Bioedukasi
Jurnal Biologi dan Pembelajarannya

Vol. 22 No 2, June 2024, page 218-223
e-ISSN: 2580-0094; p-ISSN:1693-3931
DOI: 10.19184/bioedu.v22i2.45484
jurnal.unej.ac.id/index.php/BIOED

Molecular identification of Cyclosorus parasiticus (L.) Farw. From Gumitir, Jember Indonesia

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Article Info
Article history:
Received January 9, 2024
Revised March 22, 2024
Accepted June 21, 2024

Keywords: (A-Z)
Cyclosorus parasiticus
DNA Barcode
Ferns
rbcL

ABSTRACT

Cyclosorus parasiticus (L.) Farw. from Mount Gumitir dominates the understory ferns vegetation along the roadside of a pine-mixed coffee plantation. Previous studies reported the metabolite profile with the flavonoid and alkaloid content. Current observations based on morphological structure of the specimen's collection indicated a high variation, therefor molecular identification was required to support the phylogenetic data of the species. The aim of the study was to identify the Cyclosorus parasiticus sample originate from Gumitir using DNA barcode. We used three primers, i.e. ribulose 1,5-biphosphate carboxylase (*rbc*L), maturase K (*mat*K), and Internal transcribed spacer 2 (*ITS*2) for molecular identification. The result showed that *Cyclosorus parasiticus* from Gumitir has a highest similarity with *Christella latipinna* (NC070300) from China with a value of Percent Identities value of 99.11% based on the *ITS*2 marker. The *rbc*L data in this study might contribute to supporting the molecular genus concept from recent Thelypteridaceae phylogeny.

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1. INTRODUCTION

Indonesia is a mega-biodiversity country because it has high biodiversity, for example, from a diversity of plants, 29,477 species have been reported (Ida et al., 2019; Maskun et al., 2021). One area that has abiotic factors suitable for ferns is Mount Gumitir. This area is located on the border of Jember and Banyuwangi Regencies and is between 700-850 meters above sea level (Setyati et al., 2021). The diversity of ferns in this area was reported by (Makhmud, 2018) found 9 families, 13 genera and 19 species. These results provide important information in studies related to systematic ferns as the earliest stage in the exploration of ferns as herbal medicines.

Ferns are a group of plants that have a high diversity of ± 1,611 species or 11.35% of the diversity in the world (Setiawan, 2022). The Thelypteridaceae family is one of the largest families of ferns consisting of ±1,200 species or 8% of all ferns (Fawcett et al., 2022). Ferns have been used as herbal medicine because they contain secondary metabolites such as alkaloids and flavonoids (Leki et al., 2022). Ferns are easy to find in tropical and subtropical forests with high humidity (Prasani et al., 2021).

Cyclosorus parasiticus (L.) Farw. synonym: *Thelypteris latipinna* (Benth.) K.Iwats.; *Christella latipinna* (Benth.) H.Lév., *Sphaerostephanos latebrosus* (Kunze) Holttum Cyclosorus parasiticus is one of the ferns that dominates on the roadside of coffee-pine plantations and has been reported to contain alkaloids and flavonoids with potential anticancer and antibacterial drugs (Setyati et al., 2020). (Powo, 2023) grouped the Cyclosorus genus into the same genus as Thelypteris and Sphaerostephanos from the Thelypteridaceae subfamily. Systematists (Holttum, 1980) grouped the Cyclosorus and Thelypteris genera in the same grub and separately from Sphaerostephanos. (Fawcett & Smith, 2021) has separated the Cyclosorus, Thelypteris and Sphaerostephanos genera into different genera. The systematics of the Thelypteridaceae subfamily is still under evaluation among systematists because of the very close morphological similarities that make it interesting to study.

Morphological identification of *C. parasiticus* has its own difficulties due to phenotypic plasticity (Grašič et al., 2020). The more stable DNA profile of this species can be used as important information in systematic studies of the Thelypteridaceae through DNA Barcoding. This method is a molecular identification based on short

DNA sequences as plant barcodes using a molecular marker. The recommended molecular marker for confirmation of fern species is riboluse-1.5-bisphosphate carboxylase (rbcL) (Su'udi and Setyati, 2022).

