Agr. Nat. Resour. 56 (2022) 597–604

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Evaluation of low-copy nuclear genes *SQD1* and *pgiC* as DNA barcodes for *Stenochlaena palustris*

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Article Info

Article history:
Received 5 November 2021
Revised 1 April 2022
Accepted 7 April 2022
Available online 30 June 2022

Keywords:
DNA barcoding,
Fern,
pgiC,
SQD1,
Stenochlaena palustris

Abstract

Importance of the work: Identifying potential low-copy nuclear genes as DNA barcodes is needed because there is little genetic information on *Stenochlaena palustris*.

Objectives: To sequence the nuclear genes *SQD1* and *pgiC* and to evaluate their suitability as DNA barcodes for *S. palustris*.

Materials & Methods: *SQD1* and *pgiC* were amplified using the DNA of 19 *S. palustris* samples collected from five locations in Central Kalimantan, Indonesia. The polymerase chain reaction (PCR) products were sequenced using direct Sanger sequencing methods and the chromatograms were examined. The resulting nucleotides were compared within *S. palustris* and with the known *SQD1* and *pgiC* sequences of other ferns.

Results: Direct sequencing showed that *SQD1* was present as a single copy and the nucleotide sequence was highly conserved among *S. palustris* samples. It also showed nucleotide variations compared with *SQD1* from other ferns. On the other hand, *pgiC* produced a single copy of sequence in seven samples but multiple PCR products in the remaining samples. Based on results of the seven samples, *pgiC* was conserved within *S. palustris*. Nucleotide variations were observed between *pgiC* from *S. palustris* and that from other *Blechnaceae* ferns.

Main finding: This was the first report on the *SQD1* and *pgiC* sequence of *S. palustris*. The availability of both sequences will support species identification of *S. palustris*, in addition to using chloroplast genes.

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https://doi.org/10.34044/j.anres.2022.56.3.15

Introduction

DNA barcoding analysis is useful for species identification in addition to morphological characters examination as DNA barcoding utilizes a portion of the gene sequence that is conserved within species, such as chloroplast genes or low-copy nuclear genes (Wang et al. 2016). For most ferns, chloroplast gene information is available but nuclear gene information is still rare due to the lack of fern genome sequence information. However, chloroplast genes are inherited maternally; therefore, they cannot be used to detect the polyploidy and hybridization that often occur in ferns (Rothfels et al., 2013; Wang et al., 2016). In contrast, nuclear genes are inherited from both parents and so can be used to detect both events (Wang et al., 2016). In addition, chloroplast gene sequences sometimes are highly conserved among closely related fern species, often making it difficult to differentiate species based on the chloroplast genes only (Rothfels et al., 2013). Thus, nuclear genes can provide complementary information to chloroplast genes to assist with species identification.

Low-copy nuclear genes often present in genomes as a single copy making it easy to determine the sequences through direct sequencing method. For example, Rothfels et al. (2013) developed primer sets to amplify 20 regions of the low-copy nuclear genes in 10 ferns. The primer sets were tested using 19 fern species, namely *Alsophila podophylla, Dicksonia sellowiana*, *Lindsaea lancea* (L.) Bedd., *Saccoloma inaequale* (Kunze), *Adiantum aleuticum* (Rupr.), *A. pedatum* L., *Cheilanthes covillei*, *Cryptogramma acrostichoides*, *C. acrostichoides*, *Dennstaedtia punctilobula* (Michx.), *Dryopteris intermedia* (Muhl. ex Willd.), *D. intermedia* (Muhl. ex Willd.), *Polypodium amorphum*, *P. glycyrrhiza*, *Athyrium filix-femina* (L.), *Cystopteris bulbifera* (L.), *C. protrusa* (Weath.), *Thelypteris noveboracensis* (L.) and *Woodsia ilvensis* (L.). Some of these sequences have been used for fern phylogenetic analysis (Rothfels et al., 2015) as well as identification of closely related ferns belonging to the Blechnaceae (Cardenas et al., 2019) and *Adiantum* (Wang et al., 2016).

