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Morphological and preliminary molecular data on freshwater red algae in Tam Dao National Park, Vietnam

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ABSTRACT

Rhodophyta is one of the oldest and largest groups of algae and is dominated by marine species, while freshwater species are relatively scarce. Although the diversity and distribution of rhodophytes in freshwater plants have been studied worldwide, there are few published records of freshwater red algae in Vietnam. Therefore, this study aimed to preliminarily investigate the morphology and analyze the phylogeny of freshwater red algae in Vietnam. This report provides information about red algal specimens collected in July 2018 and 2022 in the Tam Dao National Park watershed forests. Morphological analysis of the specimens revealed similar characteristics to those of *Batrachospermum*; however, phylogenetic analysis of the Tam Dao specimens and *Batrachospermum* placed them into different clades. Data relating to both morphological and molecular characteristics suggest that Tam Dao specimens may represent a new clade within Batrachospermaceae. **Key words:** Batrachospermum, Batrachospermales, Rhodophyta, Thoreales, Freshwater red algae

INTRODUCTION

- 2 Red algae (Rhodophyta) are among the earliest and
- ³ largest groups of multicellular algae and can be found
- 4 in both oceans and freshwater bodies. While ma-
- ⁵ rine species make up the majority of the Rhodophyta
 ⁶ division, freshwater red algae account for less than
 ⁷ 5%, with two-thirds of all known species belonging
 ⁸ to Batrachospermales and Florideophyceae¹. Fresh⁹ water rhodophytes are distinguished from others by
 ¹⁰ unique characteristics, as the predominant photosyn¹¹ thetic pigments in their chloroplasts are phycocyanin
 ¹² or phycoerythrin, resulting in cells that appear blue
 ¹³ or red in color. Research on the distribution of red
 ¹⁴ algae has shown that freshwater species occur mainly
 ¹⁵ in oligotrophic running waters. The presence of these
 ¹⁶ bacteria primarily indicates that water reservoirs con-
- tain low nutrient concentrations and are minimally
 contaminated².

In recent years, the number of research studies on 19 freshwater red algae has increased globally due to the 20 21 necessity of obtaining molecular knowledge to study the phylogeny and taxonomy of taxa that were pre-22 viously based solely on morphological features. The 23 combination of morphological and molecular data 24 has significantly contributed to a better understand-25 26 ing of the relationships among genera and species 27 within those genera. A notable outcome of this com-28 bined approach was the proposal of six orders: Balbianiales, Thoreales, Acrochaetiales, Batrachospermales, 30 Nemaliales, and Palmariales. Another significant

collaborative effort among freshwater red algal ex-31 perts led to a comprehensive phylogenetic analysis of the order Batrachospermales, incorporating members from genera and sections of Batrachospermum 34 with the aim of establishing better-defined taxonomic 35 categories, especially to address the issue of paraphyly 36 within the genus Batrachospermum. These collabora-37 tions led to the suggestion of numerous new genera, 38 including Kumanoa, Sheathia, Torularia, Virescentia, Acarposporophycos, Visia, Montagnia, and Paludicola. Under this revised classification approach, Kumanoa 41 and Sheathia were the two genera with the highest 42 numbers of species within the freshwater red algae¹. 43 Despite significant advancements in rhodophyte tax-44 onomy worldwide, there is limited published infor-45 mation regarding the distribution of red algae in freshwater bodies in Vietnam. Notifications about 47 the occurrence of freshwater red algae in Vietnam 48 are primarily conveyed through oral communication 49 among researchers, often supplemented by occasional 50 observations in certain national parks. The Checklist 51 of Plant Species of Vietnam (2001) records only one species, Batrachospermum moniliforme, within sev-53 eral national parks, such as Ba Be National Park (Bac 54 Kan), Phia Oac – Phia Den National Park (Cao Bang), 55 Tam Dao National Park (Vinh Phuc), and Chu Mom 56 Ray National Park (Kon Tum)³. In 2010, Nguyen 57 Thuy Lien reported the presence of two species of 58 freshwater rhodophytes in the Ma Da area within 59

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- ⁶⁰ the Dong Nai Biosphere Reserve in southern Viet⁶¹ nam⁴. However, these occurrence records are rela⁶² tively vague and lack parameters for molecular analy⁶³ sis.
- ⁶⁴ From that perspective, in this report, we present in-
- 65 formation about red-algal samples collected from the
- 66 Tam Dao National Park watershed forests during an-
- 67 nual summer schools in 2018 and 2022. This study
- 68 aimed to provide morphological data and molecular
- ⁶⁹ evidence for phylogenetic analysis, with the goal of ⁷⁰ clarifying the taxonomy of freshwater rhodophytes in
- 71 Vietnam.

72 MATERIALS AND METHODS

73 Sample collection

Samples were collected from the headwater streams of Tam Dao National Park at coordinates 21°49'61.71" 75 North latitude and 105°62'69.93" East longitude. 76 Sample collection took place in two phases: on July 77 21st, 2018 (TD18), and on July 17th, 2022 (TD22). 78 The collected algae were divided for various purposes, 79 including DNA extraction, morphological examina-80 tion, and herbarium voucher preparation. Specimens 81 intended for molecular analyses were rapidly dried 82 using silica gel, while those for morphological and anatomical observations were preserved in 5% for-85 malin. Voucher herbarium specimens have been de-

86 posited at the VNU University of Science Herbarium.

