Biota: Jurnal Ilmiah Ilmu-Ilmu Hayati, Vol. 10(1): 71-79, Februari 2025
p-ISSN 2527-3221, e-ISSN 2527-323X, https://ojs.uajy.ac.id/index.php/biota/issue/view/498
DOI: 10.24002/biota.v10i1.8906

The Role of Chloroplast DNA Markers (*psbA*) in Maintaining the Position of Lemnoideae

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Abstract

The duckweed plant group belongs to the botanical family Araceae and has the potential as a food source. It is difficult to classify and the plants were originally grouped as the Lemnaceae family. However, current molecular taxonomy studies revealed the integration of Lemnaceae into the Araceae family, thus becoming the Lemnoideae subfamily. It is necessary to strengthen the position of Lemnoideae in Araceae using molecular marker *psbA* from chloroplast DNA This study aimed to determine the role of the *psbA* chloroplast DNA marker in regulating the position of Lemnoideae. A total of 41 sequences of the *psbA* gene taken from the species from seven subfamilies in Araceae and one outgroup were collected from the NCBI GenBank and then arranged in FASTA format. Sequence data was then aligned by ClustalX, and phylogenetic trees were reconstructed using PAUP and MEGA. From the resulting phylogenetic trees, it can be conferred that the Lemnoideae subfamily does not form a monophyletic group. Thereby, this in silico study using psbA markers concludes the position ofthe Lemnoideae subfamily in the family Araceae and we recommend not separating the plant group from the family Araceae.

Keywords: Araceae, Lemnoideae, molecular taxonomy, phylogenetic, *psbA*

Submitted : 16 April 2024 ; Revisied : 8 January 2025 ; Accepted : 15 January 2025

Introduction

The Araceae family consists of approximately 102 genera and 3300 species and is one of the most structurally diverse groups of monocots (Choi et al., 2017). The subfamily Lemnoideae (duckweed) within Araceae displays morphological, habitus, and life cycle traits that are unique from other Araceae. (Grayum, 1990). Plants within this group are aquatic plants and are considered to be one of the fastest-growing angiosperms (Ziegler et al., 2015). This characteristic gives the potential of this plant group as a food source due to its high productivity in biomass (Sońtaet al., 2019). However, despite its potential, the duckweed group is still difficult to classify using morphological data, due to size reduction and high complexity due to various structural modifications (Acosta et al., 2021).

Initially, Lemnoideae was grouped as the separate family of Lemnaceae, supported by a determinationkey, mainly based on morphological characteristics (Bog et al., 2020). However, theconfidence in the phylogenetic tree is not strongly supported when using traditional morphological markers as the main basis (Rothwell et al., 2004). The emergence of molecular taxonomy complemented the morphology-based system and helped forming the more accurate systematics of the duckweed group, establishing its stronger position in the phylogenetic tree and revealing the relationship between Lemnaceae and Araceae (Al-Dakhil et al., 2021). Through molecular data, modern taxonomy has changed the status of the Lemnaceae family to a subfamily Lemnoideae within the family of Araceae to preserve the monophyletic nature of the group (Cusimanoet al., 2011). Therefore, the Angiosperm Phylogeny Group (APG) began

How to Cite : Sururi, Z. F., Nururrahmani, A., Sihombing, M., E., & Hidayat, T. (2025). The Role of Chloroplast DNA Markers (psbA) in Maintaining the Position of Lemnoideae. Jurnal Ilmiah Ilmu-Ilmu Hayati 10(1):71-79.

recommending the fusion of Lemnaceae with Araceae, conforming to the molecular-based taxonomy (Angiosperm Phylogeny Group, 1998).

Although the Lemnaceae family has already been dissolved, the use of 'Lemnaceae' remains quite prevalent in scientific literature. Starting from the early 2000s, the use of Lemnoideae taxa in scientific publications began to increase, in accordance with the recommendation from the APG. However, Lemnaceae taxa are still used more frequently in scientific publications, especially in studies that specifically address the duckweed group (Tipperyet al., 2021). Thus, there is still a need for molecular analysis that can strengthen the position of Lemnoideae in the Araceae family, whichis further supported by the growing scientific research on the potential of duckweed plants in various fields (Bog et al., 2019).