Stenochlaena palustris is a climbing fern species belonging to the Blechnaceae family and the Eupolypod II clade (Irawan et al., 2006; Perrie et al., 2014). It can be found in India, Southeast Asia and Australia to Polynesia (Perrie et al., 2014). It is known as kalakai in Indonesia or as midin/lembiding in Malaysia (Irawan et al., 2006; Chai, 2016). *S. palustris* grows naturally in open, acidic, swamp areas, along riversides, in natural and secondary forests, in oil palm plantations and residential areas and along roadsides (Irawan et al., 2006; Rahmawati et al., 2017). The young, sterile fronds of *S. palustris* are used as a vegetable and in traditional medicines in Southeast Asia (Fig. 1, Chai et al., 2012). In addition to its function as a vegetable, leaves of the fern are used as traditional medicine to treat fever, toothache, skin diseases, ulcers and stomach-ache (Chai et al., 2012). The young, sterile fronds contain high contents of polyphenols, anthocyanin, hydroxycinnamic acids and folic acid compared to other plant parts (Irawan et al., 2006; Chai et al., 2012; Chai, 2016). The water fraction extract of *S. palustris* mature fronds contains an α-glucosidase inhibitor (AGI) that is useful in preventing diabetes (Chai et al., 2015). Recently, kaempferol 3-O-β- glucopyranoside (astragalin) was identified as the responsible bioactive AGI compound (Gunawan-Puteri et al., 2021). *S. palustris* has been categorized as a potential plant species that can be used to restore degraded peatland (Budiman et al., 2020). There are approximately 14 million ha of critically degraded land in Indonesia (Leksono et al., 2021); therefore, the high adaptability of *S. palustris* to various habitats and its nutritional content make it a potential food source on such marginal land. However, due to land

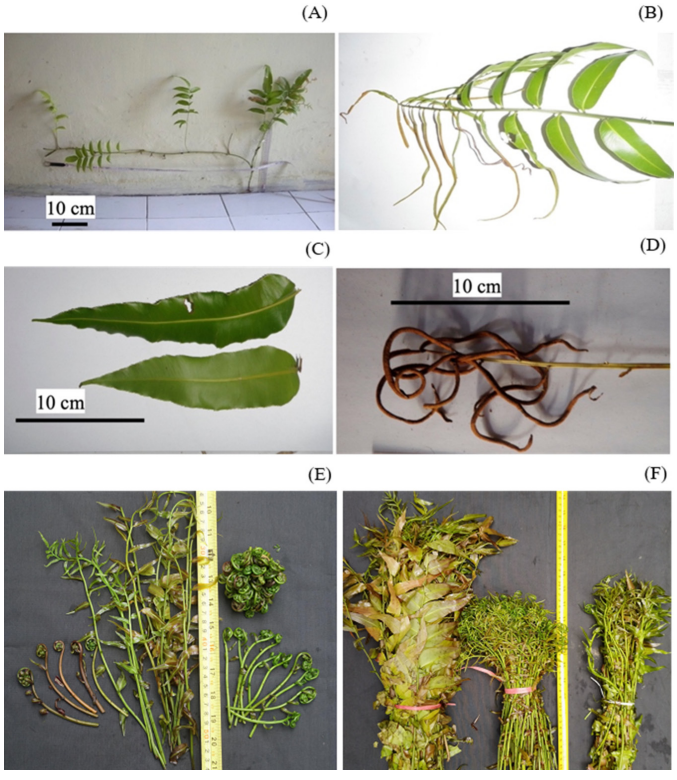

Fig. 1 *Stenochlaena palustris*: (A) whole plant; (B) leaves with spores; (C) mature leaves; (D) spores; (E) young leaves; (F) young leaves (bundle on left) and fronds of *S. palustris* sold in market by a collector

development in the Central Kalimantan region, many of natural habitats of *S. palustris* are being lost; consequently, its genetic resources are also diminishing. It is important to identify any remaining or new *S. palustris* populations and to promote the conservation of its genetic resources. However, without sufficient taxonomic knowledge, it is difficult to identify *S. palustris*, particularly young seedlings.

