



The complete chloroplast genome of *Zoysia macrostachya* (Poaceae): Insights into intraspecific variations and species delimitation of the *Zoysia* species

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ABSTRACT: The complete chloroplast genome of *Zoysia macrostachya* Franch. & Sav. isolated in Korea is 135,902 bp long (GC ratio is 38.4%) and has four subregions; 81,546 bp of large single-copy (36.3%) and 12,586 bp of small single-copy (32.7%) regions are separated by 20,885 bp of inverted repeat (44.1%) regions, including 130 genes (83 protein-coding genes, eight rRNAs, and 39 tRNAs). Thirty-nine single nucleotide polymorphisms and 11 insertions and deletion (INDEL) regions were identified from two *Z. macrostachya* chloroplast genomes, the smallest among other *Zoysia* species. Phylogenetic trees show that two *Z. macrostachya* chloroplast genomes are clustered into a single clade. However, we found some incongruity with regard to the phylogenetic position of the *Z. macrostachya* clade. Our chloroplast genome provides insights into intraspecific variations and species delimitation issues pertaining to the *Zoysia* species.

Keywords: chloroplast genome, low-level intraspecific variations, Poaceae, *Zoysia macrostachya*

Zoysia macrostachya Steud. is a perennial plant species, majorly distributed in Korea, Japan, and east costal of China (Soreng et al., 2015; Park et al., 2020a). *Zoysia macrostachya* is a warm season grass having C-4 photosynthetic system (Moser et al., 2004), which can grow well under the high temperature. Till now, no commercial cultivars of *Z. macrostachya* has been developed (Loch et al., 2017; Wang et al., 2020), which is different from *Zoysia japonica* (Chai and Kim, 2000; Ge et al., 2006; Sun et al., 2010) and *Zoysia matrella* (Bae et al., 2008; Choi et al., 2017). Because *Z. macrostachya* has more resistance to salt than *Z. japonica* and *Z. matrella* (Loch et al., 2017), it is highly valuable as a breeding copy for land reclamation or coastal areas. Due to recently sequenced chloroplast genomes of *Zoysia* (Tanaka et al., 2016; Lee and Park, 2021a; Lee and Park, 2021b),

intraspecific variations which have been utilized for developing markers (Li et al., 2020) and understanding phylogenetic relationship (Park et al., 2020b; Park et al., 2021a) can be investigated along with *Zoysia* species. Here, we completed the complete chloroplast genome of *Z. macrostachya* to understand intraspecific variations as well as species boundary.

Materials and Methods

Plant material

We isolated the *Z. macrostachya* in the Subtropical Horticulture Research Institute, Jeju city, Korea (36.83914N, 127.17096E) for conserving natural isolate in Jejudo island in Korea. A voucher and isolated DNA was deposited in the InfoBoss Cyber Herbarium (IN, the voucher number IB-01097).

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DNA extraction and mitochondrial genome determination

Its total DNA was extracted from fresh leaf by using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Genome sequencing was performed using NovaSeq6000 at Macrogen Inc., Korea, and *de novo* assembly was done by Velvet v1.2.10 (Zerbino and Birney, 2008) and GapCloser v1.12 (Zhao et al., 2011). Assembled sequences were modified and confirmed by BWA v0.7.17 (Li, 2013) and SAMtools v1.9 (Li et al., 2009). Circular form was confirmed by connecting both ends using GapCloser v1.12. All analyses were conducted in the Genome Information System (<http://geis.infoboss.co.kr/>) used in the previous studies (Kim et al., 2021a, 2021b; Park et al., 2019a, 2019d, 2021f).

Genome annotation was conducted based on the *Z. macrostachya* chloroplast reported previously (NC_042189) (Cheon et al., 2021) with Geneious Prime 2020.2.4 (Biomatters Ltd, Auckland, New Zealand). A circular map of *Z. macrostachya* chloroplast genome was drawn using OGDRAW v1.31 (Greiner et al., 2019).

Identification of intraspecific variations

Single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were identified from the pairwise sequence alignment of the two *Z. macrostachya* chloroplast genomes conducted by MAFFT 7.450 (Katoh and Standley, 2013) with ‘Find variations/SNPs’ implemented in Geneious Prime 2020.2.4 (Biomatters Ltd., Auckland, New Zealand), which has been used in the previous studies investigating intraspecific variations on organelle genomes (Park et al., 2020c, 2021b, 2021e). INDEL region was defined as the continuous INDELs.

