Research paper

The complete plastome sequences of five Aponogeton species (Aponogetonaceae): Insights into the structural organization and mutational hotspots

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\textbf{A B S T R A C T}

Members of the aquatic plant genus Aponogeton are widely used commercially in aquariums because of their variable leaf shape and unique inflorescences. However, due to extensive similarity between species in this genus, morphological characters are generally inadequate for taxonomic classification. Currently, molecular makers available for taxonomic and phylogenetic studies of Aponogeton are limited. One approach to clarifying relationships between species in these complex groups is to use divergence hotspot regions within the genome. Here, we sequenced and analyzed the plastomes of five Aponogeton species collected from China, Zambia, and Kenya, and subsequently screened these plastomes for divergent DNA hotspots. The five plastomes are circular structures with sizes ranging from 154,167 bp to 154,860 bp. The Large and the Small Single Copies are separated by two Inverted Repeats. One hundred and thirteen unique genes were identified including 79 protein-coding, 30 tRNA, and four rRNA genes. We found that the most abundant repeats in all but one species were mononucleotide repeats (A/T) and that there were 23 potential RNA ending sites. Interestingly, a ~3 kb inversion, which includes the accD gene, was detected within the Asian species of Aponogeton. The inversion may be related to more frequent exchanges between this region and the nuclear genome. Furthermore, we detected mutational hotspot sites among the five Aponogeton species. Three of these hotspots are intergenic spacer regions (accD-psaI, rbcL-accD and trnH-GUG-psbA) that might be suitable for use as barcodes to resolve intra-generic relationships. We also identified four highly variable protein-coding genes (ccsA, rpl22, rps16 and ycf1) may be used as barcodes to resolve the higher-level phylogenies. Our study will provide valuable molecular resources for the taxonomic and phylogenomic study of the complex genus Aponogeton.

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1. Introduction

The freshwater cape-pondweed family Aponogetonaceae comprises approximately 60 species in the sole genus Aponogeton (Chen et al., 2015; Manawaduge et al., 2016), which are predominantly distributed in tropical and subtropical regions of Africa (~33 species), Asia (~14 species), and Australia (~13 species) (Manawaduge et al., 2016). Because of their variable leaf shape and unique inflorescences, Aponogeton species have become commercially valuable aquarium plants (Les et al., 2005; Dauphinee et al., 2015) that have been cultivated, in some cases (e.g., Aponogeton madagascariensis),
for nearly two centuries (Manawaduge and Yakandawala, 2018). Despite their commercial value, the phylogenetic relationships between members of this genus remain unclear. In recent years, taxonomic revisions and newly described Aponogeton species have relied on morphometric analysis (Yadav, 2017); however, the extensive morphological similarity between Aponogeton species, combined with their highly simplified reproductive structures and extreme phenotypic plasticity, present significant challenges for using morphological data to understand the interspecific relationships within Aponogeton (e.g., Grimsson et al., 2014).

Previous studies have used molecular markers to elucidate the phylogenetic relationships within Aponogeton (Les et al., 2005). For instance, two plastid regions (trnK 5′ intron, matK) and nuclear DNA (nrITS) have been used to identify the relationships among Australian species of Aponogeton, and have shown that these species have recently undergone relatively rapid speciation. In addition, nrITS and the plastid trnK 5′ intron with an adjacent portion of the matK coding region have been used to systematically study the phylogeny and biogeography of the family Aponogetonaceae, although some clades displayed low support.

In fact, to date, species classification within Aponogeton has been based on only a few DNA regions (i.e., nrITS, matK, rbcL), which have been unable to resolve most relationships within the genus, especially those that involve relatively recent or rapidly divergent species (Chen et al., 2015).

Whole chloroplast (cp) genomes have been extensively used in plant phylogeny, biogeography, and species discrimination (Moore et al., 2010; Shaw et al., 2014). Plastome sequences are maternally inherited, lack recombination, and hold more variable sites, which have the potential to resolve complicated evolutionary relationships (Huang et al., 2017; Ji et al., 2019). Notably, the sequences display significant variation in the divergence rate between coding and non-coding regions, which is crucial in resolving the phylogenetic relationships at different taxonomic levels (Niu et al., 2017; Zhang et al., 2017). The majority of cp sequences consist of one large single-copy region (LSC), a pair of inverted repeats (IRA/b), and one small single-copy region (SSC). Their sizes range from 100 to 200 kb due to the genome rearrangements, i.e., IR expansion/contractions, insertions/deletions, gene duplication and transposition (Hong et al., 2017). Comparative analysis of cp sequences provides information on the structural organization of chloroplasts and is vital for an in-depth understanding chloroplast evolution (Burke et al., 2016; Li et al., 2017). In addition, comparison of plastid genomes can lead to the identification of mutational hotspots, which can be used for taxonomic, phylogenetic, and population genetic studies (Ahmed et al., 2013; Downie and Jansen, 2015).

