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PLASTOME REPORT

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# Sequencing of the complete plastome of the thermal adder's-tongue fern, *Ophioglossum thermale* Kom. (Ophioglossaceae)

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**ABSTRACT**

The Ophioglossaceae family, one of the oldest orders of extant ferns, exhibits diverse morphological and chromosomal characteristics. This study presents the first complete plastome sequence of thermal adder's-tongue fern (*Ophioglossum thermale*), a species renowned for its antioxidant properties in traditional Chinese medicine. Our analyses revealed 27 simple sequence repeats (SSRs) in the plastome, with variations in SSR frequencies compared to related genera. Our phylogenetic analyses placed *O. thermale* within the *Ophioglossum* s.s. clade, supporting previous studies and suggesting polyphyly within the genus *Ophioglossum* based on the sensu PPG I system. The enlarged noncoding regions in fern organelles (ENRFOs) resulting from foreign DNA insertions in *O. thermale* were identified in the *ycf2-trnH* and *trnT-trnfM* regions, similar to other *Ophioglossum* species. ENRFOs were found at the LSC and SSC, but not in IRs in Ophioglossaceae. Consequently, foreign DNA insertions and lineage-specific SSRs shed light on plastome evolution in the Ophioglossaceae family.

## Introduction

The Ophioglossaceae family, one of the oldest orders of extant ferns, is cosmopolitan (Zhang and Zhang 2022). The members of this family are known to possess the highest recorded chromosome numbers of any organism analyzed thus far, resulting from continuous polyploidizations (Khandelwal 1990). The highly unique morphology of these ferns has also puzzled botanists for decades, as its frond features both fertile (sporophore) and sterile (trophophore) segments. However, although the monophyly of the family is strongly supported by both morphological characters and molecular data (Hauk et al. 2003; Shinohara et al. 2013; Knie et al. 2015), debates persist regarding the relationships among the four subfamilies (Shinohara et al. 2013; Kim and Kim 2018) and the number of genera within the family (Wagner 1990; PPG I 2016; Zhang and Zhang 2022).

Within the Ophioglossaceae family, the genus *Ophioglossum* L. encompasses 41 species (PPG I 2016). Among these, the thermal adder's-tongue fern (Ophioglossum thermale Kom. 1914), which is the focus of this study, can be distinguished from other species within the genus by its narrow sterile lamina and narrowly cuneate base (Zhang et al. 2013). Previously, this fern was known to be distributed across China, Korea, Japan, Taiwan, and Russia (Zhang et al. 2013). However, recent findings have expanded its distribution to include India (Kachhiyapatel et al. 2018).

Due to its antioxidant and anti-inflammatory properties, *Ophioglossum thermale* is commonly used in traditional Chinese medicine (Zhang et al. 2012).

This study presents the first sequencing of the complete plastome of *O. thermale*, aiming to explore the plastome characteristics within the genus *Ophioglossum* to enhance our understanding of plastome evolution within this ancient lineage.



**Figure 1.** Photograph of *Ophioglossum thermale* taken by Sang Hee Park at the collection site. This plant is terrestrial. The frond has a narrow sterile lamina and a narrowly cuneate base. The fertile frond is immature.