Currently, species identification methods available for *S. palustris* are based on the observation of morphological characters or sequence analysis of chloroplast genes, such as *rbcL*, *rpoC1*, *psbA-trnH*, *trnG-trnR*, *trnL-trnF*, *rps4-trnS* and *atpA* (Perrie et al., 2014). The current study used the *SQD1* and *pgiC* primers based on information in Rothfels et al. (2013) and they were evaluated for their potential as DNA barcodes for *S. palustris*. *SQD1* and *pgiC* were selected because they amplified well, were present as a single copy and did not require cloning to determine their sequences in most fern taxa (Rothfels et al., 2013). *SQD1* is a gene expressing sulfoquinovosyldiacylglycerol-1 that is involved in the synthesis of sulfoquinovosyldiacylglycerol, a sulfolipid found in chloroplast membranes (Sato, 2004). The primer set EMSQDE1F6 and EMSQDE1R4 amplified a 530 bp region of the *SQD1* gene from 15 fern taxa tested in Rothfels et al. (2013). Another target is *pgiC*, a gene encoding phosphoglucose isomerase that has been extensively used as a nuclear marker in ferns. For example, the *pgiC* primer sets used in Rothfels et al. (2013) amplified 9 out of 15 fern taxa and the length of the PCR products varied among taxa. Cardenas et al. (2019) sequenced the *pgiC* region of 52 *Salpichlaena* specimens belonging to the Blechnaceae fern family and 11 outgroup specimens. *S. palustris* was included as an outgroup specimen in that study; however, the *pgiC* sequence was not reported for *S. palustris*.

The current study evaluated *SQD1* and *pgiC* for their ability as barcodes to identify *S. palustris* from other fern taxa, specifically based on the nucleotide variations between *S. palustris* and other fern taxa.

Materials and Methods

Plant materials and DNA extraction

Young leaves were collected from *S. palustris* populations growing in five locations in Central Kalimantan, Indonesia in January 2019 that represented different peat soil types available in the Central Kalimantan region (Table 1). Peatland types are based on their thickness and their decomposition rate. Peat thickness is divided into four classes, namely shallow (less than 1 m), medium (1–2 m), thick (2–3 m) and very thick (> 3 m). Based on the decomposition rate, the peatland locations in the current study can be categorized into two types: 1) hemic, where soil materials are half decomposed, as indicated by a bulk density in the range 0.07–0.18 g/mL, the fiber content is normally between one-third and two-thirds of the volume before rubbing and the maximum water content when saturated is in the range 450–850%; and 2) sapric, where soil materials are most highly decomposed, as indicated by a bulk density of more than 0.2 g/mL the fiber content averages less than one-third of the volume before rubbing and the maximum water content when saturated normally is less than 450% on an oven-dry basis (Boelter, 1969). The sampling locations were approximately 20–30 km apart. In addition to land development surrounding Palangkaraya city, the region also has experienced many peatland fires under El Nino conditions. For example, during 2002–2011, there were more than 400 fire hotspots annually (Yulianti and Hayasaka, 2013) in the peatland regions in Central Kalimantan. *S. palustris* was one of ferns that re-established after the peatland fires (Yulianti, 2018). Although there is no molecular information, it was postulated for the current study that the plant samples were from different populations. Thus, 3–4 plants were selected from each location for sequencing analysis. Plant material from the same location had been previously identified at the Herbarium Bogoriense, Research Center for Biology, Cibinong, Indonesia

Table 1 Sample information

No	Location	Latitude	Longitude	Peat type	Sample number
1	Kasongan, Katingan Regency	1°43’15.0”S	113°19’34.8”E	Hemic, medium	K1.1, K1.2, K1.3, K1.4
2	Sei Gohong, Bukit Batu	1°55’14.9”S	113°35’23.4”E	Hemic, shallow	K2.1, K2.2, K2.3, K2.4
3	University of Palangka Raya	2°12’48.4”S	113°54’02.8”E	Sapric, shallow	K3.1, K3.2, K3.3, K3.5
4	Pilang Village, Pulang Pisau Regency	2°26’10.9”S	114°10’24.2”E	Hemic, very thick	K4.1, K4.4, K4.6
5	Tanjung Taruna, Pulang Pisau Regency	2°21’15.2”S	114°05’56.9”E	Hemic, thick	K5.1, K5.2, K5.3, K5.10

