Hidden diversity of *Salvinia* (Salviniaceae) revealed by molecular identification in a Neotropical floodplain

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Key words:
- aquatic macrophyte
- fern
- molecular markers
- species delimitation

Abstract

Salvinia species are important to aquatic ecosystems but challenging to identify in the field due to their morphological similarities. In the upper Paraná River basin, *Salvinia auriculata*, *Salvinia herzogii*, and *Salvinia minima* are among the most abundant aquatic plants. For this reason, this study aimed to confirm the identification of these species using molecular markers ITS (nuclear) and *trnL-trnF* (chloroplast). Genetic distances, phylogenetic analysis, and molecular delimitation were applied to group sequences into Molecular Operational Taxonomic Units (MOTUs). Results showed that *S. auriculata* formed a single MOTU, while *S. herzogii* clustered with *S. molesta* from GenBank, indicating a new occurrence of *S. molesta* in the region. One *S. minima* specimen was confirmed, while another was identified as *S. natans*, marking the first record of this species in Brazil. The identification of *S. herzogii* as *S. molesta* accentuate the challenge of distinguishing these species without the sorophore. The presence of *S. natans* in sympatry with *S. minima* is concerning due to its invasive potential. The study highlights the importance of molecular techniques in distinguishing morphologically similar species.

Palavras-chave:
- macrófitas aquáticas
- samambaias
- marcadores moleculares
- delimitação de espécies

Resumo

As espécies do gênero *Salvinia* são importantes para os ecossistemas aquáticos, mas a sua identificação em campo é difícil devido às suas semelhanças morfológicas. Na bacia do alto Rio Paraná, *Salvinia auriculata*, *Salvinia herzogii* e *Salvinia minima* estão entre as macrófitas aquáticas mais abundantes. Este estudo teve como objetivo confirmar a identificação dessas espécies utilizando os marcadores moleculares ITS (nuclear) e *trnL-trnF* (cloroplastidial). Foram analisadas distâncias genéticas, filogenia e delimitação molecular para agrupar as sequências em Unidades Taxonômicas Operacionais Moleculares (MOTUs). Os resultados mostraram que *S. auriculata* formou um único MOTU, enquanto *S. herzogii* se agrupou com *S. molesta* do GenBank, indicando uma nova ocorrência de *S. molesta* na região. Um espécime de *S. minima* foi confirmado, enquanto outro foi identificado como *S. natans*, representando o primeiro registro dessa espécie no Brasil em áreas naturais. A identificação de *S. herzogii* como *S. molesta* ressalta o desafio de distinguir essas espécies na ausência do soróforo. A presença de *S. natans* em simpatria com *S. minima* é preocupante devido ao seu potencial invasor. O estudo destaca a importância das técnicas moleculares na distinção de espécies morfologicamente semelhantes e no registro de novas ocorrências.

Original Papers

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Introduction

Salvinia (Salviniaceae) is a heterosporous monilophyte with approximately 12 species distributed worldwide, being South America the most diverse region for *Salvinia*, with about 10 species occurring in Brazil (Mitchell & Thomas 1972; Miranda & Schwartsburd 2019). *Salvinia* are free-floating aquatic macrophytes with two floating leaves and one submerged leaf per node. The floating leaves are densely covered with trichomes, which make them highly water-repellent, while the submerged leaf bears the reproductive structure known as the sorophore (Nagalingum *et al*. 2008). The arrangement of sorophore is a key trait for accurately identifying species, although trichome shape and floating leaf morphology can also aid in this task (Nagalingum *et al*. 2008; Miranda & Schwartsburd 2019).

Trichomes are among the most notable vegetative structures in *Salvinia* due to their diverse arrangements, shapes, and sizes, making them valuable morphological characters for distinguishing species and forming taxonomic groups (Barthlott *et al*. 2009; van Valkenburg *et al*. 2023). One example of this is the *Salvinia auriculata* complex, which includes four taxa: *Salvinia auriculata* Aubl., *Salvinia biloba* Raddi, *Salvinia herzogii* de la Sota, and *Salvinia molesta* D. Mitch. (Mitchell & Thomas 1972). These taxa are grouped within the *Salvinia auriculata* complex because they possess four trichomes fused at the apex, forming a distinctive "eggbeater" shape (Mitchell & Thomas 1972). However, the taxa within this complex exhibit strikingly similar vegetative structures, which can lead to misidentification.

The occurrence of *Salvinia* species and other macrophytes has been monitored in the upper Paraná River basin, particularly in the upper Paraná River floodplain (planície de inundação do alto Rio Paraná - PIAP) by a long-term Ecological Research Program. During this monitoring, *Salvinia herzogii*, *S. auriculata*, and *Salvinia minima* Baker have frequently been recorded (Souza *et al*. 2017), but with uncertain identification due to their close morphological similarities. In these environments, *Salvinia* is one of the most abundant macrophytes (Casatti *et al*. 2003; Souza *et al*. 2017), playing significant ecological roles in supporting communities of Ostracod, Protists, and fishes (Pelicice & Agostinho 2006; Alves *et al*. 2010; Higuti & Martens 2014). Another area adjacent to the PIAP where *Salvinia* is abundant and ecologically important is the Rosana's Reservoir (Casatti *et al*. 2003; Pelicice & Agostinho 2006; Ferrareze *et al*. 2014). Some studies in this area have not identified *Salvinia* individuals at the species level (Casatti *et al*. 2003; Pelicice & Agostinho 2006; Ferrareze *et al*. 2014), while others refer to *Salvinia* in the reservoir as *S. herzogii* (Thomaz *et al*. 2012; Silveira & Harthman 2024). Despite the ecological importance of this genus in these environments, no studies have been dedicated exclusively to confirm species identities in the region. Additionally, it is rare to find *Salvinia* individuals with sorophore, which compromises accurate species identification.