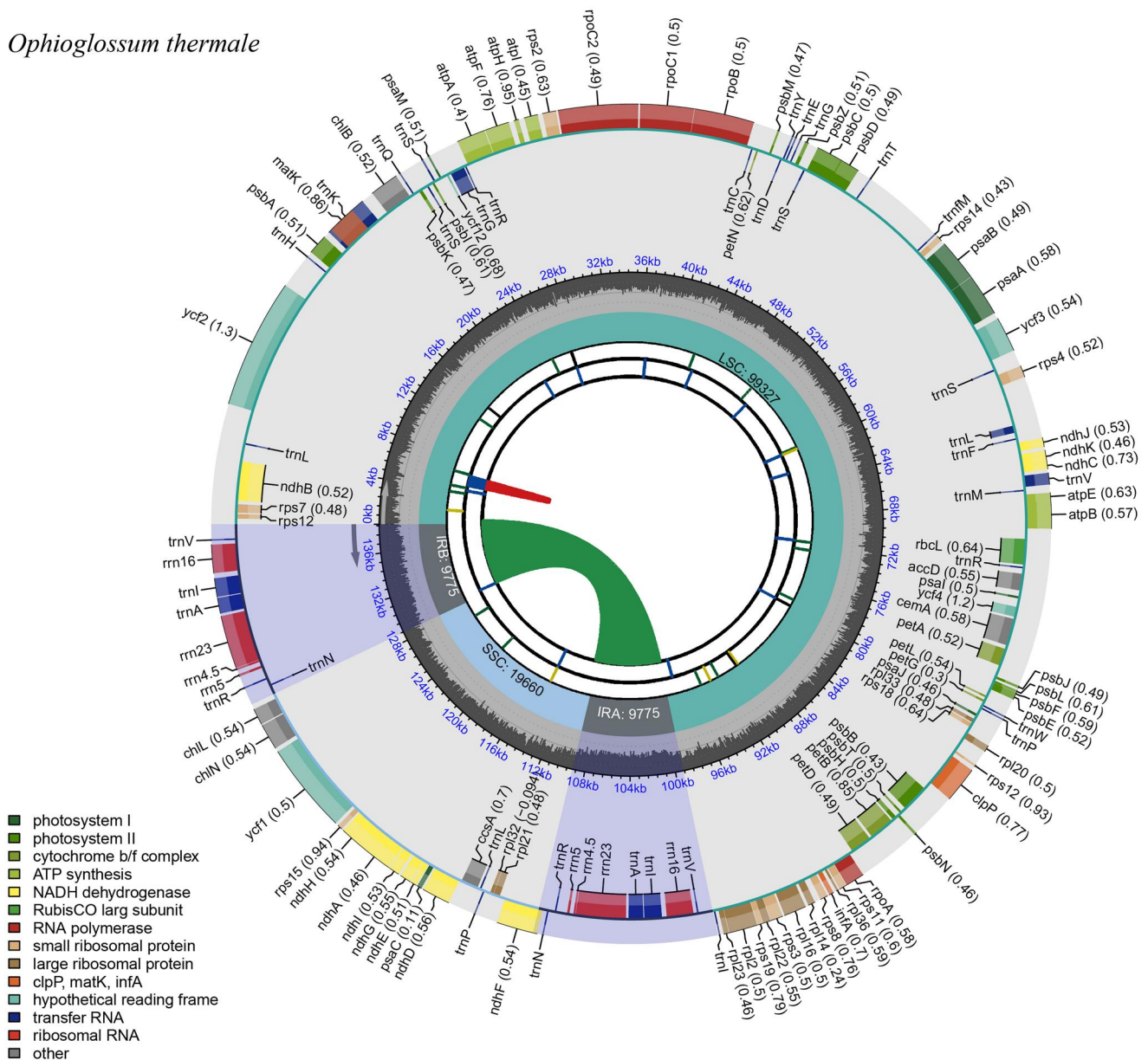**CONTACT** Hyoung Tae Kim ✉ htkim0922@knu.ac.kr 🏫 Department of Crop Science, Kyungpook National University, Sangju, Kyungpook, South Korea

**Figure 2.** Complete plastome map of *O. thermale*, containing six tracks. From the center, the first track shows the dispersed repeats exhibiting direct (red) and palindromic (green) repeats. The second and third tracks show the long and short tandem repeats, respectively. The regional composition of the genome, containing a large single copy, a small single copy, and two inverted regions, are identified on the fourth track. The guanine-cytosine content along the genome is plotted in the fifth track. The genes are shown on the outer sixth track.

## Materials and methods

*Ophioglossum thermale* was collected from Jeonju-si, Korea (35°50′29.8″N 127°03′52.7″E) in August 2023 by Jung Sung Kim, Hyoung Tae Kim, Mi Jeong Moon, and Sang Hee Park and transplanted in the Chungbuk National University's greenhouse (Figure 1). The specimen was deposited at the Chungbuk National University Herbarium (contact: Jung Sung Kim, jungsung@chungbuk.ac.kr) under the voucher number CBNU2023-71A.