(No. 252/IPH.1.01/If.07/II/2019) (Gunawan-Puteri et al., 2021). The collected leaves were freeze-dried before DNA extraction. DNA was extracted using a 2% cetyl trimethyl ammonium bromide buffer following the procedure described in Doyle and Doyle (1990). The quality and quantity of the DNA samples were examined using Nanodrop and gel electrophoresis. The DNA samples were diluted to 30–40 ng/µL concentration for PCR.

Polymerase chain reaction conditions

The PCR reaction mixture contained: 30–40 ng DNA, 1× ExTaq buffer, 0.4 mM of each dNTP, 0.5 µM each of the forward and reverse primers and 0.05 unit of ExTaq (TaKaRa, Japan). All primers used in this study are summarized in Table 2. Primers for *SQD1* were from Rothfels et al. (2013), whereas the primers for *pgiC* were designed based on *Salpichlaena* sequences from Cardenas et al. (2019).

The PCR conditions for *SQD1* and *pgiC* were: first stage of 20 cycles of denaturation (95 °C for 15 s), annealing from 48 °C to 68 °C (30 s) raised for 1 °C/cycle, elongation at 72 °C for 1 min; second stage consisting of 10 cycles of denaturation (98 °C for 15 s), annealing (50 °C or 55 °C, 30 s) and elongation (72 °C for 1 min). The reactions were held at 72 °C for 7 min for final elongation. The PCR conditions for *rbcL* were: first denaturation at 95 °C for 5 mins, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (54 °C, 30 s) and elongation (72 °C for 90 s). The reactions were held at 72 °C for 7 min for final elongation and kept in final storage at 10 °C.

The PCR products were checked using gel electrophoresis with 1% agarose gel for 30 min at 100 V. The gels were stained in ethidium bromide or SafeLook Green NucleicAcid Stain (FujiFilm; Japan) solution for 30 min and the PCR bands were examined under ultraviolet light.

Sanger sequencing and analysis

The PCR products were cleaned of excess primers and additional bands using ExoSAP-it Express (ThermoFisher; Japan) following the manufacturer's procedure or using the gel extraction-ethanol precipitation method. Subsequently, the purified PCR products were amplified using a PCR primer and ABI BigDye Terminator v3.1 (Applied Biosystem; Japan) following the manufacturer's protocol. For *SQD1,* sequencing was conducted using the primer EMSQD1ER4, whereas the *pgiC* sequencing was conducted using the primer pgic-F. The samples were sequenced using the ABI PRISM® 3130 Genetic Analyzer (Applied Biosystem; Japan) facility at Hokkaido University, Japan. The quality of the sequence electropherograms was checked using the FinchTV version 1.5.0 software (Geospiza Inc.; USA).

All nucleotide sequences produced in this study were deposited in the National Center for Biotechnology Information (NCBI) Genbank database (https://www.ncbi.nlm.nih.gov). Details of samples and their corresponding Genbank accession numbers are summarized in Table 3.

Comparison of SQD1 and pgiC sequences of S. palustris with other fern species

Alignment was performed using the 'Create Alignment' function in the CLC Sequence Viewer 8 software (Qiagen; the Netherlands). The *SQD1* sequences were compared to known *SQD1* sequences reported in Rothfels et al. (2013) and Rothfels et al. (2015). The *pgiC* sequence was compared to *Salpichlaena volubilis*, S. *papyrus* sp. nov., *S. volubilis* subsp. *Thalassica*, *S. volubilis* subsp. *amazonica*, *S. hookeriana* (Kuntze) Alston, *S. hybrida*, *S. volubilis* subsp. *volubilis*, *Blechnum francii* Rosenst., *Blechnum cartilagineum* Sw., *Sadleria cyatheoides*, and *Woodwardia unigemmata* from Cardenas et al. (2019) and sequences of fern taxa analyzed in Rothfels et al. (2013). For future crosschecking, the GenBank accession numbers of sequences used in this study are shown on the phylogenetic trees and alignments (Fig. 3, Fig. 4, Fig. S2, Fig. S4).