Molecular markers have helped in species delimitation for plants with few diagnostic morphological characters, as in the case of *Salvinia* (Hollingsworth *et al*. 2011; Perkins 2019). Molecular species delimitation methods test hypotheses based on genetic (species as entities sharing nucleotide sequence similarities) and phylogenetic (species as entities sharing a common ancestor in phylogeny) concepts, resulting in Molecular Operational Taxonomic Units (MOTUs) (Blaxter *et al*. 2005). For example, the Assemble Species by Automatic Partition (ASAP) method (Puillandre *et al*. 2021) uses genetic concepts to delimit species based on genetic distance, while the Poisson Tree Processes (PTP) method uses phylogenetic concepts to delimit species based on phylogenetic positioning (Zhang *et al*. 2013). Both methods have been useful in delineating morphologically similar plant species (Ji *et al*. 2021; Jiang *et al*. 2024).

Studies utilizing molecular markers for *Salvinia* taxonomy and systematics are scarce, as most of them have used *Salvinia* in a broader context of monilophytes (Hasebe *et al*. 1995; Pryer 1999; Shen *et al*. 2018) or as an outgroup for *Azolla*, a sister-genus of *Salvinia* (Reid *et al*. 2006; Metzgar *et al*. 2007). To our knowledge, only three studies have exclusively used molecular markers to study *Salvinia* species: two used cpDNA markers to examine phylogenetic relationships within the genus (Nagalingum *et al*. 2008; Machado *et al*. 2016), and one used plastome markers to trace the origin of invasive populations of *S. molesta* in the United States (Holt Jr. *et al*. 2023). Thus, molecular delimitation methods for distinguishing *Salvinia* species have not yet been applied, which could provide valuable data for understanding relationships among morphologically similar species and reveal cryptic species.

For *Salvinia* species occurring in the upper Paraná River basin (*S. auriculata*, *S. herzogii*, and *S. minima*), we suspect that the main problem lies in identifying *S. herzogii*. According to Miranda & Schwartsburd (2019), *S. herzogii* closely resembles *S. biloba* and *S. molesta*, and they can only be distinguished by subtle differences in leaf incision and submerged leaf length in the absence of sorophore. Furthermore, the close phylogenetic positioning between *S. herzogii* and *S. biloba*, and the possibility that one of these species may be the progenitor of *S. molesta* (Miranda & Schwartsburd 2019; Holt Jr. *et al*. 2023), could explain the difficulty in morphological delimitation, which may also be reflected at the molecular level. On the other hand, although *S. auriculata* belongs to the same

morphological complex as *S. herzogii*, *S. biloba*, and *S. molesta*, its larger leaf dimensions and the absence of leaf incision are distinguishing traits that allow for its identification even in the absence of sorophore. Likewise, *S. minima* is characterized by its smaller leaves and an ungulate-like trichome type (four unconnected trichomes at the apex), which differentiates this specie from both *S. auriculata* and *S. herzogii* (see Miranda & Schwartsburd 2019 for details on morphological differences among *Salvinia* species in Brazil).

Therefore, we aim to use the nuclear DNA (nrDNA) Internal Transcribed Spacer (ITS) and cpDNA *trnL-trnF* markers, both widely used in molecular identification and phylogenetic studies in plants (Hollingsworth *et al*. 2011; Lucio *et al*. 2019; Scorsim *et al*. 2024) to confirm the identification of *S. auriculata*, *S. herzogii*, and *S. minima* in the PIAP and Rosana's Reservoir, both located in the upper Paraná River basin. This study analyzed genetic distance, phylogenetic positioning, and applies molecular species delimitation tests.

Material and Methods

Salvinia's samples

Salvinia specimens were collected in the upper Paraná River floodplain (*Planície de inundação do alto Rio Paraná* PIAP, 22°38′–22°57′S and 53°05′–53°36′W) located in the boundary between northwest of Paraná state and southeast of Mato Grosso do Sul state, and in the Rosana's Reservoir (22°36′7″S, 52°52′20″W) localized in São Paulo state, between September 2022 and June 2023 (Fig. 1a-b). The PIAP constitute the Site 6 of the National Long-Term Ecological Research Program (*Programa Ecológico de Longa Duração* PELD, Site 6), and is the only stretch free of dams of the upper Paraná River basin, extending 230 kilometers, and reaching up to 20 kilometers in width (Agostinho & Zalewski 1996), where the presence of *S. auriculata*, *S. herzogii*, and *S. minima* has been recorded (Souza *et al*. 2017). The Rosana's Reservoir is part of a hydroelectric power plant that contains extensive shallow areas along the main channel and tributaries, forming suitable habitats for colonization by aquatic macrophytes, with *S. herzogii* being the species from this genus present in the area and one of the most abundant among other macrophyte species (Thomaz *et al*. 2012).

In these environments, 9 specimens were collected and identified in the field as *S. auriculata*, *S. herzogii*, and *S. minima* (Tab. 1). Access to the genetic heritage of these specimens was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen - registration number A76372D). The collection of biological material was authorized by the Sistema de Autorização e Informação da Biodiversidade (Biodiversity Information and Authorization Sistem - SISBIO) under 52596-5 license.