Genomic DNA was extracted from a fresh leaf using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. High-throughput sequencing was conducted using an Illumina NovaSeq 6000 sequencer. A total of 145,801,412 paired-end raw reads were obtained, each comprising 151 bp, resulting in a total of

22,016,013,212 bp in read bases. The raw data were trimmed using Trimmomatic 0.39 with the following options: LEADING:10, TRAILING:10, SLIDINGWINDOW:4:20, and MINLEN:50 (Bolger et al. 2014). The trimmed reads, accounting for a total of 4,545,065,458 bp in read bases, were assembled using the reference genome of *Ophioglossum californicum* (NC_020147) to construct the complete plastome sequence following the protocol described by Kim and Chase (2017), with the exception of the read-trimming stage. Genes were annotated and compared with the genes of *O. californicum* using Geneious Prime (Kearse et al. 2012). The putative RNA editing sites were investigated using the ReFernment R package (Robison and Wolf 2019). The complete plastome sequence was submitted to the National Center for Biotechnology Information (NCBI) database under the accession number PP588400. The associated BioProject, SRA, and
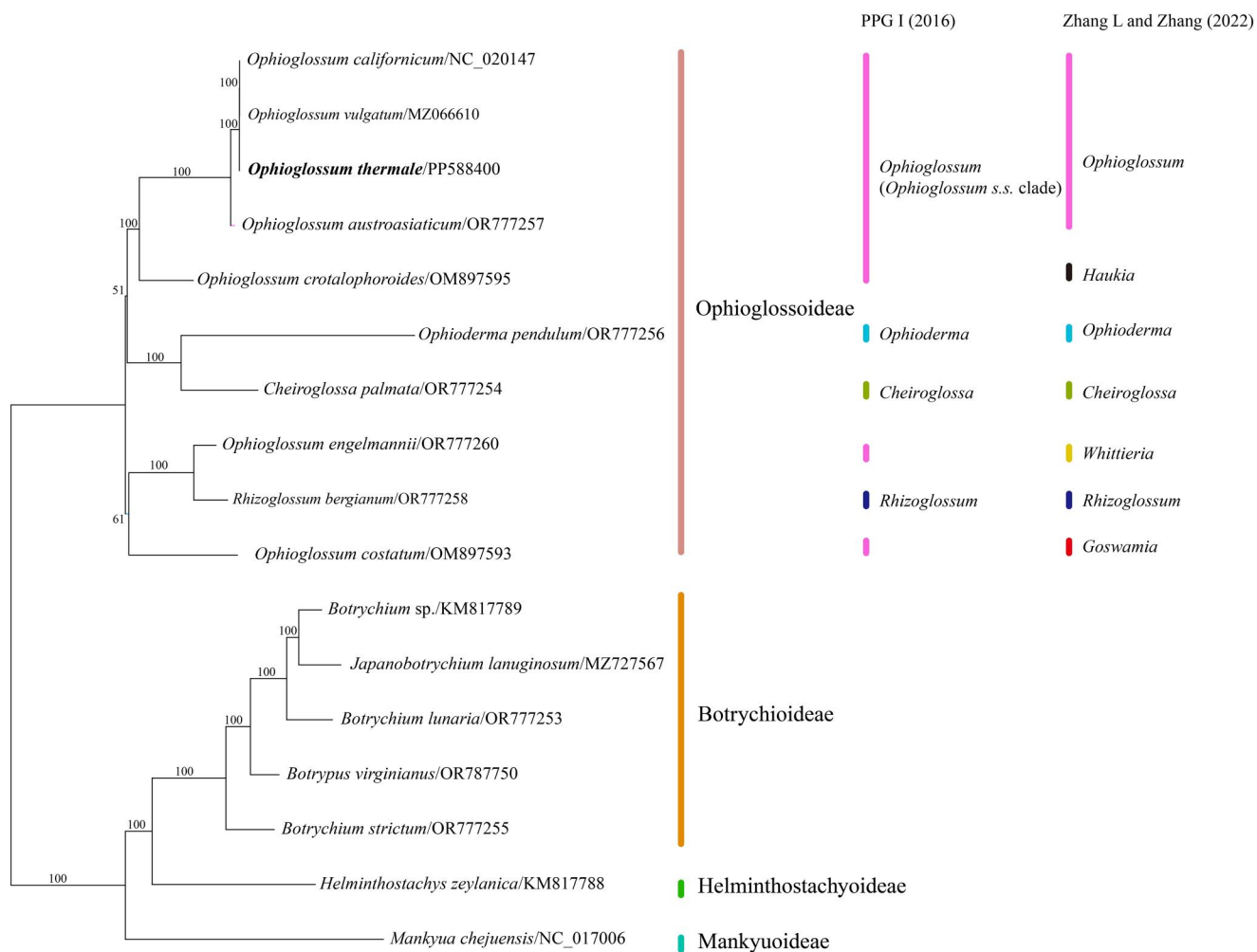
**Figure 3.** Maximum-likelihood tree based on the 84 chloroplast genes. The numbers on the nodes represent the bootstrap support values. The phylogenetic position of *Ophioglossum thermale* was investigated using nine Ophioglossoideae species. The outgroup taxa included *Mankyua chejuensis*, *Helminthostachys zeylanica*, and five Botrichioideae species. The following sequences were used: OR777253 (*Botrychium lunaria*), KM817789 (*Botrychium* sp.), OR777259 (*Botrychium strictum*), OR785750 (*Botrypus virginianus*), OR777254 (*Cheiroglossa palmata*), KM817788 (*Helminthostachys zeylanica*), MZ727567 (*Japanobotrychum lanuginosum*), NC_017006 (*Mankyua chejuensis*), OR777256 (*Ophioderma pendulum*), OR777257 (*Ophioglossum austroasiaticum*), NC_020147 (*Ophioglossum californicum*), OM897593 (*Ophioglossum costatum*), OM897595 (*Ophioglossum crotalophoroides*), OR777260 (*Ophioglossum engelmannii*), PP588400 (*Ophioglossum thermale*), MZ066610 (*Ophioglossum vulgatum*), OR777258 (*Rhizoglossum bergianum*). The color bars refer to different taxonomic groups.