The close morphological similarity of each of the Thelypteridaceae subfamily genera requires re-identification of the morphology and the need for additional information from the DNA barcode profile to obtain more valid results. The results of this study can later be used to study systematics related to the concept of the genus of the Thelypteridaceae subfamily adopted by Fawcett & Smith (2021). In addition, the results of this study can also be used as a reference for further research in avoiding mislabelling and authentication if one day this species has the potential to be used as herbal medicine in the future.

2. RESEARCH METHOD

Plant material

The specimen of *Ciclosorus parasiticus* (L.) Farw was collected from Mount Gumitir, Jember, East Java, Indonesia. The location of the specimen habitat and field identification was followed previous research (Makhmud, 2018). Voucher specimens were stored at herbarium Jemberiense, The University of Jember and live specimens were planted in the Botanical Garden of Biology Department, Faculty of Mathematics and Sciences, The University of Jember, Indonesia.

Morphological identification

The plants habitus and fertile frond containing sori were used for specimen identification. Species names and authors were verified using the Plants of the World Online database (http://www.plantsoftheworldonline.org/). One set of complete specimens was transferred to the pteridologist expert (Wita Wardani, National Research and Innovation Agency) for verification of the specimen identification.

Molecular identification

DNA extraction was conducted on the leaves (0.2 g), which were crushed into a fine powder using a mortar and pestle, and CTAB buffer. In the final stage, the genomic DNA (pellet) was dissolved in a resuspension (RE) buffer and stored for further use. PCR analysis and sequencing were performed using primers *mat*K, *rbc*L dan *ITS*2 from Bionerr. The PCR reaction mixture was prepared by adding 6 µL of ddH2O, 2 µL of DNA template, 2 µL of primer set, and 10 µL of AccuPower® PCR PreMix (Bioneer, Korea) to a total volume of 20 µL. The amplification reaction was carried out in three steps: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min 30 s, and final extension at 72 °C for 5 min. The PCR product underwent separation in a 1.25% agarose gel electrophoresis containing EtBr and was visualized under a UV transilluminator. Subsequently, the PCR product was sent to 1st BASE (Singapore) for purification and sequencing. The genome data was stored in NCBI with ID OR421274.1. The sequencing results were edited using BioEdit and then subjected to BLAST (The Basic Local Alignment Search Information (www.ncbi.nlm.nih.gov). The phylogenetic analysis was performed by processing the sequences in each region using ClustalX 2.1. The phylogenetic tree was then reconstructed using MEGA 11 (Molecular Evolutionary Genetics Analysis) with Neighbour-Joining Tree and Maximum Likelihood with bootstrapping 1000 times (Su'udi and Setyati, 2022).

3. RESULT AND DISCUSSION

Morphological charecteristic and identification

Cyclosorus parasiticus from Mount Gumitir had a high morphological similarity with *Sphaerostephanos latebrosus* from china (Brach, 2006). The morphological character as the key characteristic of *S. latebrosus* was the proximal pinnae gradually shortened; pinnae almost glabrous abaxially. Fawcett & Smith (2021) described the rhizomes of the Sphaerostephanos genera as mostly suberect or erect, but noted that the rhizomes of Cyclosorus are very long-creeping and branching. Identification of pteridologist expert to the herbarium speciment confirmed the species was *Sphaerostephanos latebrosus* (Kunze ex Mett.) Holttum. The nomenclature of *S. latebrosus* in Plants of the World Online (POWO) was synonym of *Thelypteris latebrosa* (Kunze) C.F.Reed. The specimen picture of *Cyclosorus parasiticus* was provided in Figure 1 and the specie description was provided below.