Table 2 Primer sequences for polymerase chain reaction and DNA sequencing

Gene	Primer name	Sequence (5' → 3')
Sulfoquinovosyldiacylglycerol 1 (*SQD1*)	EMSQD1E1F6	GCAAGGGTACHAAGGTHATGATCATAGG
	EMSQD1ER4	GCGTGARTCRTGCACTTTGCTRAGATG
Glucose-6-phosphate isomerase (*pgiC*)	pgic-F	GAGTGTTTGGAATGTCTCATTCCTTT
	pgic-R	GTTCCAGGTTCCCCGAAGTC
Ribulose-1,5-biphosphate carboxylase/oxygenase large subunit (*rbcL)*	rbcL-F	GTTGGATTCAAAGCTGGTGTCAAAG
	rbcL-internal	ACCGCCTCATGGTATTCAAGT
	rbcL-R	CTTACTAGCTTCACGAATAATTTC

Table 3 Sample numbers and corresponding Genbank accession numbers

Sample	Genbank accession number		
	rbcL	*pgiC*	*SQD1*
K1.1	MT416741	OK945948	MT707727
K1.2	MT416743	OK945949	-
K1.3	MT416738	-	MT707728
K1.4	-	-	MT707729
K2.1	MT416739	OK945950	MT707730
K2.2	MT416742	OK945953	-
K2.3	-	-	MT707731
K2.4	MT416735	-	MT707732
K3.1	-	-	MT707733
K3.2	MT416740	-	-
K3.3	MT416727	OK945952	MT707734
K3.5	MT416728	-	MT707735
K4.1	MT416736	-	-
K4.4	MT416729	-	MT707736
K4.6	MT416730	-	MT707737
K5.1	MT416731	-	-
K5.2	MT416737	OK945951	MT707738
K5.3	MT416732	-	MT707739
K5.10	MT416733	OK945954	MT707740

Phylogenetic tree construction for SQD1 and pgiC

The pylogenetic tree was constructed using the raxmlGUI 2.0.6 package (Edler et al., 2020), based on the "maximum likelihood analysis (ML) method + transfer bootstrap expectation + consensus" option, with 100 runs and 100 replications. The resulting tree was viewed using the FigTree v1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

SQD1 evaluation

The *SQD1* primers amplified 14 samples and produced a PCR product of approximately 600 bp in length (Fig. S1). Direct sequencing produced a clean single chromatogram (Fig. 2). There was no nucleotide variation observed among the Central Kalimantan samples, indicating that the *SQD1* sequence was highly conserved in the *S. palustris* from this region (Fig. S2). Since there is not much available information of the *SQD1* sequence from Blechnaceae ferns, *SQD1* from *S. palustris* was compared to that of several ferns studied in Rothfels et al. (2015). The *SQD1* region is relatively conserved across the fern genus, meaning there is no large insertion-deletion shown (Fig. S2). Nevertheless, *SQD1* sequence alignment showed that there were nucleotide variations that will be useful for species identification and phylogenetic studies (Fig. S2). *S. palustris* belongs to the Eupolypod II clade. The phylogenetic tree constructed based on the maximum likelihood method of raxmlGUI 2.0.6 showed that *S. palustris* could be grouped correctly into Eupolypod II using the *SQD1* sequences obtained in the current study (Fig. 3).

pgiC evaluation

Two PCR products, approximately 360 and 600 bp long, were produced based on the primers and PCR conditions set in the current study (Fig. S3). The 600 bp PCR product was amplified only in a subset of samples; thus, further analysis focused on the 360 bp PCR product. Direct sequencing of 360 bp PCR products only showed clean chromatograms in 7 out of the 14 samples tested (data not shown). The remaining samples showed multiple chromatograms. Alignment of the seven clean sequences showed that there were no nucleotide variations