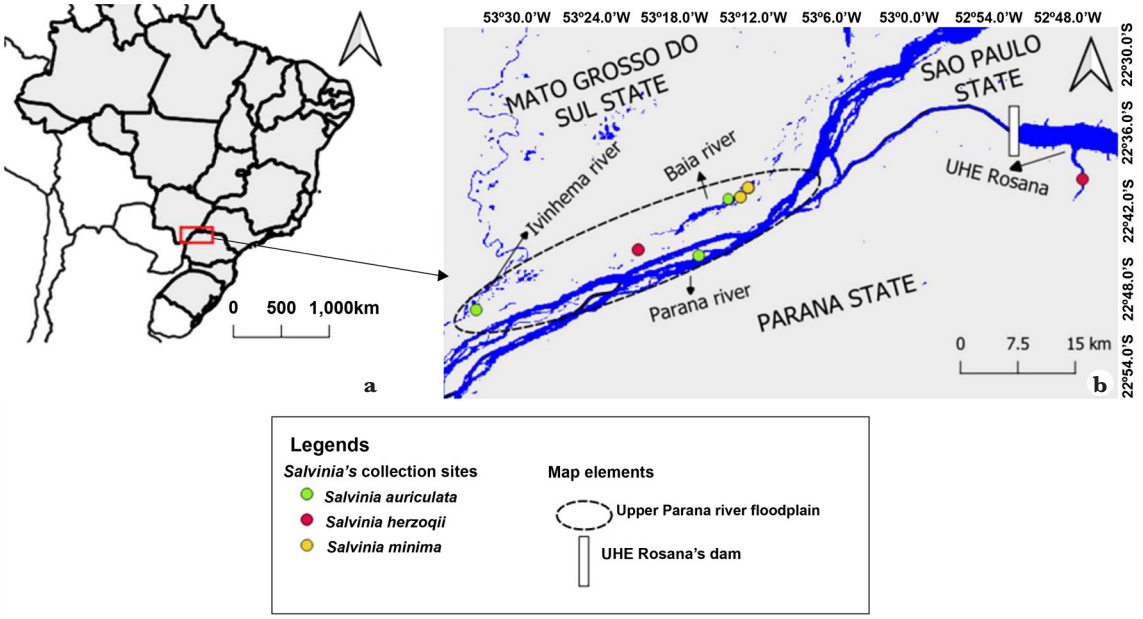

Figure 1 – a-b. Map showing the sampled sites of *Salvinia* specimens in the upper Paraná river floodplain and in the Rosana's reservoir – a. Brazil; b. sites in the Paraná River floodplain and in the Rosana's reservoir where the *Salvinia's* specimens were collected for the DNA extraction and which PCR was successfully conducted. UHE = Hydroelectric basin. The specimens showed in the map legends refers to the field identification.

Table 1 – *Salvinia's* specimens collected in the upper Paraná river floodplain and in the Rosana's reservoir with their coordinates. The species showed in the table refers to the field identification. ID = Specimens code for genetic analysis; MS = Mato Grosso do Sul state; PR = Parana state.

Herbarium voucher	ID	Point of collection	Coordinates
		Salvinia auriculata	
HUEM40705	S1	MS, Taquarussu, Baía river	22°40'55.1"S; 53°12'56.7"W
HUEM40710	S2	MS, Taquarussu, Baía river	22°40'13.1"S; 53°12'19.5"W
HUEM40793	S3	MS, Taquarussu, Baía river	22°44'57.5"S; 53°20'45.9"W
HUEM40709	S4	MS, Taquarussu, Ivinhema river, Pato's lagoon	22°49'33.66"S; 53°33"9.9"W
HUEM40712	S5	PR, Porto Rico, Paraná river, ressaco do Leopoldo	22°45'24"S; 53°16'7,98"W
		Salvinia herzogii	
HUEM40707	S6	MS, Taquarussu, Baía river	22°44'57.5"S; 53°20'45.9"W
HUEM40708	S7	PR, Diamante do Norte, Rosana's reservoir	22°39'32.9"S; 52°46'46.4"W
		Salvinia minima	
HUEM40111	S8	MS, Taquarussu, Baía river	22°40'55.1"S; 53°12'56.7"W
HUEM40706	S9	MS, Taquarussu, Baía river	22°40'11.2"S; 53°12'21.6"W

The *Salvinia* specimens collected in this study and used for genetic analyses were prepared using standard herbarium techniques: labelled and numbered, then placed between folded newspaper sheets. The drying of pressed materials was conducted in ovens at 50 °C, with periodic checks to ensure complete drying. The specimens were then mounted as herbarium sheets after drying and registered in the State University of Maringá Herbarium (HUEM).

DNA extraction and quantification

For molecular analysis, leaf tissue samples of *Salvinia* collected in the field were preserved in Falcon tubes with TE buffer at pH 8.0 [Tris-HCl (1M), EDTA (0.5mM)]. Total DNA was extracted using the Promega Wizard® Genomic DNA Extraction Kit according to the manufacturer's instructions, with liquid nitrogen for mechanical leaf maceration. The concentration of the extracted DNA was estimated using a NanoDrop™ Lite spectrophotometer.