In the study of phylogenetic relationships between taxa in plants, plastid DNA sequence data is more often used, including in the study of Araceae (Henriquezet al., 2014). Most angiosperm plastid genes are highly conserved, however, there are deletions, inversions, and rearrangements in some lineages (Gruzdevet al., 2019). Further research into these genetic variations could provide valuable insights into the evolutionary history of a taxonomic group and establish its relationship to another groups (Raman et al., 2023). Sequences of a conserved gene from multiple species within and outside the group are often used to generate phylogenetic trees, as it can be assumed that organisms with similar sequences are closely related (Lozano-Fernandez, 2022).

The *psbA* gene, which encodes the D1 protein in photosystem II of the chloroplast, is one of the most conserved genes, making it suitable for use in phylogenetic studies (Sen et al., 2012). This characteristic allows the gene to be utilized for phylogenetic analysis, as homologous sequences can be reliably aligned and compared across a wide range of taxa (Zhang et al., 2018). Several recent studies have utilized *psbA*gene as the genetic marker in phylogenetic analysis in different taxa (Liu et al., 2023; Zhang et al., 2023; Chen et al., 2024).However, there has been no phylogenetic study of Araceae using *psbA* gene, especially regarding the position of Lemnoideae in the Araceae family.

A previous study used the several other plastid genes to study the position of Lemnoideae, and argued for separating Lemnoideae from the Araceae family into a separate family (Tippery et al., 2021). This discovery provided an intriguing starting point to explore the position of Lemnoideae and use another conserved gene to establish another opinion. Therefore, a new study using plastid gene*psbA* can be complementary and provide new opinions about the position of Lemnoideae. As such, this study aims to determine the role of the chloroplast DNA marker *psbA* in determining the position of Lemnoideae.

Methods

The research used was descriptive qualitative with an *in silico* approach to analyze the position of Lemnoideae in the Araceae family.An*in silico*approach to analyze the plant taxonomic position and genetic variation has been carried out through other research (Pratiwi et al., 2023; Chika &Zahro, 2024). The analysis was carried out from the grouping generated from the phylogenetic tree reconstructed using the *psbA* plastid gene. The DNA barcode *psb*A plastid gene also has been used in previous study(Chika &Zahro, 2024; Ho et al., 2023). In this study, the hardware used was a laptop with Intel® Core™ i3-6006U CPU @ 2.00 GHz processor specifications, 8.00 GB RAM, and Windows 10 Pro 64-bit as the operating system. The software used was Notepad, ClustalX 1.83, PAUP 4.0, MEGA 4, and TreeView 1.6.6. The *in silico* study protocols refer to the previous study (Dzikrina et al., 2023; Ekajaya et al., 2023).

Collection of Sample DNA Sequences

For this study, DNA sequences from representatives of each subfamily in Araceae were collected from the NCBI GenBank (https://www.ncbi.nlm.nih.gov/) by writing the name of the species along with the desired marker or gene (Example: *Lemna minor psbA*). The DNA sequences obtained were saved into the Notepad application and organized in FASTA format (.*txt).