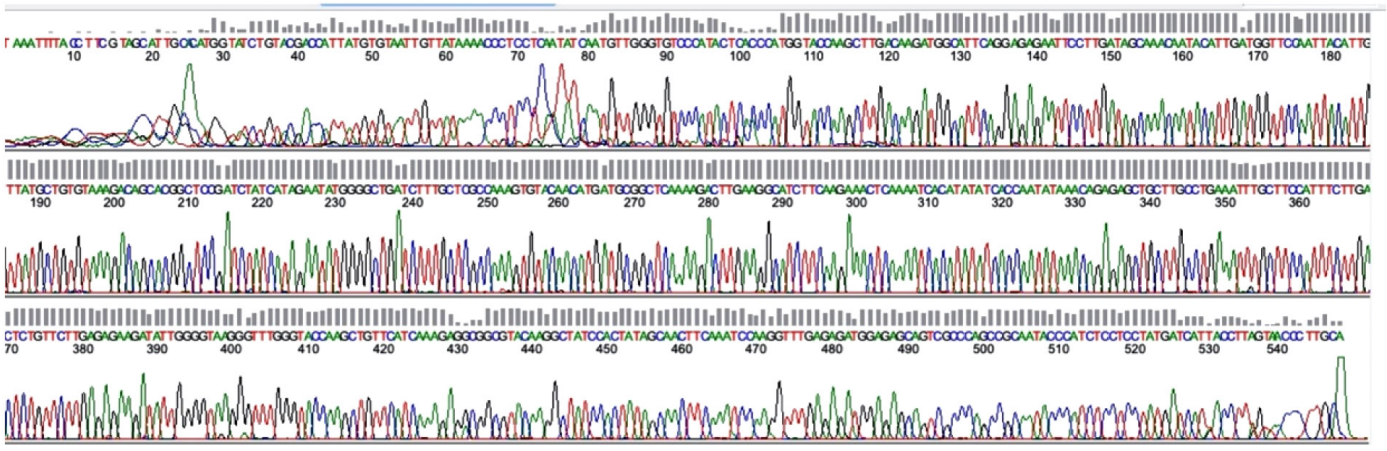

Fig. 2 Example of a *SQD1* chromatogram from *Stenochlaena palustris*

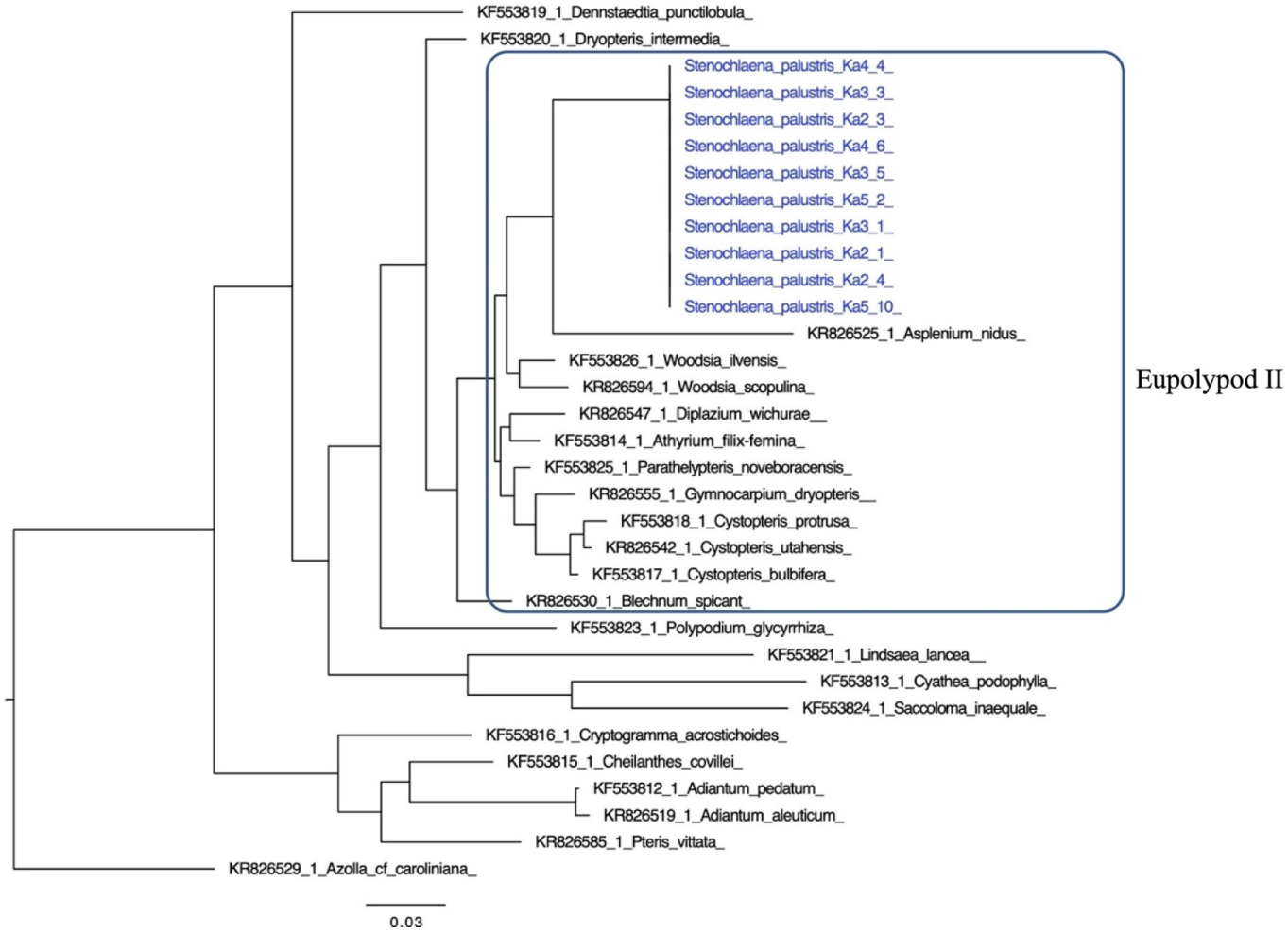

Fig. 3 Phylogenetic tree constructed based on *SQD1* sequences using maximum likelihood method in raxmlGUI 2.0.6 and viewed in FigTree v1.4.4, where scale bar represents branch length. each accession sequence is shown as a GenBank accession number and species name, with *SQD1* sequences obtained in the current study shown in blue and the blue box shows ferns belonging to Eupolypod II clade

in the *pgiC* sequences, indicating that the *pgiC* sequences were also highly conserved among the Central Kalimantan *S. palustris* samples (Fig. S4). Subsequently, the *pgiC* sequence from *S. palustris* was compared to the *pgiC* sequences from ferns belong to the Blechnaceae family, such as *Salpichlaena volubilis, S. hookeriana, S. papyrus, S. volubilis* (Cardenas et al., 2019). There were adequate nucleotide variations observed between *S. palustris* and other ferns belonging to the Blechnaceae (Fig. S2). From the phylogenetic tree analysis, *S. palustris* could be grouped correctly into the Blechnaceae family, based on the *pgiC* sequence obtained in the current study (Fig. 4).

Discussion

Based on currently available information, this was the first report on the *SQD1* and *pgiC* sequences from *S. palustris*. The availability of both sequences will help *S. palustris* species identification, as well as phylogenetic analysis involving *S. palustris*. *SQD1* is promising as a DNA barcode for *S. palustris* because it is conserved within *S. palustris* samples but shows nucleotide variations compared to *SQD1* from other fern taxa. In addition, it is easy-to-use barcode because it was amplified as a single sequence and it is possible to determine the sequence through direct Sanger sequencing. However, more information is needed to determine whether the *SQD1* sequence could be used to distinguish *S. palustris* from other ferns belonging to the genus *Stenochlaena*.