Amplification

In this study, we analyzed the ITS (internal transcribed spacer) and *trnL-trnF loci* (spacer between the chloroplast genes $tRNA^{Leu}$ and $tRNA^{Phe}$). Both regions were partially amplified using the primers ITS 5a-F (5'-TATCATTTAGAGGAAGGAG-3') and ITS 4-R (5'-GCATATCAATAAGCGGAGGA-3') (Baldwin 1992) for ITS, and *trn-c-F* (5'-GGAAATCGGTAGACGCTACG-3') and *trn-f-R* (5'-ATTTGAACTGGTGACACGAG-3') (Reid *et al*. 2006) for *trnL-trnF*. Polymerase chain reaction (PCR) was conducted in a total volume of 25 μL, containing 10 ng of DNA, 1x Tris-KCl reaction buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5 mM $MgCl_2$, primers (2.5 μM each), dNTPs (0.1 mM each), and 0.5 U Platinum™ Taq DNA Polymerase.

The amplification protocol for the ITS *locus* consisted of 35 cycles, with denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and polymerization at 72 °C for 1 minute. After the 35 cycles, a final extension was performed at 72 °C for 5 minutes to complete fragment extension. For the *trnL-trnF* locus, the amplification protocol started with 4 minutes at 92 °C, followed by 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 59 °C for 30 seconds, and polymerization at 72 °C for 2 minutes, with a final extension of 10 minutes at 72 °C to complete fragment extension. PCR products were then analyzed in 1% agarose gel and purified following the protocol by Rosenthal *et al*. (1993). Sequencing reactions were carried out using the BigDye Terminator kit. Sequencing was performed by a private company using an AB-3500 automated sequencer.

Sequences edition, alignment and genetic distance

The nucleotide sequences of *Salvinia* from this study were edited using the BioEdit software (Hall 1999). After editing, we included all available *Salvinia* sequences from GenBank for the ITS (4 sequences) and *trnL-trnF* (12 sequences) regions in

the analyses, and the *Salvinia* sequences from the work of Machado *et al*. (2016) that are not available in Genbank but were provided by the authors [see Tab. S1 (available on supplementary material <https://doi.org/10.6084/m9.figshare.29156225.v1>) for details about the sequences used in this work]. All sequences were aligned by the Clustal W implemented in MEGA11 software, where we also calculated the corrected genetic distance using the Kimura 2-parameter (K2P) model (Tamura *et al*. 2013). Additionally, the *Salvinia* sequences from this study were submitted to the BLASTn tool on the NCBI platform (<https://www.ncbi.nlm.nih.gov/genbank/>).

Phylogenetic analysis, molecular species delimitation and haplotypic diversity

To evaluate the phylogenetic position of *Salvinia* species from PIAP and the Rosana reservoir concerning other species within the genus, a gene tree was constructed using the Maximum Likelihood statistical method on the IQTREE online server (<http://iqtree.cibiv.univie.ac.at/>), in which the optimal nucleotide substitution model was also determined. We selected 1,000 ultrafast bootstraps (Minh *et al*. 2013) to assess branch support. *Azolla* sequences (JX297320 for ITS and HQ909788 for *trnL-trnF*) were chosen as outgroups. The resulting topology was visualized and edited on the iTOL online server (<https://itol.embl.de/>).

All *Salvinia* sequences for the *trnL-trnF* and ITS markers were employed to perform molecular species delimitation tests, with the resulting clusters grouped into Molecular Operational Taxonomic Units (MOTU). We selected two molecular species delimitation methods which differ in their methodologies to compare their congruences and identify the best-fitting hypothesis for our dataset: Assemble Species by Automatic Partition (ASAP, <https://bioinfo.mnhn.fr/abi/public/asap/asapold.html>) (Puillandre *et al*. 2021), based on genetic distance; and the Poisson Tree Processes (PTP) method, performed on the server <https://species.h-its.org/ptp/>, a tree-based approach (Zhang *et al*. 2013). Additionally, to determine whether sequences within the same MOTU exhibit sufficient divergence to be segregated by nucleotide composition, we performed a haplotypic characterization using DnaSP v5 software (Librado & Rozas 2009).

Results

BLASTn and genetic distance

We obtained a fragment of 722 base pairs (bp) for *trnL-trnF* and 682 bp for ITS after edition and alignment. PCR amplification was more successful for the *trnL-trnF* marker, with at least two individuals of each *Salvinia* species collected to be successfully amplified. For ITS, two individuals of *S. auriculata* and two individuals of *S. minima* were successfully amplified.

According to BLASTn results, identity percentages generally remained above ~98% for both markers used in the analyzed specimens. The exception was the specimens identified in the field as *S. auriculata*, which showed maximum identity percentages of ~94% with *S. molesta* sequences (ITS) and ~94% to ~97% with *S. oblongifolia* sequences (*trnL-trnF*). For specimens identified as *S. herzogii*, both individuals (S6 and S7) showed identity percentages of 99.40% and 98.68%, respectively, with *S. molesta* sequences for *trnL-trnF*. Regarding *S. minima*, individual S8 exhibited high nucleotide similarity with other *S. minima* sequences in GenBank for both ITS (98.64%) and *trnL-trnF* (99.15%), while individual S9 showed high nucleotide similarity with *S. natans* for ITS (99.20%). Photographs of some *Salvinia* specimens analyzed in this study are available in Figure S1 (available on supplementary material <https://doi.org/10.6084/m9.figshare.29156225.v1>).

Concerning the K2P genetic distance for ITS (Tab. 2), individuals morphologically identified as *S. auriculata* (S1 and S5) showed values exceeding 15% in relation to other analyzed species and differed from each other by 3.2%. The *S. minima* individuals (S8 and S9) displayed a genetic distance of 13.8% from each other. The specimen S8 differed by 0.8% from *S. minima* (DQ522290.1), while specimen S9 had zero genetic distance from *S. natans* (AF448791).

