Intergenic spacers; IR: Inverted repeat; ITS: Internal transcribed
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All newly annotated plastomes in this study are available from the National Center for Biotechnology Information (NCBI) (accession numbers: OK326868-OK326871). The associated BioProject, BioSample numbers, and SRA are PRJNA771243, SAMN22263148-SAMN22263151, and SRR16469678-SRR16469681.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Z-Z L, Q-F W, and J-M C conceived and designed the study. Z-Z L, AWG and SL performed de novo assembly, genome annotation, phylogenetic and other analyses. Z-Z L drafted the manuscript. Z-Z L, KM, J-MC collected the leaf materials. Z-Z L, AWG, and SL, EA revised the manuscript. All authors approved the final manuscript.

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**Figures**
Figure 1

Plastome maps of Hydrocharis species and Limnobium laevigatum. The arrowhead shows the inverted region.
Inverted Repeats

Figure 2

Comparison of the boundary of Hydrocharis species and Limnobium laevigatum

Figure 3
Phylogenomic and biogeographic analysis of Hydrocharis. Asterisks represent bootstrap support = 100/posterior probability = 1.00; the first two values represent unpartitioned data, and the last two values represent partitioned data. Triangles indicate the fossil calibration nodes and numbers below branches refer to the mean divergent time estimates. Error bars indicate 95% highest posterior distributions. a-b, H. chevalieri; c, L. laevigatum; and d, H. dubia.

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