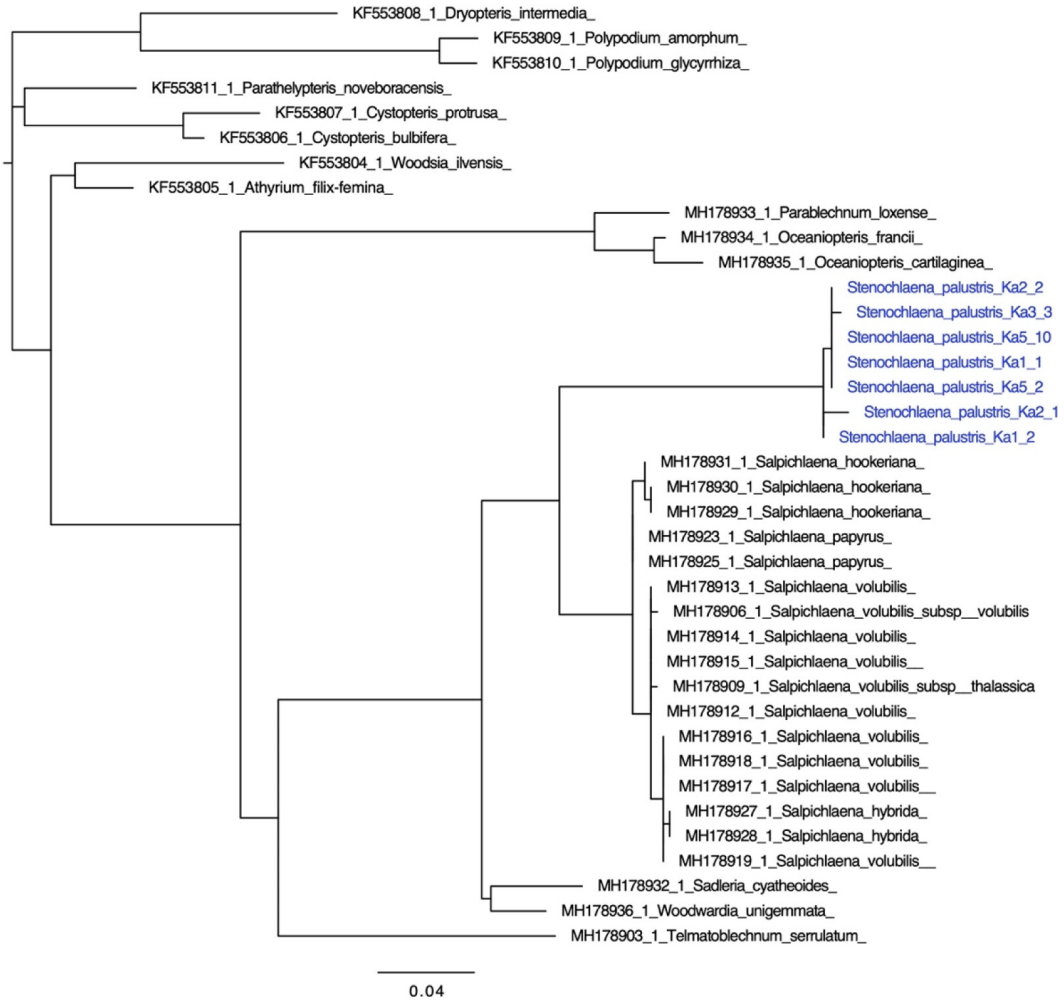

Fig. 4 Phylogenetic tree constructed based on *pgiC* sequences using maximum likelihood method in raxmlGUI 2.0.6 and viewed in FigTree v1.4.4, where scale bar represents branch length, each accession sequence is shown as GenBank accession number and species name, with *pgiC* sequences (blue color) obtained in the current study shown as corresponding sample number

Previously, *pgiC* has been used as a nuclear marker for ferns (Ishikawa et al., 2002; Juslen et al., 2011; Cardenas et al., 2019). Cardenas et al. (2019) determined the *pgiC* sequence of 52 specimens from 7 *Salpichlaena* species. The number of samples per species varied, from 3 samples for *S. hybrida* sp. nov to 20 samples for *S. papyrus* sp. Nov (Cardenas et al., 2019). The primers used in the current study produced a single sequence in seven samples but produced multiple sequences in the remaining samples. Optimization of the PCR conditions, such as determining the right annealing temperature, and gene cloning will help to determine the *pgiC* sequence for *S. palustris*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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