For *trnL-trnF* (Tab. 3), the minimum K2P genetic distance between individuals identified as *S. auriculata* (S1~S5) and the other sequences analyzed in our study is 1.4%, while the distance among *S. auriculata* individuals ranges from 0 to 1%. Specimens S6 and S7 identified as *S. herzogii* had zero genetic distance from the three *S. molesta* sequences in GenBank (EU269688, EU269689, and MW017464) and showed a value of 0.6% compared to the *S. herzogii* sequence from Machado *et al*. (2016). The *S. minima* specimen S2 had a genetic distance of 0.2% and 0.6% with *S. minima* sequences in GenBank (EU269687 and EU269686) and an unidentified sequence (DQ066503). It is noteworthy that the genetic distance values calculated for *trnL-trnF* were both intra and interspecifically lower than ITS.

Phylogenetic analysis and molecular delimitation

The best nucleotide substitution models were TPM3+F+I and HKY+F+G4, selected for the *trnL-trnF* and ITS gene trees, respectively (Fig.

Table 2 – Genetic distance matrix calculated by Kimura-2-parameter (K2P) model between the sequences of *Salvinia* from the upper Paraná basin (bold) and other sequences available in Genbank for ITS region.

Sequences	1	2	3	4	5	6	7	8
1 PP125760 *Salvinia* S1								
2 PP125761 *Salvinia* S5	0,032							
3 MF111106.1 *Salvinia molesta*	0,164	0,151						
4 DQ522291.1 *Salvinia oblongifolia*	0,219	0,203	0,082					
5 PP125762 *Salvinia* S9	0,270	0,253	0,176	0,191				
6 AF448791.1 *Salvinia natans*	0,270	0,253	0,176	0,191	0,000			
7 DQ522290.1 *Salvinia minima*	0,294	0,285	0,179	0,187	0,148	0,148		
8 PP125763 *Salvinia* S8	0,289	0,280	0,174	0,176	0,138	0,138	0,008	
9 JX297320.2 *Azolla caroliniana*	0,642	0,651	0,615	0,612	0,594	0,594	0,591	0,592

2a-b). It is important to note that sequences for *S. herzogii* and *S. cucullata* are only represented for the *trnL-trnF* marker due to data availability in public databases.

In the tree based on the *trnL-trnF* region (Fig. 2a), the *S. minima* sequences from GenBank and *Salvinia* S8 were grouped into the same clade and defined as a single MOTU with three haplotypes. The specimens identified in the field as *S. herzogii* (S6 and S7) were clustered with *S. molesta* sequences from GenBank and *S. herzogii* from Machado *et al*. (2016) into a single MOTU, with *S. herzogii* Machado *et al*. (2016) representing a unique haplotype. Finally, a clade was formed exclusively by the specimens identified in the field as *S. auriculata* (S1 to S5) and *S. auriculata* from Machado *et al*. (2016). In this case, two MOTUs were detected: one consisting of the *Salvinia* S1 sequence, and the other containing the remaining sequences from the group.

In the gene tree based on the ITS region (Fig. 2b), the sequences from specimens identified in the field as *S. minima* (S8 and S9) were placed separately. *Salvinia* S9 and *S. natans* (AF448791) were clustered together into a single MOTU, sharing the same haplotype. The sequences from *Salvinia* S8 and *S. minima* (DQ522290) also formed a single MOTU, but with two unique haplotypes. This suggests that the genetic variation is not sufficient to distinguish them as separate species. Molecular delimitation methods identified *S. oblongifolia*, *S. molesta*, and two sequences from specimens identified in the field as *S. auriculata* (S1 and S5) as distinct MOTUs. Although there is genetic variation between S1 and S5 evidenced by the presence of two unique haplotypes, both molecular delimitation methods grouped them into a single MOTU, indicating that this variation is not enough to treat them as separate species on a molecular level.

Discussion

In this study, we used the nuclear (ITS) and chloroplast (*trnL-trnF*) molecular markers to identify *Salvinia* species occurring in the upper Paraná River basin, specifically in the floodplain environments of the upper Paraná River and the Rosana's reservoir. The results confirmed the field identification of one of the *S. minima* specimens. However, although there is evidence that the molecular data support the previous identification of the *S. auriculata* specimens by forming a single MOTU and exhibiting higher genetic distances from other sequences than from each other, it is not possible to confirm their molecular identification, as no other *S. auriculata* sequences are available in GenBank for comparison. Nonetheless, the results revealed new occurrences, enriching the knowledge about the diversity of this genus in the region. This is because two specimens identified in the field as *S. herzogii* were molecularly delimited as *S. molesta*, and one of the specimens identified in the field as *S. minima* is genetically identical to sequences of *S. natans*.