Phylogenetic analysis

Maximum-Likelihood (ML), Neighbor-Joining (NJ), and Bayesian inference (BI) phylogenetic trees were constructed based on the multiple sequence alignment of ten *Zoysia* chloroplast genomes by MAFFT v7.450 (Katoh and Standley, 2013). The NJ and ML tree were reconstructed in MEGA X with 10,000 and 1,000 bootstrap repeats, respectively (Kumar et al., 2018). In the ML analysis, a heuristic search was used with nearest-neighbor interchange branch swapping, TVM + F + R4 model, and uniform rates among sites. All other options used the default settings. The posterior probability of each node was estimated by BI using MrBayes v3.2.6 (Ronquist et al., 2012) plug-in implemented in Geneious Prime 2020.2.4 (Biomatters Ltd., Auckland, New Zealand). The HKY85 model with gamma rates was used as a molecular model. A Markov chain Monte Carlo algorithm was employed for 1,100,000

generations, sampling trees every 200 generations, with four chains running simultaneously. Trees from the first 100,000 generations were discarded as burn-in.

Data Availability Statement

Chloroplast genome sequence can be accessed via accession number MZ233426 in GenBank of NCBI at <https://www.ncbi.nlm.nih.gov>. The associated BioProject, SRA, and Bio-Sample numbers are PRJNA730583, SAMN19236164, and SRR14572437, respectively.

Results and Discussion

The chloroplast genome of *Z. macrostachya* (GenBank accession no. MZ233426) is 135,902 bp (GC ratio, 38.4%) and has four subregions: 81,546 bp of large single copy (LSC; 36.3%) and 12,586 bp of small single copy (SSC; 32.7%) regions are separated by 20,885 bp of inverted repeat (IR; 44.1%) (Fig. 1). Lengths of the complete *Zoysia* chloroplast genomes displayed 135,810 bp to 135,904 bp, similar to those of *Triticum*, *Oryza*, and *Avena* genera (Ogihara et al., 2000; Wambugu et al., 2015; Liu et al., 2020) and displayed the short length among available Poaceae chloroplast genomes (from 129,905 bp [*Rytidosperma semiannulare* (Labill.) Connor & Edgar; NC_036701] to 162,086 bp [*Paspalum ionanthum* Chase; NC_039464] (Burke et al., 2018)). It contains 130 genes (83 protein-coding genes, eight rRNAs, 39 tRNAs, and one pseudogene); 19 genes (seven protein-coding genes, four rRNAs, and eight tRNAs) are duplicated in inverted repeat regions (Fig. 1). One pseudogene is a partial *ndhF*, same to that of the previously reported chloroplast genome of *Z. macrostachya* (Cheon et al., 2021).

Thirty-nine SNPs and 11 INDEL regions (18 bp in total) were identified from pairwise alignment of two *Z. macrostachya* chloroplast genomes. The longest INDEL region is 6 bp long and two INDEL regions are 2-bp. Number of SNPs identified in *Z. macrostachya* is greater in number than that of *Z. matrella* (28 SNPs) (Lee and Park, 2021a); while number of the INDEL regions is smaller. In addition, both are smaller than those of *Z. japonica* (68 SNPs and 24 INDEL regions) (Lee and Park, 2021b), indicating that intraspecific variations of *Z. macrostachya* is the smallest among *Zoysia* species. These numbers are also smaller than those identified between the samples isolated in Korea: e.g., *Campanula takesimana* (Park et al., 2021a), *Pseudostellaria palibiniana* (Kim et al., 2019), *Daphne genkwa* (Yoo et al., 2021), *Abeliophyllum distichum* (Min et al., 2019; Park et al., 2019b, 2019c, 2021d), *Chenopodium album* (Park et al., 2021c), and *Pyrus ussuriensis* (Cho et al., 2019).