Table 1. Collection of Plant DNA Samples

No.	Taxon	Species	Accession Number
1	Lemnoideae	*Lemnaaequinoctialis*	GU454502
2	Lemnoideae	*Lemnagibba*	GU454507
3	Lemnoideae	*Lemna minor*	GU454516
4	Lemnoideae	*Lemnaminuta*	GU454527
5	Lemnoideae	*Lemnaturionifera*	GU454525
6	Lemnoideae	*Lemnatrisulca*	GU454522
7	Lemnoideae	*Lemnavaldiviana*	GU454529
8	Lemnoideae	*Spirodela intermedia*	GU454484
9	Lemnoideae	*Spirodelapolyrhiza*	GU454493
10	Lemnoideae	*Wolffia arrhiza*	GU454553
11	Lemnoideae	*Wolffia borealis*	GU454556
12	Lemnoideae	*Wolffia brasiliensis*	GU454557
13	Lemnoideae	*Wolffia elongata*	GU454566
14	Lemnoideae	*Wolffia globosa*	GU454573
15	Lemnoideae	*Wolffia microscopica*	GU454574
16	Lemnoideae	*Wolffiellagladiata*	GU454535
17	Lemnoideae	*Wolffiellalingulata*	GU454537
18	Lemnoideae	*Wolffiellaneotropica*	GU454544
19	Lemnoideae	*Wolffiella oblonga*	GU454549
20	Lemnoideae	*Wolffiella rotunda*	GU454551
21	Monsteroideae	*Monstera deliciosa*	KC241914
22	Monsteroideae	*Spatiphyllumphryniifolium*	KC241916
23	Zamioculcadoideae	*Gonatopusboivinii*	OL312313
24	Zamioculcadoideae	*Zamioculcaszamiifolia*	KC241914
25	Aroideae	*Alocasia macorrhizos*	JN406928
26	Aroideae	*Carlephytonglaucophyllum*	OL312166
27	Aroideae	*Colocasia esculenta*	GU135448
28	Aroideae	*Pistia stratiotes*	GU135357
29	Aroideae	*Schismatoglottisneoguineensis*	MH749318
30	Aroideae	*Syngonium podophyllum*	GU135446
31	Pothoideae	*Anthurium durandii*	JX894799
32	Pothoideae	*Anthurium parasiticum*	JX894861
33	Pothoideae	*Anthurium scandens*	JX894875
34	Pothoideae	*Anthurium verapazense*	JX894888
35	Pothoideae	*Pothos chinensis*	JX894785
36	Pothoideae	*Pothos scandens*	JX894789
37	Pothoideae	*Pothosjunghuhnii*	JX894786
38	Orontioideae	*Lysichiton americanus*	KC241913
39	Orontioideae	*Orontium aquaticum*	OL312085
40	Orontioideae	*Symplocarpus foetidus*	KC241912
41	Gymnostachydoideae	*Gymnostachys anceps*	KM895275
		Outgroup	
42	Acoraceae	*Acorus americanus*	DQ008896

The grouping of subfamilies in Araceae used in this study refers to the grouping of eight subfamilies, namely Gymnostachydoideae, Orontioideae, Lemnoideae, Pothoideae, Monsteroideae, Lasioideae, Zamioculcadoideae, and Aroidea (Cabrera et al., 2008). Data on representative species from each subfamily as well as accession numbers were organized in a sample collection table (Table 1). Data from seven subfamilies of Araceae were used, namely Gymnostachydoideae, Orontioideae, Lemnoideae, Pothoideae, Monsteroideae, Zamioculcadoideae, and Aroideae, while the *psbA* sequence data for the subfamily Lasioideae were not available in the NCBI GenBank.

Alignment of Sample DNA Sequences

All DNA sequences that have been obtained from NCBI GenBank were aligned using the ClustalX 1.83 application. Alignment of DNA sequences aims to determine the level of homology between species (Yang et al., 2020). Gaps indicate insertions and deletions as missing data.

Phylogenetic Tree Reconstruction

DNA sequence data that have been obtained from the alignment results are then used for phylogenetic tree reconstruction using PAUP 4.0 and MEGA 4 software to determine the level of kinship of each plant group (Inyang et al., 2022). Phylogenetic tree analysis in both software uses the maximum parsimony method and bootstrap analysis of 1000 replicates. In PAUP, the consistency index (CI) and retention index (RI) values for the consensus tree are known, while the phylogenetic tree resulting from PAUP analysis is displayed using the TreeView 1.6 application (Ihya et al., 2020).