Molecular identification

The specimens identified as *S. auriculata* and the other sequences in this study show the highest genetic distance compared to the intraspecific distance for the species. Thus, although there is no molecular data for *S. auriculata* available in the database, the high genetic distance in relation to the other groups corroborates the distinction of *S. auriculata* with the other analyzed species. While *S. auriculata* is part of a species complex with *S. molesta*, *S. herzogii*, and *S. biloba*, sharing the characteristic eggbeater shaped trichomes, it can be easily distinguished from the others by its larger

Table 3 – Genetic distance matrix calculated by Kimura-2-parameter (K2P) model between the sequences of *Salvinia* from the upper Parana basin (bold) and other sequences available in Genbank for *trnL-trnF* region.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 PP156675 *Salvinia* S1																						
2 PP156677 *Salvinia* S3	0,010																					
3 PP156678 *Salvinia* S2	0,008	0,002																				
4 PP156674 *Salvinia* S4	0,008	0,002	0,000																			
5 PP156673 *Salvinia* S5	0,008	0,002	0,000	0,000																		
6 *Salvinia auriculata* Machado *et al.* 2016	0,008	0,002	0,000	0,000	0,000																	
7 *Salvinia herzogi*i Machado *et al.* 2016	0,036	0,030	0,028	0,028	0,028	0,028																
8 EU269688 *Salvinia molesta*	0,034	0,028	0,026	0,026	0,026	0,026	0,006															
9 EU269689 *Salvinia molesta*	0,034	0,028	0,026	0,026	0,026	0,026	0,006	0,000														
10 MW017464 *Salvinia molesta*	0,034	0,028	0,026	0,026	0,026	0,026	0,006	0,000	0,000													
11 PP156672 *Salvinia* S6	0,034	0,028	0,026	0,026	0,026	0,026	0,006	0,000	0,000	0,000												
12 PP156679 *Salvinia* S7	0,034	0,028	0,026	0,026	0,026	0,026	0,006	0,000	0,000	0,000	0,000											
13 AY651839 *Salvinia oblongifolia*	0,022	0,016	0,014	0,014	0,014	0,014	0,034	0,032	0,032	0,032	0,032	0,032										
14 EU269692 *Salvinia oblongifolia*	0,020	0,014	0,012	0,012	0,012	0,012	0,032	0,030	0,030	0,030	0,030	0,030	0,002									
15 EU269693 *Salvinia oblongifolia*	0,020	0,014	0,012	0,012	0,012	0,012	0,032	0,030	0,030	0,030	0,030	0,030	0,002	0,000								
16 EU269687 *Salvinia minima*	0,048	0,042	0,040	0,040	0,040	0,040	0,046	0,044	0,044	0,044	0,044	0,044	0,048	0,046	0,046							
17 EU269686 *Salvinia minima*	0,048	0,042	0,040	0,040	0,040	0,040	0,046	0,044	0,044	0,044	0,044	0,044	0,048	0,046	0,046	0,004						
18 DQ066503 *Salvinia* sp.	0,048	0,042	0,040	0,040	0,040	0,040	0,046	0,044	0,044	0,044	0,044	0,044	0,048	0,046	0,046	0,004	0,000					
19 PP156676 *Salvinia* S8	0,050	0,044	0,042	0,042	0,042	0,042	0,048	0,046	0,046	0,046	0,046	0,046	0,046	0,044	0,044	0,006	0,002	0,002				
20 EU269690 *Salvinia natans*	0,104	0,098	0,095	0,095	0,095	0,095	0,104	0,102	0,102	0,102	0,102	0,102	0,100	0,097	0,097	0,095	0,095	0,095	0,097			
21 EU269691 *Salvinia natans*	0,107	0,100	0,098	0,098	0,098	0,098	0,106	0,104	0,104	0,104	0,104	0,104	0,102	0,100	0,100	0,097	0,097	0,097	0,100	0,002		
22 MF177095 *Salvinia cucullata*	0,129	0,122	0,120	0,120	0,120	0,120	0,134	0,131	0,131	0,131	0,131	0,131	0,127	0,127	0,127	0,113	0,118	0,118	0,120	0,091	0,093	
23 HQ909788 *Azolla cristata*	0,351	0,342	0,338	0,338	0,338	0,338	0,347	0,344	0,344	0,344	0,344	0,344	0,339	0,336	0,336	0,325	0,322	0,322	0,322	0,317	0,317	0,336

size and the absence of an incision at the leaf apex (Miranda & Schwartsburd 2019), which facilitates its identification in the field, even in the absence of sorophore. The haplotype diversity found among *S. auriculata* sequences for cpDNA may indicate that somatic mutations are being fixed in the population (Scorsim *et al*. 2023; Lucio *et al*. 2024).

On the other hand, the molecular data did not confirm the field identification of the *S. herzogii* specimens (S6 and S7), as both sequences were placed within the same MOTU as *S. molesta* sequences from GenBank. According to Miranda & Schwartsburd (2019), *S. biloba*, *S. molesta*, and *S. herzogii* are very similar in vegetative structures. In *S. biloba*, the leaf apex of floating leaves has an incision that reaches 1/2 of the leaf length, with short papillae on the abaxial side and 3 or 4 short trichomes at the apex, while the adaxial side is glabrous. Both *S. herzogii* and *S. molesta* are somewhat distinct from *S. biloba*, but vegetatively identical to each other: their leaf apex of floating leaves incision reaches 1/3 of the leaf length, they possess short papillae, and 3 or 4 trichomes fused at the apex. The sorophore is the only feature that allows their distinction (Miranda & Schwartsburd 2019). Therefore, differentiating *S. herzogii* and *S. molesta* in the field is virtually impossible in the absence of sorophore.