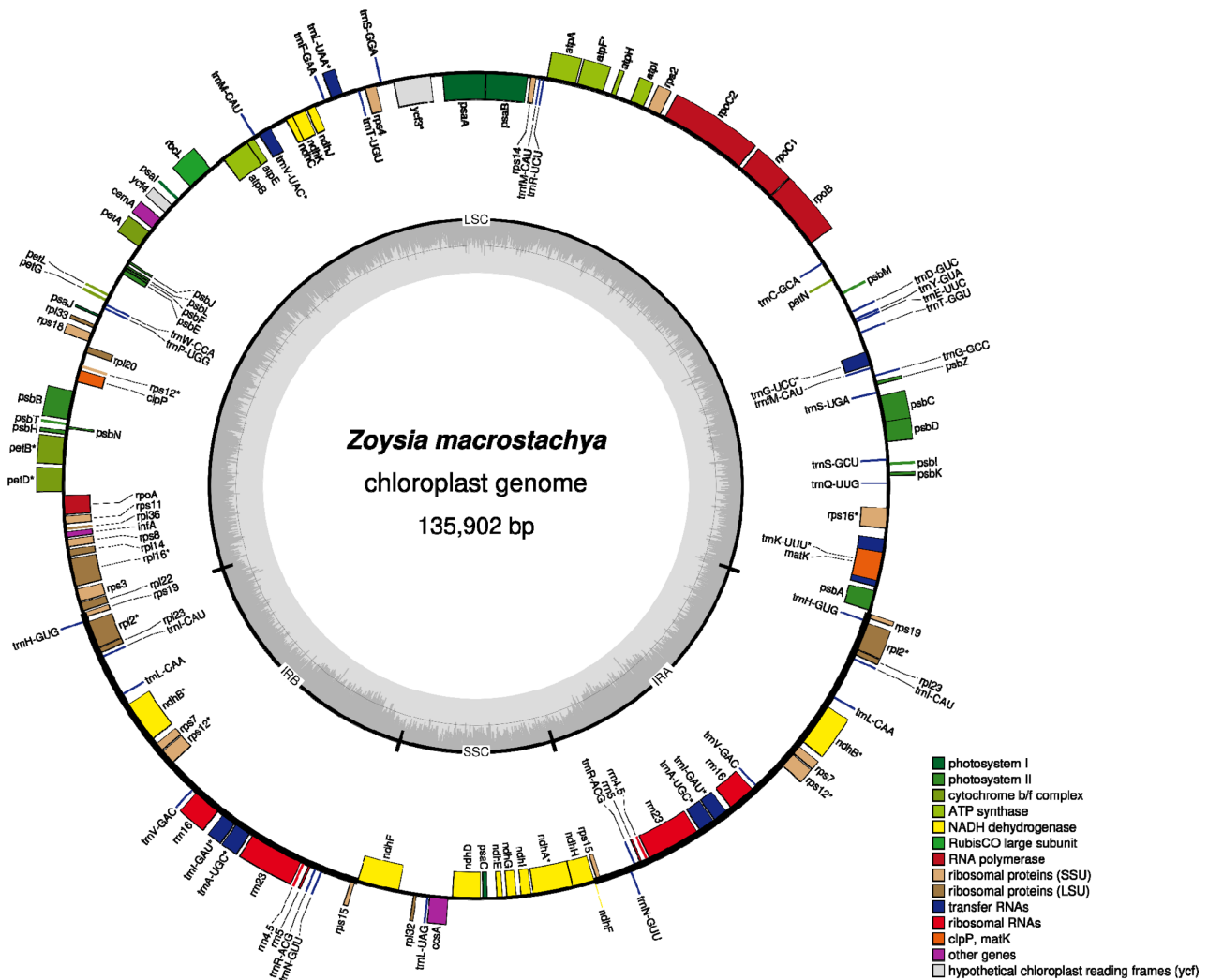


Fig. 1. Circular map of chloroplast genome of *Zoysia macrostachya* isolated in Korea. Gene shown outside are transcribed clockwise, and inside the circle are transcribed counter clockwise. Genes are color-coded to distinguish different functional groups. The dark grey and the light grey plot in the inner circle correspond to the GC content and AT content, respectively.

Two and one non-synonymous SNPs were found in *psaB* and *psbK*, respectively; while two, one, and one synonymous SNPs were identified in *rpoC2*, *infA*, and *ccsA*, respectively. In addition, one SNP was in *trnQ*, displaying 20.51% SNPs are in genic region. Number of genes containing SNPs in *Z. macrostachya* is smaller than that of *Z. japonica*, exhibiting different genes: *rpoB*, *rpoC2*, *atpB*, and *ndhA* contain one synonymous SNP each, *petA* covers three synonymous SNPs, *rpoC1* and *atpF* have one non-synonymous SNP each (Lee and Park, 2021b). This distribution of intraspecific SNPs in the genic region can be developed as intraspecific molecular markers with additional experiments of validation. In addition, *ycf1* which exhibits high nucleotide diversity in various plant species (Jiang et al., 2017; Park and Oh, 2020; Loeuille et al.,

2021) was not found in this chloroplast genome, congruent to the other *Zoysia* chloroplast genomes (Tanaka et al., 2016; Cheon et al., 2021; Lee and Park, 2021a, 2021b).