One study has sequenced the whole chloroplast genome of one Aponogeton species (A. distachyos) (Ross et al., 2015). However, the complete plastome was not assembled or annotated. Thus, the structural variations of plastomes within Aponogeton remain unknown. Furthermore, no mutational hotspots have been reported for Aponogeton species. In this study, the complete plastomes of five Aponogeton species are reported. We sequenced these plastomes and compared their structural variations with other Alismatales to 1) provide insights into the evolution of Aponogeton and 2) identify mutational hotspot regions within species that would provide molecular resources for future in-depth taxonomic, phylogenetic, and population genetic research within Aponogeton.

2. Materials and methods

2.1. Plant sampling, high-throughput sequencing, and assembly

During 2018–2019, fresh young leaves of five Aponogeton species were collected from China, Zambia, and Kenya (Table 1), then dried in silica gel. The voucher specimens were deposited at the Herbarium of Wuhan Botanical Garden, Chinese Academy of Sciences (Table 1). High quality total genomic DNA was isolated from 25 mg of dry material using the Modified 2 × CTAB DNA extraction method (Doyle, 1987). A 350-bp DNA sequencing library was constructed for all samples, using TruSeq DNA sample preparation kits (Illumina, San Diego, CA, USA). Genome skimming was performed with the 150-bp pair-end reads using the Illumina Hiseq 2500 platform at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The clean data (~5 Gb) were filtered by the Fastp software (Chen et al., 2018) with default settings, and all high-quality reads were assembled using NOVOplasty 2.7.2 with K-mer 31–39 (Dierckxsens et al., 2017) and Potamogeton perforatus (NC_029814) as the seed and reference genome. The contigs were remapped to the reference genome using Geneious 5.6.4 (Biomatters Ltd., Auckland, New Zealand).

2.2. Plastome annotation and identification of simple sequence repeats

The program GeSeq (Tillich et al., 2017) and Plastid Genome Annotator (PGA; Qu et al., 2019) were used to annotate all genes with reference Potamogeton perforatus (NC_029814). For the start/stop codons, intron boundaries and tRNA genes were further adjusted manually. The circular cp maps of all plastomes were generated and displayed using OGDRAW v.1.2 (Tillich et al., 2017). Simple sequence repeats (SSRs) were detected using MISA-web (MicroSatellite; https://webblast.ipk-gatersleben.de/misa/index.php) with minimal repeat numbers of 10, 5, 4, 3, 3, and 3 for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively. The five plastomes were deposited in the GenBank under accession numbers MN266476-MN266480.

2.3. Comparative analyses and identification of divergence hotspots

To identify potential differences of structure and sequence in the plastomes of Aponogeton, ProgressiveMAUVE (Darling et al., 2004) was used to detect the variations between Aponogeton and the closely related genus Potamogeton. Expansions and contractions of IR regions were analyzed by comparing the positions of the LSC/IR and SSC/IR junctions and their adjacent genes using IRscope (Amiryousefi et al., 2018).

RNA editing among the five newly sequenced plastomes of Aponogeton in this study was analyzed using the online program Predictive RNA Editor for Plants (PREP) Suite (Mower, 2009). The cutoff value was set at 0.8, and the available 35 reference cp genomes were used to detect RNA editing sites in the cp sequences. To explore divergence hotspots within Aponogeton, we extracted protein-coding genes and intergenic spacers and aligned sequences using MAFFT 7.0 (Katoh and Standley, 2013). Sequentially, DnaSP version 6.10 (Rozas et al., 2017) was used to assess nucleotide variability (Pi) in all these regions (>200bp).