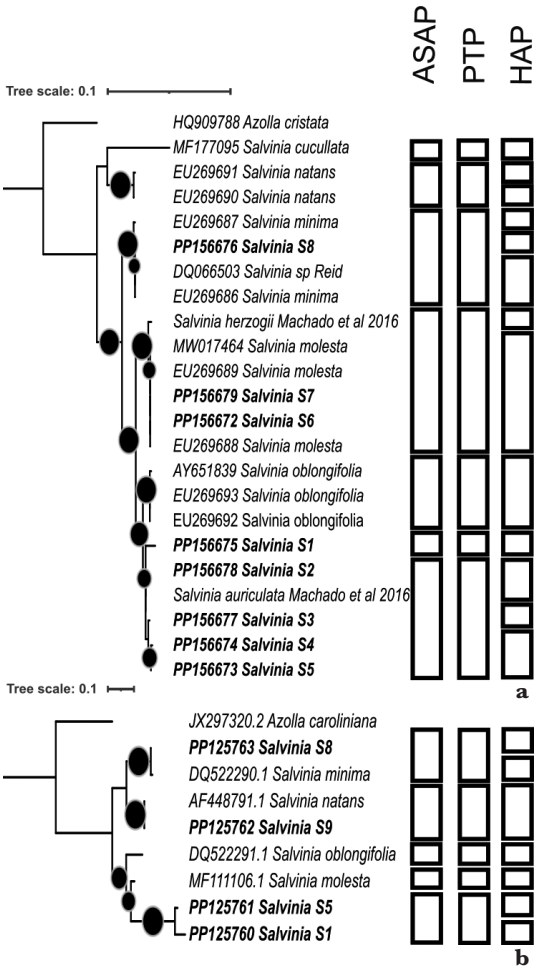

Figure 2 – a-b. Gene tree and molecular operational taxonomic units built for *Salvinia* based on *trnL-trnF* (a) and ITS (b) regions for individuals sampled in the upper Paraná river basin (bold) and other *Salvinia* sequences available in GenBank. Black circles represent a range of bootstrap values from 70 (minimum circle size) to 100 (maximum circle size). ASAP = Assemble Species by Automatic Partitioning; PTP = Poisson Tree processes; HAP = haplotype.

Even assuming the accuracy of the species identification in genetic databases, the locality of the *S. molesta* specimens in GenBank (Costa Rica and India; Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.29156225.v1>) is inconsistent with the current knowledge of the geographical distribution of *S. herzogii* and *S. biloba*, both of which are endemic to southeastern Brazil (Miranda & Schwartsburd 2019), whereas *S. molesta* is an invasive species widely distributed across the globe (Holt Jr. *et al*. 2023). Thus, there is evidence to confirm the occurrence of *S. molesta* in the PIAP and in the Rosana's reservoir. However, it is not possible to assert that all specimens previously identified as *S. herzogii* are *S. molesta* due to the lack of collections, especially of individuals with sorophore.

Additionally, the sequences of *Salvinia* S6 and S7 are genetically identical to other *S. molesta* sequences in GenBank, despite their geographical distance. This genetic uniformity may be explained by the hypothesis that *S. molesta* is a hybrid species that reproduces exclusively in vegetative ways, as it does not produce viable spores (Loyal & Grewal 1996). Another issue to consider is that the sequence of the *S. herzogii* specimen from Machado *et al*. (2016) forms a distinct haplotype from *S. molesta* but still belongs to the same MOTU. The morphological similarity, sympatric geographical distribution, and phylogenetic proximity between *S. herzogii* and *S. molesta* (Machado *et al*. 2016; Miranda & Schwartsburd 2019) suggest that a very recent speciation process may have occurred in this group's evolutionary history, making it difficult to distinguish them both morphologically and molecularly (Holt Jr. *et al*. 2023). However, it is not possible to confirm the identification of *S. herzogii* sequence from Machado *et al*. (2016).

Finally, the molecular delimitation results for the specimens *Salvinia* S8 and S9, identified in the field as *S. minima*, provided new insights. While *Salvinia* S8 was molecularly identified as *S. minima* for both ITS and *trnL-trnF*, *Salvinia* S9 was molecularly delimited as *S. natans* based on ITS.

The confusion in field identification between these species had not been previously addressed because, to our knowledge, this is the first record of *S. natans* in Brazilian natural areas and occurring in sympatry with *S. minima*. The commonly used morphological characters to distinguish *S. minima* from other *Salvinia* species in the field are the ungulate-type trichomes (four free trichomes at the apex; Miranda & Schwartsburd 2019) and their smaller size. However, *S. natans* also possesses ungulate type trichomes and similar dimensions to *S. minima* (Barthlott *et al*. 2009; Miranda & Schwartsburd 2019; World Flora Online, <https://www.worldfloraonline.org/taxon/wfo-0001117304>), making it relatively difficult to distinguish them in the field.

ITS with more intra-interspecific distance than trnL-trnF

The use of molecular markers, combined with new species delimitation tools, has been instrumental in recognizing species that are difficult to identify based solely on morphological traits (Hollingsworth *et al*. 2011), as is the case with *Salvinia*. However, unlike animals where the COI molecular marker serves as an effective DNA barcode, the complex evolutionary history of plants, which involves hybridizations and polyploidy, makes it challenging to establish universal molecular markers (Hollingsworth *et al*. 2011). In terms of nuclear genome usage, the ITS region is the most employed in molecular studies of aquatic plants (Scorsim *et al*. 2024) and is widely used in plant molecular phylogenetic studies, molecular discrimination and identification, as well as assessing the genetic variability within populations (Diamante *et al*. 2020; Scorsim *et al*. 2023; Lucio *et al*. 2024).