Bio-Sample numbers are PRJNA1093248, SRR28535686, and SAMN40650121, respectively. Furthermore, a plastome map was generated using CPGView (Liu et al. 2023); http://www.1kmpg.cn/cpgview/).

One plastome sequence from *Mankyua chejuensis* (NC_017006), *Helminthostachys zeylanica* (KM817788), five Botrichioideae species (*Japanobotrychum lanuginosum*, MZ727567; *Botrychium lunaria*, OR777253; *Botrychium* sp., KM817789; *Botrychium strictum*, OR777259; *Botrypus virginianus*, OR785750), and nine Ophioglossoideae species (*Ophioderma pendulum*, OR777256; *Ophioglossum austroasiaticum*, OR777257; *Ophioglossum californicum*, NC_020147; *Ophioglossum costatum*, OM897593; *Ophioglossum crotalophoroides*, OM897595; *Ophioglossum engelmannii*, OR777260; *Ophioglossum vulgatum*, MZ066610; *Rhizoglossum bergianum*, OR777258; *Cheiroglossa palmata*, OR777254) were downloaded from the GenBank database. The downloaded plastome sequences within the Ophioglossaceae family were aligned using MAFFT (Katoh et al. 2002). Gaps within the plastome sequence alignment were calculated to confirm enlarged noncoding regions using the "pegas,"' "biostrings,"' and "'dplyr"' packages (Paradis 2010; Pagès et al. 2019; Wickham et al. 2023) in R 4.3.1 (R Core Team 2023), and simple sequence repeats (SSRs) of plastomes in Ophioglossaceae were detected using MISA (Beier et al. 2017). All graphs were generated using the "'ggplot2" R package (Villanueva and Chen 2019).

To determine the phylogenetic position of *O. thermale* within the genus *Ophioglossum*, each of the 84 coding genes was aligned separately using MAFFT (Katoh et al. 2002). Afterward, each alignment was trimmed using trimAl to remove poorly aligned regions, resulting in a single concatenated sequence alignment encompassing 71,699 bp (Capella-Gutiérrez et al. 2009). The partition model for each gene was selected using ModelFinder with edge-proportional partitions and merge options (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017). A phylogenetic tree based on maximum likelihood analysis was constructed using IQ-Tree 2

(Minh et al. 2020) with 10,000 ultrafast bootstraps (Hoang et al. 2018).