2.4. Phylogenetic analysis

The phylogenetic position of the five Aponogeton species, as well as their relationship with other species in the order Alismatales, was analyzed by constructing phylogenetic trees based on protein-
coding genes from 30 species. 25 downloaded from the NCBI database (Table S1). Among these species, three (Acorus calamus, A. americanus, and A. gramineus) were set as outgroups. Maximum-likelihood (ML) analyses and Bayesian inference (BI) were implemented based on 69 commonly shared protein-coding genes using IQ-TREE (Nguyen et al., 2015) and MrBayes version 3.2.3 (Ronquist likelihood (ML) analyses and Bayesian inference (BI) were implemented based on 69 commonly shared protein-coding genes using IQ-TREE (Nguyen et al., 2015) and MrBayes version 3.2.3 (Ronquist et al., 2012), respectively. The best fit model GTR + I + G was selected using jModelTest 2.1.7 (Darriba et al., 2012). For ML analysis, 1000 bootstrap replicates (BS) were used to calculate the bootstrap values. Following two independent Markov Chain Monte Carlo chains, BI analyses were evaluated for ten million generations, with trees sampled every five thousand generations. The first 25% of trees were discarded as burn-in, and the remaining trees were used to construct a consensus tree to calculate the posterior probabilities (PP). To resolve phylogenetic issues within Aponogeton at different taxonomic levels, we carried out ML and BI of genomic plastomes occurred in the first or second codon positions. Nucleotide changes observed in all the Aponogeton species were from cytidine (C) to uridine (U). Twenty-three editing sites were predicted in all Aponogeton species (Table S2). The highest number of potential RNA editing sites was in the ndhB gene, which had nine sites in all species except in A. lakschonensis (eight sites). The ndhD gene also had a high number of RNA editing sites (nine) in A. abyssinicus and A. undulatus. Predicted RNA editing sites were similar in all species except in ten genes (ccsA, ndhA, ndhB, ndhD, ndhF, rpoC1, rpoC2, rps8, rps16, and ycf3), that varied from species to species (Table S2).

3. Results

3.1. General plastome features

The full-length chloroplast genome sequences for the five Aponogeton species examined in this study ranged from 154,167 bp (A. undulatus) to 154,860 bp (A. abyssinicus) (Table 1). The Aponogeton plastomes possess the quadripartite structure typical of most land plant cp genomes (Fig. 1). In addition, each of the five plastomes had 113 unique genes, including four unique rRNA genes, 30 unique tRNA genes, and 79 unique protein-coding genes. Sixteen genes had a single intron, while the genes clpP and ycf3 contained two introns each. The IR regions of each of the cp sequences had 16 genes, which included five protein-coding genes, seven tRNA, and four rRNA genes. The average gene density of all plastomes was the most abundant in all the species, except in A. desertorum (21). In A. desertorum, the most abundant SSRs were dinucleotides (22). In the Aponogeton cp genomes examined only two hexanucleotides were present. Of these, one hexanucleotide was found in A. undulatus, whereas the other was found in A. desertorum. Pentanucleotide repeat motifs were only found in A. desertorum (3) and A. abyssinicus (1) (Fig. 2).

Predicted RNA editing sites in Aponogeton plastomes occurred in the first or second codon positions. Nucleotide changes observed in all the Aponogeton species were from cytidine (C) to uridine (U). Twenty-three editing sites were predicted in all Aponogeton species (Table S2). The highest number of potential RNA editing sites was in the ndhB gene, which had nine sites in all species except in A. lakschonensis (eight sites). The ndhD gene also had a high number of RNA editing sites (nine) in A. abyssinicus and A. undulatus. Predicted RNA editing sites were similar in all species except in ten genes (ccsA, ndhA, ndhB, ndhD, ndhF, rpoC1, rpoC2, rps8, rps16, and ycf3), that varied from species to species (Table S2).

3.2. Comparative analyses and identification of divergence hotspot

Comparative analyses among the five Aponogeton species and P. perfoliatus revealed relatively conserved structures. However, a ~3 kb inversion that included the accD gene was detected within the Asian species of Aponogeton (Figs. 1 and 3). Plastid genome sequences contain regions that are highly variable (genetic divergence hotspots), which are present in coding and non-coding regions. Hotspot regions are useful for screening suitable loci to resolve phylogenetic relationships among closely related species and developing DNA markers for population genetic studies. When we compared sequence variation among five Aponogeton species, we found that intergenic spacer sequences were more variable than protein-coding genes. We also found that four protein-coding genes (ccsA, rpl22, rps16 and ycf1) and three intergenic spacer regions (accD-psal, rbcL-accD and trnH-GUC-psbA) exhibited high nucleotide variability (>(0.02/0.04) and can be used as DNA barcodes at different taxonomic levels (Fig. 4). Comparative sequence analysis of the five Aponogeton species revealed that IR region size was highly conserved between species, ranging from 24,307 bp (A. undulatus) to 24,453 bp (A. desertorum) (Fig. 5). The JLB (LSC/IRb) border was similar in all plastomes between rps19 and rpl2 genes. However, the JSA (SSC/IRA) border
differed profoundly among the studied plastomes. The JSB (IRb/SSC) border of A. desertorum, A. abyssinicus, and Aponogeton rehmanii created a ycf1 pseudogene of 145 bp, 133 bp, and 145 bp, respectively. In A. lakhonensis and A. undulatus, the entire ycf1 gene was located in the SSC region, whereas in P. perfoliatus, the ycf1 gene was located in the IR region. In further contrast to P. perfoliatus, the JSA (SSC/IRa) border of all Aponogeton plastomes was located between the rps19 and rpl2 genes. The JLA (IRa/LSC) border was generally located between rps2 and trnH.