Figure 1. Morphological Characteristics of the *Cyclosorus parasiticus* from Gumitir, Indonesia: a) habitus, b) pinnae, c) Sori

Plant description: Terrestrial; 50-70 cm tall; Rhizome Suberect up to 12 cm diameter; Frond clustered; Stipes 6-7 cm, dark stramineous, bases with sparse brown lanceolate scales; Laminae 39-50 × 13-20 cm, bases gradually narrowed, apices acuminate, oblong to elliptic, Laminae papery, reddish brown when dried, adaxially with sparse short acicular hairs along costae and veins, abaxial surface with yellow sessile spherical glands throughout and almost glabrous; Pinnae 12-16 pairs, a long narrowly triangular terminal pinnae 6.2 × 1.8 cm, longer than lateral pinnae, acute at apex; veinlets 6-8 pairs, proximal pair anastomosing; Proximal pinnae gradually shortened; pinnae almost glabrous abaxially; Middle pinnae lanceolate, 10-18 × 1-2 cm, bases broadly cuneate to truncate, lobed 1/2-2/3 toward costae, apices acuminate. Aerophores beneath pinna bases are slightly swollen; Segments (Pinnule) 20-30 pairs on middle pinnae; Sori orbicular; Indusia glabrous, sometimes glandular; Sporangia bearing yellow spherical glands on capsules; Spores light brown, densely echinulate.

Molecular Identification

The DNA *C. parasiticus* were amplified by PCR method using three primers: *mat*K, *rbc*L, and *ITS*2 with only the *rbc*L primer produced a band with a size of approximately 500 bp (Figure 2). This suggests that the matK and ITS2 primers were unable to amplify the target DNA of *C. Parasiticus*, as no band was formed. rbcL was the most common DNA Barcode through plant (Nitta and Chambers, 2022). This finding is consistent with the research conducted by Trujillo et al. (2021), which reported a 97% success rate for *rbc*L amplification in the Thelypteridaceae family, while *mat*K amplification was unsuccessful.

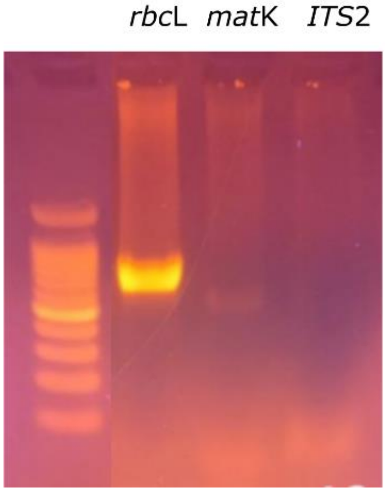

Figure 2. PCR amplification of *C. parasiticus* DNA with three primers

The result of the BLAST analysis of *C. parasiticus* rbcL presented the highest homology with 5 species from genus of Christella. The highest similarity was observed with *Christella latipinna* from China (NC_070300.1) with a value of Per. Identity is 99.11% and Query Cover value is 99 % (Table 1). Sequence similarity of >99 % indicates that the two species are very closely related and even belong to the same species. A Query Cover value of >99 % indicates a large number of *C. parasiticus* nucleotide lengths that are consistent with the BLAST results (Trujillo et al., 2021). Fraser-Jenkins et al. (2018) purposed the genera Thelypteris and Christella are synonyms (Powo, 2023). These results also support the concept of a new genus based on molecular (Fawcett et al., 2021) that the Sphaerostephanos genus from evolutionary history comes from the Christella genus which has separated so that the BLAST results of the two have high similarities.

Tabel 1. BLAST results from the rbcL gene for *C. parasiticus* from Gumitir. The BLAST similarity value was selected based on the highest identity percentage (PI) query cover (Q)

No	Name	Accession Number	Per. Ident (%)	Query Cover (%)	E-value	Source
1	*Christella latipinna*	NC_070300.1	99.11	99	0.0	China
2	*Christella appendiculata*	NC_035842.1	98.93	99	0.0	China
3	*Christella parasitica*	NC_070301.1	98.93	99	0.0	China
4	*Christella acuminata*	NC_070299.1	98.93	99	0.0	China
5	*Christella dentata*	MN159455.1	98.75	96	0.0	China