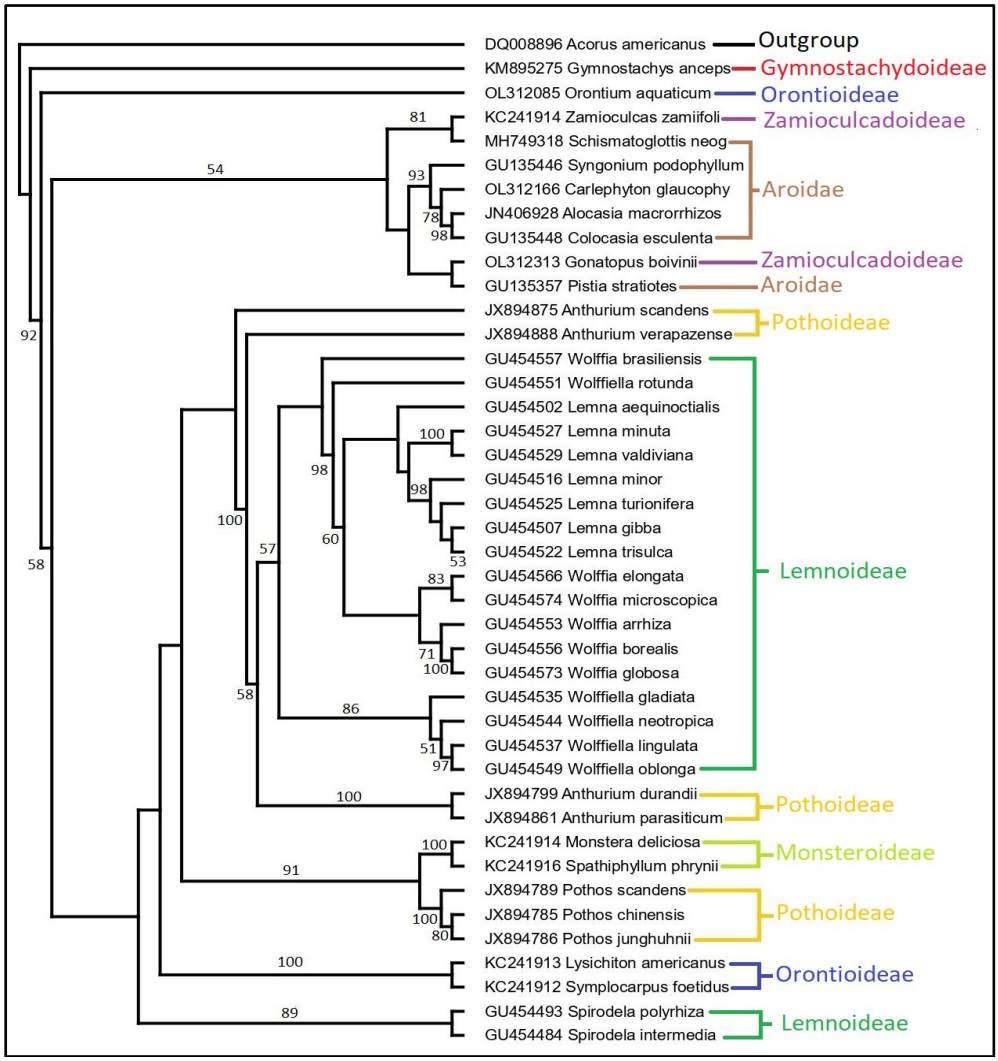

Figure1. Phylogenetic tree reconstruction results of the Araceae family based on psbA marker using PAUP 4.0.

Results and Discussion

Before the phylogenetic tree was reconstructed, an analysis was carried out using the PAUP 4.0 program with 389 characters, which included 43 constant characters, 43 uninformative characters, and 303 informative characters. The number of the characters reflects the number of base pairs in the DNA sequence. The amount of the characters used in this research does not constitute the complete sequence of *psbA*gene due to varying availability of the gene data, therefore partial gene analysis is performed instead. A partial gene analysis can still produce phylogenetic trees needed to establish a taxon's placement in the tree (Ekajaya et al., 2023).

The results of the analysis using the maximum parsimony method, obtained a phylogenetic tree with a consistency index (CI) = 0.569 and a retention index (RI) = 0.765. In addition, from 1000 replicates, the bootstrap value of the majority of consensus trees was also obtained at 50%. The results of the phylogenetic tree reconstruction using PAUP 4.0 are shown in Figure 1.