In the analysis presented, the ITS region of nuclear ribosomal DNA showed higher intra- and interspecific distance values compared to the chloroplast marker *trnL-trnF*. This indicates that ITS is a better candidate as a DNA barcode for *Salvinia* than *trnL-trnF*, as higher intra- and interspecific distance values enhance the discriminatory power of the molecular marker to identify closely related species (Hollingsworth *et al*. 2011).

Incongruences between cpDNA and nrDNA gene tree

The phylogenetic reconstruction of *Salvinia* species using ITS and *trnL-trnF* reveals divergent topologies. On one hand, our *trnL-trnF* gene tree aligns with previous cpDNA and plastome-based phylogenies, supporting the division of *Salvinia* into distinct Neotropical and Eurasian clades (Naglagium *et al*. 2008; Machado *et al*. 2016; Holt Jr. *et al*. 2023). Additionally, our findings confirm the lack of phylogenetic support for the *Salvinia auriculata* complex based on *trnL-trnF*, since *S. auriculata* clustered with *S. oblongifolia*, and *S. molesta* and *S. herzogii* formed a separate clade (Machado *et al*. 2016). On the other hand, our ITS gene tree supports the monophyly of the *Salvinia auriculata* complex, placing *S. auriculata* and *S. molesta* in a distinct clade from *S. oblongifolia*. Furthermore, *Salvinia* species did not form biogeographically cohesive clades in the ITS gene tree, as *S. minima* and *S. natans* were grouped together.

Due to the biparental inheritance of the nuclear genome, the ITS marker can detect genetic recombination and hybridization events (Grimm & Denk 2008). Consequently, incongruences between nuclear and plastid gene trees are common (Toro-Nuñez *et al*. 2013; Favro *et al*. 2022). Possible explanations for these discrepancies include hybridization or incomplete lineage sorting (Wang *et al*. 2014). The comparison between the ITS and *trnL-trnF* gene trees suggests that hybridization has played a significant role in the evolutionary history of this genus. However, the limited availability of molecular data for ITS, compared to plastid regions, constrains deeper inferences about the impact of hybridization on *Salvinia* evolution.

Ecological implications

The use of molecular data revealed that the two individuals of *S. herzogii* analyzed in the PIAP and Rosana's reservoir are *S. molesta*. In the same way, one of the two individuals of *S. minima* studied in PIAP was molecularly identified as *S. natans*. These findings suggest that previous known taxonomic diversity of the genus in upper Paraná River has been underestimated, underscoring the importance of using molecular markers to aid in species identification - an approach with significant ecological implications for the genus in this region.

For instance, the presence of *S. molesta* presents opportunities to study its ecological characteristics. As previously mentioned, this species is considered an aggressive invader in many regions worldwide (Martin *et al*. 2018; Holt Jr. *et al*. 2023). Therefore, studying how *S. molesta* interacts with co-occurring macrophyte species and other aquatic organisms, as well as exploring genetic aspects (such as haplotype diversity) in its native habitat, will provide valuable data for comparing these same ecological and genetic attributes in *S. molesta* populations from introduced areas. Such studies can help clarify the species invasion mechanisms and improve the development of appropriate management strategies.

However, the presence of *S. natans* in the PIAP is a concerning new occurrence, as this species is native to Europe and Asia and can be considered exotic (Zutshi & Vass 1971; POWO 2025). Even some Brazilian studies have reported the use of this species

from cultivated populations in research centers for experimental purposes (Lima *et al*. 2016), this is the first study to report its presence in natural areas with support of molecular data. In experimental studies, *S. natans* seems to be a strong competitor under conditions of high nutrient concentration and elevated temperatures, being a potentially invasive species (Netten *et al*. 2010; Zhang *et al*. 2020). Consequently, its presence in the PIAP, a tropical region where temperatures are higher than in its native temperate areas, coupled with the constant anthropogenic pressure in flooded environments that negatively affect ecosystem functions (Moi *et al*. 2022), makes *S. natans* a potentially invasive species in the floodplain. Therefore, observational studies are needed to determine if this species occurs in other environments of the upper Paraná River basin or other aquatic ecosystems in Brazil, as well as experimental studies to understand its impacts on the local aquatic community.

In conclusion, this study contributes to the understanding of aquatic plant diversity in the upper Paraná River basin, revealing, through molecular delimitation, new occurrences of *S. natans* and *S. molesta*. It is important to highlight that the presence of *S. natans* is also the first record for Brazil in natural areas, marking a concerning new occurrence of an exotic and potentially invasive species. Among the markers used, the nuclear marker (ITS) can be considered a better DNA barcode than the chloroplast marker (*trnL-trnF*) for *Salvinia*, due to its higher intraspecific and interspecific distance. By using both markers, this study also provides the first molecular species delimitation analysis and a comparison between nuclear and plastidial gene tree for the genus, which proved to be a useful method for assisting in field identification of *Salvinia* and revealing important insights about the importance of hybridization for the genus. Additionally, we provided the first sequencing data for *S. auriculata* specimens in GenBank for both nuclear and chloroplast markers. Unfortunately, due to difficulties in amplification, it was not possible to obtain *trnL-trnF* and ITS fragments for all samples, which affects the final conclusions of the phylogenetic analysis. These, along with other sequencing data generated in this study, will be valuable for future investigations into the genetic analysis of *Salvinia*.

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Data Availability Statement

The molecular data generated and analyzed during the current study are available in the GenBank repository (<https://www.ncbi.nlm.nih.gov/genbank/>), and the *Salvinia* specimens sampled can be found in SpeciesLink database (<https://specieslink.net/>). Further data can be obtained through the corresponding author, Adrian Cesar da Silva.

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