The evolutionary history of *C. parasiticus* from Gumitir was inferred by using the Maximum Likelihood method and Tamura-Nei model. The maximum likelihood approach aims to identify the tree topologies and branch lengths that offer the highest probability of observing the sequence data (Lin, et al., 2023). The tree with the highest log likelihood (-972.67). The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 20 nucleotide sequences. There were a total of 408 positions in the final dataset. The results of phylogenetic reconstruction based on the rbcL sequence show that *C. parasiticus* forms a cluster with *Christella latipina* (NC_070300.1) from China (Figure 1). These results show that these species are closely related. The validation level of the booster value of 51 % shows a fine value in both NJ tree and Maximum likely hood. According to Kusumaningsih et al (2021) the bootstrap value of a phytogenic tree, its validity can be accepted if the bootstrap value is >50 %. Comparison to the genus representative of global Thelypteridaceae phylogenetic (Fawcett et al, 2021) the rbcL did not show monophyletic of the genus Sphaerostephanos, Thelypteris, Cristella, and Cyclosorus. The bootstrap value among those genus was high (> 90 %). Both trees put the Sphaerostephanos in outer cluster of other three genus. The *rbc*L markers have limitations in species-level detection in ferns might related to the rearrangement of chloroplast in the ferns ancestor (Nitta and Chamber, 2022).

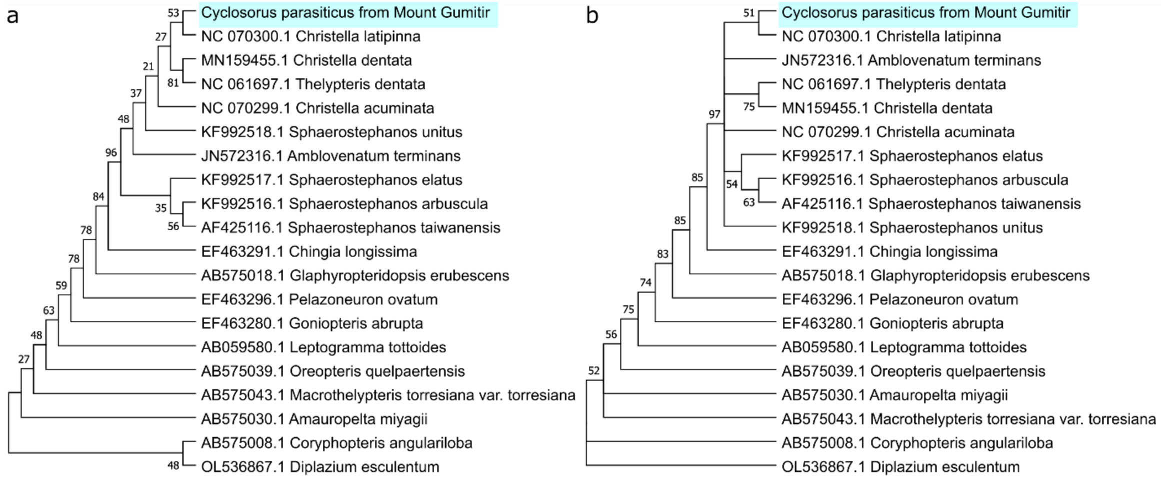

Figure 2. Phylogenetic tree of *rbc*L sequences of *C. parasiticus*: a. Neighbour-Joining, b. Maximum Likelihood

4. CONCLUSION

The morphological and molecular identification of *C. parasiticus* from Gumitir provided the close relationship to the *Sphaerostephanos latebrosus* (Kunze) Holttum and *Thelypteris latebrosa* (Kunze) C.F.Reed. The DNA barcode suggested the highest similarity of our sample to the reference data of *Christella latipinna* (NC070300) from China with a value of Per. Identities 99.11%. Since the systematic of Thelypteridaceae was still under evaluation. The result of our study might contribute to supporting the molecular new genus concept adopted by Fawcett & Smith (2021).

5. ACKNOWLEDGEMENT

This research was funded by The University of Jember, under the grant scheme KERIS-DIMAS 2023 to the Symplast research group of Laboratory Botany, Biology Department, The University of Jember.

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