3.3. Phylogenetic analysis

The phylogenetic tree topologies reconstructed from ML and BI analyses were identical (Fig. 6). The five Aponogeton species formed a monophyletic cluster with a robust bootstrap value (BS = 100, PS = 1.00) (Fig. 6). The Asian species, A. undulatus and A. lakhonensis, which had an inverted region containing the gene accD, clustered together close to the African species, A. desertorum and A. abyssinicus, with strong support (BS = 100, PS = 1.00). A. rehmanii was in a sister clade to all the other species. The families Zosteraceae and Potamogetonaceae were recovered as sister to Aponogetonaceae with full support (BS = 100, PS = 1.00). Next, we extracted the identified divergence hotspot regions from 30 studied species. All species included in the data set contained four protein-coding genes, while only 19 species shared the three intergenic spacer regions (Fig. 6). The ML and BI trees generated using hotspots revealed stable topologies, which, with the exception of the placement of Tolifieldiaceae, were consistent with those of the phylogenetic trees based on shared protein-coding genes. Phylogenetic trees based on intergenic spacer region hotspots indicated that Tolifieldiaceae was the root of the Alismatales. Even so, the phylogenetic relationships of the five
Fig. 3. Synteny and the chloroplast genome structure detected in *Aponogeton* genomes using the Mauve multiple-genome alignment program. The yellow box is the inverted regions.

Fig. 4. Nucleotide diversity hotspot regions in *Aponogeton*. 
**Aponogeton** species examined in this study were consistent in all data sets (Fig. 6).

4. **Discussion**

4.1. **Comparative analysis of Aponogeton plastomes**

Our study revealed that the general gene content and arrangement within the five *Aponogeton* plastomes showed a high degree of collinearity. However, a single region containing the *accD* gene was inverted in Asian species (*A. undulatus* and *A. lakhonenensis*), but not in African species (*A. desertorum*, *A. abyssinicus*, and *A. rehmanii*). This inversion has not been found in any of the Alismatales chloroplast genomes sequenced to date. In a related species, *Potamogeton perfoliatus*, similar inversions were detected in the *rbcL-trnM-CAU* region (Luo et al., 2016), which is adjacent to the *accD* gene. The *accD* gene plays a vital role in regulating and controlling the biosynthesis of *de novo* fatty acids in most plants (Ku et al., 2013; Straub et al., 2014). *accD* is usually indispensable for leaf growth and development (Kode et al., 2005), as well as seed oil production (Madoka et al., 2002). Furthermore, in many plant species, *accD* exhibits a highly variable structure, possibly explained by transfers from the nuclear genome (Sudianto and Chaw, 2019). In this study, we also detected a high level of nucleotide variability in this *accD* gene region, which might be due to frequent exchanges between this region and the nuclear genome (Sudianto and Chaw, 2019). The existence of the inversion, which is conspicuously missing in the African species, might be related to the evolutionary history of this genus. However, understanding the evolutionary history of these changes requires more comprehensive sampling within *Aponogeton*.

**Fig. 5.** Comparison of the LSC, SSC and IR border regions among five *Aponogeton* species and *Potamogeton perfoliatus*.

| Species              | Length (bp) | LSC Border | SSC Border | IR Border |
|----------------------|-------------|------------|------------|-----------|
| *Aponogeton desertorum* | 154,590     | LSC: 85,787 | SSC: 6438   | IRb: 20,023 |
| *Aponogeton abyssinicus* | 154,562     | LSC: 85,756 | SSC: 6438   | IRb: 20,023 |
| *Aponogeton lakhonenensis* | 154,860     | LSC: 85,420 | SSC: 6438   | IRb: 20,023 |
| *Aponogeton undulatus* | 154,167     | LSC: 85,754 | SSC: 6438   | IRb: 20,023 |
| *Aponogeton rehmanii*  | 154,528     | LSC: 85,671 | SSC: 6438   | IRb: 20,023 |