Phylogenetic tree reconstruction using MEGA 4 also involved 1000 replicates. From the reconstruction, the bootstrap value of the majority of the consensus tree was also obtained at 50%. The results of the phylogenetic tree reconstruction using MEGA 4 are shown in Figure 2.

Phylogenetic trees serve as powerful tools in elucidating the evolutionary relationships among species and their common ancestors (Su'udi, 2019). Phylogenetic analysis is based on the idea that organisms are dynamic entities and have different nucleotide sequence, so it can be used to study the genetic diversity and kinship relationships of organisms (Budiarsaet al., 2019). Therefore, by analyzing certain genetic markers, researchers can reconstruct phylogenetic trees to depict the evolutionary history of various taxa (Choi et al., 2019).

In the case of the Araceae family, the Lemnoideae subfamily's position was investigated using *psbA*genetic markers across 41 species, with *Acorus americanus* serving as an outgroup. The *psbA* gene serves as a valuable genetic marker due to its conserved nature and functional significance (Daniell et al., 2016). Homologous character matching and gap analysis of DNA sequences between species can be analyzed through the alignment process, with gaps in sequences indicating deletions, insertions or rearrangements of genetic material of one or more sequence characters during evolution (Su'udi, 2019). By examining sequence variation in the *psbA* gene among different species of Lemnoideae, we have reconstructed evolutionary relationships and infer the phylogenetic history of duckweed plants within the broader context of the Araceae family.

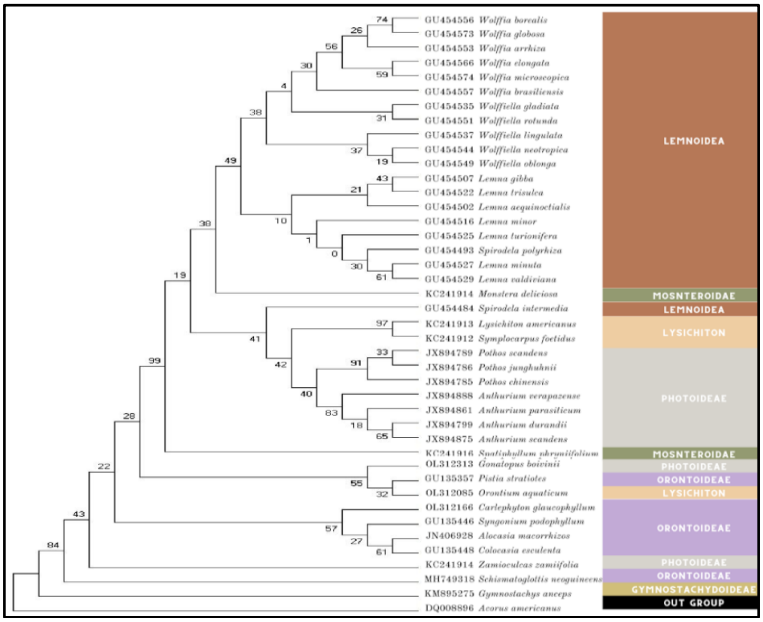

Figure2. Phylogenetic tree reconstruction results of the Araceae family based on *psbA* marker using MEGA 4.

One of the critical aspects of phylogenetic analysis is the assessment of the confidence of inferred relationships (Malik et al., 2020). Bootstrapping, a resampling technique, is commonly employed to evaluate the reliability of phylogenetic trees by assessing how variations in the dataset impact the inferred relationships (Zaharias et al., 2023). Bootstrapping measures the internal consistency of molecular data by analyzing whether slightly modifiedalignments support the same clade (Russo &Selvatti, 2018). Bootstrap values provide a measure of statistical support for specific branches in the tree, with higher values indicating greater confidence in the inferred relationships up to 100%. Bootstrap values less than 50% are not accounted for phylogenetic tree construction (Ojhaet al., 2022).