ML and BI phylogenetic trees show that our *Z. macrostachya* was clustered with previous *Z. macrostachya* with high supportive values of all trees (Fig. 1). Interestingly, the branch length in *Z. macrostachya* clade is similar to the clade containing *Z. japonica* and *Z. sinica* (Fig. 2), suggesting the possible scenario that *Z. sinica* can be considered as *Z. japonica* together with similar morphological features of the two species (Yu et al., 1974). In addition, phylogenetic position of the *Z. macrostachya* clade was not supported by the BI tree (Fig. 2) and topology of *Z. macrostachya* and *Z. macrantha* presented in Cheon et al. (2021) was not also congruent with the three trees (Fig. 2), suggesting

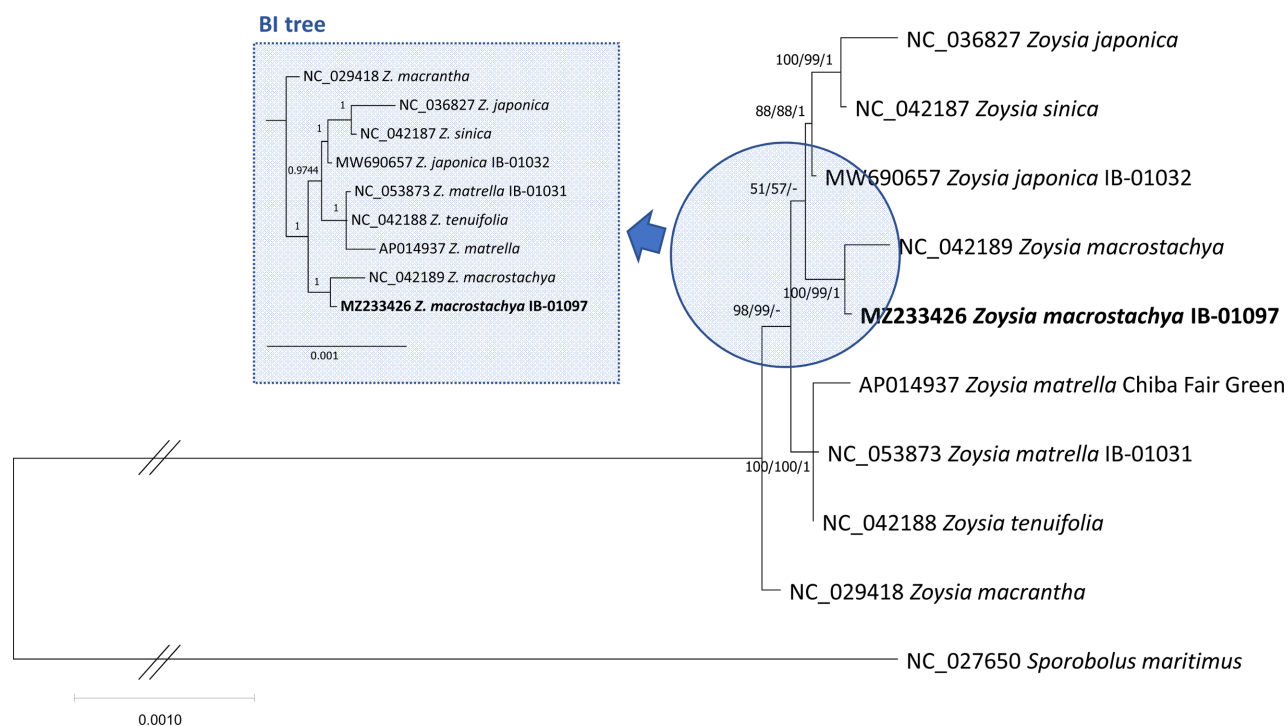


Fig. 2. Maximum-Likelihood (ML), Neighbor-Joining (NJ), and Bayesian inference (BI) phylogenetic trees of ten *Zoysia* and one outgroup complete chloroplast genomes. Phylogenetic tree was drawn based on ML tree. The numbers and hyphens above branches indicate support values of ML, NJ, and BI trees, and incongruent node in comparison to that of ML tree, respectively. BI tree was displayed separately without outgroup species which displayed too long branch length with blue colored shaded dotted rectangle.

additional chloroplast genomes of neighbor species of *Z. macrostachya* are required to clarify the phylogenetic relationship. Taken together, our chloroplast genome provides the insight of intraspecific variations and species delimitation of *Zoysia* species.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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