GC content acts as a landmark for the physical location of functional elements in the genome (Zhang et al., 2004) and is positively correlated to the rate of recombination and exon density (Freudenberg et al., 2009). We found that the plastomes of *Aponogeton* species in this study had a GC content of ~37%, which is consistent with GC content of plastomes in general. Repetitive elements play a significant role in genomic rearrangements and recombination in chloroplast genomes (Weng et al., 2013). Chloroplast microsatellites have been shown to be effective markers for population genetics, and species delimitation at lower taxonomic levels (Provan et al., 2001). Repetitive sequence analyses revealed that the five *Aponogeton* cp genomes had moderate differences in the total number and position of each repeat motif. Certain repeat motifs were unique in some species, and therefore act as potential markers for genetic diversity studies in populations of *Aponogeton*. Although the IR/SC junctions were relatively conserved, which is a typical character for monocot genome structure, the size of IR regions (24,307 bp - 24,453 bp) displayed variation within *Aponogeton*. The expansion and contraction of IR regions could be responsible for variations in plastid genome size and rearrangements (Raubeson et al., 2007; Yang et al., 2010).

A total of 23 potential RNA editing sites were identified in the five *Aponogeton* species examined in this study. Consistent with a previous study (Wang et al., 2017), we observed that most conversions led to amino acid changes from hydrophilic to hydrophobic. RNA editing that leads to changes from cytidine (C) to uradine (U) can affect the translation of start/stop codons, producing amino acid sequences different from those predicted in the genome (Takenaka et al., 2013). In addition to influencing genetic diversity, the increased hydrophobicity of proteins caused by RNA editing may interfere with self-repair mechanisms on mutated codons, de-stabilizing protein structure (He et al., 2016).

4.2. **Divergence hotspot regions**

In a previous study, Chen et al. (2015) used nrITS and two plastid regions (trnK 5’ intron and *matK*) to study the phylogenetic
relationships among the species in the genus Aponogeton. However, because these plastid regions lack a high degree of polymorphism in Aponogeton species, relationships within this genus remained unresolved. Thus, highly polymorphic DNA barcodes are desperately needed. In this study, several hotspots were detected among the five Aponogeton species, including three highly polymorphic intergenic spacer regions \((\text{accD-psaI}, \text{rbcL-accD}, \text{and trnH-GUG-psbA})\). These intergenic spacer regions are potential barcodes that can be used to resolve intragenic relationships. However, the well-documented high structural variation of Alismatales plastomes (Peredo et al., 2013; Luo et al., 2016; Xing and Guo, 2017) may limit the use of intergenic spacer regions alone. Therefore, we suggest also using four highly variable protein-coding genes \((\text{ccsA}, \text{rpl22}, \text{rps16} \text{and ycf1})\) as barcodes to resolve higher-level phylogeny, e.g., interfamilial phylogeny.

4.3. Phylogenetic value

Ross et al. (2015) had initially investigated the phylogeny of Alismatales using cp sequences. However, only one Aponogeton species was used in their study, which under-represents the genus. Here, based on five plastomes of Aponogeton, and newly detected divergence hotspot regions, we found that the genus is monophyletic, with two subdivisions which are consistent with geological evidence. One subdivision is an Asian group, including \(A. \text{undulatus}\) and \(A. \text{lakhonensis}\), whereas the second subdivision is an African group composed of \(A. \text{desertorum}\) and \(A. \text{abyssinicus}\). The base of these two clades is roughly consistent with previous studies (Chen et al., 2015; Les et al., 2005). This analysis, therefore, confirms the ability of hotspot regions detected in this study to resolve intrageneric relationships within Aponogeton.

5. Conclusion

Our study reports whole chloroplast genome sequences of five Aponogeton species. Complete Aponogeton chloroplast genomes showed a relatively high degree of structural conservation. The plastome of each Aponogeton species had a quadripartite structure and showed no significant variation in gene content or order. However, in Asian species the accD gene was inverted. Despite the relatively high degree of structural conservation, the accD gene was inverted in cp sequences of Asian species. Although more extensive
sampling is necessary to study the evolutionary patterns and in-depth phylogeny of Aponogeton, by estimating hotspot regions, we found three intragenic spacer regions (accD-psal, rbcL-accD and trnH-GUC-psbA) that can potentially be used as intragenic barcodes and four protein-coding regions (ccsA, rps22, rps16 and ycf1) with high variation that may be used as markers for intergeneric studies.

**Author contributions**

Virginia M. Mwanza, Ding-Xuan He, Yan Li, Zhi-Zhong Li designed and performed the experiments; Virginia M. Mwanza, Andrew W. Gichira, Boniface K. Ngarega, and Mwijahki J. Karichu analyzed the data; Virginia M. Mwanza and Peris W. Kamau wrote the paper; Ding-Xuan He and Zhi-Zhong Li revised the paper.

**Declaration of Competing Interest**

The authors declare that they have no conflict of interest.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2020.02.002.

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