In our analysis of the Lemnoideae subfamily within the Araceae family, the phylogenetic tree constructed using PAUP 4.0 (Figure 1) revealed a close relationship between duckweed plants and other members of the Araceae family, supported by a high bootstrap value of 92. Despite this evolutionary affinity, duckweed plants exhibit distinct morphological characteristics that differentiate them from other Araceae members (Tipperyet al., 2021). An example of such characteristics can be observed on the duckweed roots which have been reduced in its anatomical and functional aspects and can be considered vestigial (Ware et al., 2023). This is in accordance with the data on the phylogenetic tree, which illustrates that duckweed plants have their own group, namely the Lemnoideae subfamily. However, the placement of the Lemnoideae subfamily does not cluster into one, but is divided into two branches with different sizes and bootstrap values. This result suggests a potential evolutionary divergence within the subfamily. Utilizing MEGA 4 for phylogenetic tree analysis corroborated the findings from PAUP 4.0, confirming the placement of various genera within the Lemnoideae subfamily.

The genera *Wolffia*, *Wolffiella*, and *Lemna* formed a cohesive group supported by a bootstrap value of 57, indicating their close evolutionary relationship.Conversely, the genus *Spirodela* exhibited a distinct position at the base of the phylogenetic tree, supported by a bootstrap value of 89 (Figure 2). This separation aligns with observed anatomical and morphological differences between *Spirodela*and other members of the Lemnoideae subfamily (Ware et al., 2023).

The morphological characteristics of *Spirodela*, including vascular fronds, numerous roots, clear profile, and distinctive inflorescence structures, distinguish it from other genera within the Lemnoideae subfamily (Cusimanoet al., 2011). Conversely, *Lemna* exhibits vascular fronds with a single root and similar inflorescence structures to *Spirodela*, while *Wolffiella* lacks vascular fronds and roots, with a different set of inflorescence features, and*Wolffia* differs from *Wolffiella* only in the rounded fronds (Cusimanoet al., 2011). The *Spirodela* genus is thought to be the most basal and most primitive group of the duckweed group as various results of research based on morphological markers (Park et al., 2020). *Spirodela* has beenreportedto experience the lossofgenes that contributed to the development of lateral rootandroothair (An et al., 2019). These morphological differences likely reflect underlying genetic divergence among genera *Spirodela* within the Lemnoideae subfamily, contributing to their distinct evolutionary trajectories.

The determination of the position of Lemnoideae as a subfamily in the Araceae family reinforced by this study is in line with a similar study which also used chloroplast DNA marker data from *rbcL*, *matK-trnK*, and*trnL-trnF*(Cabrera et al., 2008). In line with our results, Cabrera et al. (2008) argued that the establishment of the Lemnaceae family would give the Araceae family a paraphyletic nature, so it is not advisable to establish it as a separate family. The research conducted by Tippery et al. (2021) using data on chloroplast DNA markers *matK*, *ndhF*, *rbcL*, *rps16*, and *trnL-F* stated that Lemnoideae can be grouped as a separate family from Araceae, but accompanied by the establishment of the Orontiaceae family which has members from the Orontioideae and Gymnostachydoideae subfamilies. However, this statement is not in line with our results, because grouping Orontideae and Gymnostachydoideae in the same group will still produce a paraphyletic group. In addition, the resulting Lemnoideae group is also not monophyletic. Thus, we do

not recommend the establishment of separate families of Orontiaceae and Lemnaceae from Araceae.

Conclusions

The reconstruction of phylogenetic relationships of 41 species from the Araceae family and one outgroup species *Acorus americanus* using *psbA*marker shows that the duckweed plant group is in the Araceae family within the Lemnoideae subfamily group and does not form a monophyletic group. Therefore, phylogenetic analysis using *psbA* markers can be used to strengthen the position of Lemnoideae and does not suggest separating Lemnoideae as a separate family from Araceae. Further phylogenetic analysis of the position of the Lemnoideae subfamily by increasing the number of plant samples and adding other molecular markers needs to be carried out to develop a new, much better chloroplast DNA-based classification system.

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