## Results and discussion

The coverage depth of the assembled genome ranged from 590 to 2493, with an average of 1883 (Figure S1). The complete plastome of *O. thermale* was 138,537 bp in length with two inverted repeats (IRs) of 9,775 bp between a large single copy (LSC) of 99,327 bp and a small single copy (SSC) of 19,660 bp (Figure 2). The genome contained 129 genes, including 84 protein-coding genes, eight ribosomal RNA genes, and 37 transfer RNA genes with five tRNA genes and four rRNA genes duplicated in the IR region. Among ten cis-splicing genes, eight of them (*atpF*, *ndhA*, *petB*, *petD*, *rpl16*, *rpl2*, *rpoC1*, and *rps16*) contained two exons, while two (*clpP* and *ycf3*) contained three exons (Supplementary Figure 2). Additionally, the trans-splicing *rps12* gene lost its intron between exon 2 and exon 3, similar to other Ophioglossaceae species. The genome exhibited a GC content of 42.1%. A total of 11 putative C to U RNA editing sites able to correctly modify start and stop codons were identified, and one putative U to C RNA editing site was found in the *ycf1* gene for the adjustment of an internal stop codon.

Our phylogenetic analyses positioned *O. thermale* within the *Ophioglossum s.s.* clade (Figure 3), consistent with previous phylogenetic studies (Shinohara et al. 2013; Zhang and Zhang 2022). The *Ophioglossum s.s.* clade is sister to the *Ophioglossum crotalophoroides* clade. However, the classification of *Ophioglossum* species based on the sensu PPG I system revealed that they were polyphyletic. This result supports the proposal by Zhang and Zhang (2022) to reclassify Ophioglossoideae into seven distinct genera, enhancing the phylogenetic resolution and addressing the polyphyly issue.

Foreign DNA insertions have been frequently reported in fern plastomes (Kim and Kim 2018; Robison et al. 2018; Lehtonen & Cárdenas 2019; Kim and Kim 2020). In this study, enlarged noncoding regions in fern organelles (ENRFO) resulting from foreign DNA insertions in *O. thermale* were identified in the *ycf2-trnH* and *trnT-trnfM* regions, similar to other *Ophioglossum* species (Figure S3). ENRFOs were found at the LSC and SSC, but not in IRs in Ophioglossaceae. The *ycf2-trnH*, *petN-psbM*, *trnT-trnfM*, and *trnF-ndhJ* regions were expanded in at least two species. Among these regions, foreign DNA insertions occurred in the most recent common ancestors except for *trnF-ndhJ*. Notably, the *Ophioglossum s.s.* clade appeared to be particularly vulnerable to the integration of foreign DNAs.

A total of 27 SSRs were detected in the plastome of *O. thermale*, consisting of 17 mononucleotide motifs, 9 dinucleotide motifs, and one trinucleotide SSR motif. The mononucleotide SSR frequency in Ophioglossoideae is slightly higher than that of Botrychioideae, whereas the opposite trend is observed for dinucleotide SSRs (Supplementary Figure 4). Most SSRs are lineage-specific, and some of them appear to have disappeared due to base substitutions (Supplementary Figure 5). Given the somewhat frequent distribution of SSRs

in Ophioglossaceae plastomes, this information could provide useful insights into the evolution of this family.

## Conclusions

The sequencing of *O. thermale*'s plastome provides valuable insights into the evolutionary dynamics of the Ophioglossaceae family. The identification of SSRs and foreign DNA insertions highlights the genomic diversity within *Ophioglossum* and underscores the need for further investigation into the evolution of this ancient lineage. Collectively, our findings contribute to our understanding of plastome evolution and phylogenetic relationships within Ophioglossaceae, laying the groundwork for future research on fern biology and medicinal plant genomics.

## Ethical approval

*Ophioglossum thermale* was collected from a natural habitat outside a protective area. No permission is required to collect this species. Research on this species, including the collection of plant materials, was conducted following the guidelines established by the Kyungpook National University.

## Author's contributions

HTK conceived and designed the study, generated and analyzed data, and wrote the original draft, reviewed, and edited it.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Data availability statement

The genome sequence data supporting the findings of this study are available in the NCBI GenBank database under accession number PP588400. The associated BioProject, SRA, and Bio-Sample numbers are PRJNA1093248, SRR28535686, and SAMN40650121, respectively.

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