87 Morphological analyses

The morphological characteristics were observed and documented using a Primo Star microscope with 89 an attached camera system (Carl Zeiss, Germany) 90 following the methods described by Chapuis et al. 91 (2017), Entwisle et al. (2009), Evans et al. (2017), Fischer et al. (2020), Rossignolo et al. (2020), Suzuki & Kitavama (2021), and Vis et al. (2012, 2021)^{1,5–11}. 94 Morphological characteristics, including the whorl 95 diameter, fascicle cell number, carposporophyte and carposporangium dimensions, spermatium diameter, 97 and carpogonium dimensions, were measured in 10 98 to 15 replicates. Algal identification was performed 99 based on observation features following the methods of the Freshwater Algae of North America² and Freshwater Red Algae¹. 102

103 Molecular analyses

¹⁰⁴ Silica-dried plant material was ground in 200 μ l of ¹⁰⁵ CTAB using a mortar and pestle. The solution was ¹⁰⁶ then vortexed using two glass beads (\emptyset 5 mm) for 30 ¹⁰⁷ seconds. DNA was subsequently extracted from the obtained solution following the CTAB extraction protocol⁷.

Molecular assays for sample analysis focused on mul- 110 tilocus sequence analysis (MLSA) following the methods of Lam et al., (2016)¹². In addition to rbcL, COI-5P (cox1), psaA, psbA, psaB, EF2, cob, 18S rDNA, and 113 28S rDNA, four other genes—16S rDNA, 23S rDNA, 114 tilS (tRNA(Ile)-lysidine synthase), and trpA (trypto-115 phan synthase alpha subunit)—were included in the 116 study. The typical PCR mixture (25 μ l) consisted of 117 2X OneTaq[®] DNA Polymerase (New England Biolabs, 118 USA), 0.5 µM primers, and 2 µl of DNA. Thermocy-119 cler incubation was carried out using a Mastercycler® Nexus-PCR Thermal Cycler (Eppendorf, Germany) 121 following the following general conditions: 94 °C for 122 2 min; 35 cycles of 94 °C for 40 s, 60 °C for 45 s, and 123 68 °C for 90 s; and 1 cycle at 68 °C for 5 min, followed 124 by holding at 4 °C. The integrity of the PCR products 125 was assessed by electrophoresis for 45 min at 90 V in 126 1% (w/v) agarose gels in TAE buffer. DNA sequencing 127 was performed by 1st BASE (Axil Scientific Pte. Ltd., 128 Singapore). 129

Phylogenetic analyses

The sequences were submitted to GenBank and analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov /Blast.cgi). The individual genes and a concatenated alignment were subjected to maximum likelihood (ML) analysis using PHYML in the Geneious Prime^{*} 2023 plugins with 1000 bootstrap support replicates. 137

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RESULTS

Morphological characteristics

The specimen was undisturbed by human activity and 140 consisted of freshwater streams with shallow rocky 141 soil that were lightly shaded (Figure 1a). The collected 142 thalli ranged from olive-green in full sunlight to dark 143 blue–green in shaded areas. The morphological characteristics of the specimens from both phases of collection were similar. 146

Macroscopic gametophytic thalli are olive-green in 147 color, heterotrichous, and uniaxial, with whorls of 148 branches that give rise to a bead-like appearance on 149 the thallus. The thallus lacks an outer cortex and has a 150 mucilaginous consistency. The thalli are monoecious, 151 very mucilaginous, 5 to 17 cm in height, and 600 to 152 1000 μ m wide. They are abundantly and irregularly 153 branched and appear olive green (Figure 1b). Carposporophytes are small and pedunculate. The whorls 155 are ellipsoidal and separated, contiguous and more 156 $_{157}$ or less compressed, measuring 50 to 100 μm in di-158 ameter, with 1 to 2 peripheral carposporophytes (indicated by the arrow in Figure 1c). The carpogonial 159 branch is composed of cells that are similar in shape 160 and size to primary fascicle cells and pedunculate car-161 posporophytes. Primary branchlets are abundantly 162 branched, with 10 to 13 cell stories. The proximal cells 163 are cylindrical, measuring 8 to 10 μ m wide and 50 to 90 µm long. Carpogonium-bearing branches are 60 165 to 90 μ m long and consist of 8 to 11 cells arising from 166 pericentral cells. Carposporangia are ovoidal, measuring 10 to 12 μ m wide and 19 to 22 μ m long. Car-168 posporophytes are spherical, with a diameter of 100 169 to 150 μ m (indicated by the arrow in Figure 1e). The 170 spermatangia are spherical, with 3 to 10 cell stories 171 (indicated by the arrow in Figure 1f). Carpogonium-172 bearing branches are 60 to 100 μ m long and consist 173 of 8 to 11 cells (Figure 1d). The trichogyne is 15 μ m 174 long with a spermatium (Figure 1g). 175

176 Molecular analyses

This study designed and utilized 13 pairs of primers following previous studies and based on red algal data 178 from the NCBI. However, only two fragments of 18S 179 rDNA and trpA showed bands in agarose gels, mea-180 suring 1500 and 1000 base pairs, respectively. Amplifications for 18S rDNA and trpA were performed 182 through PCRs using the following primers: 18S 183 rDNA.RA. F (5'- CACCTGGTTGATCCTGCCAG -3'), 18S rDNA.RA. R (5'- CTTGTAAGCGTGGGT-CATCAG -3'), trpA.RA. F (5'- TCCCTTTCACG-186 187 GAGGTAACG -3'), and trpA.RA. R (5'- ACTCTAC-CATTGAGTTAGCAACC -3'). Alignment searches on NCBI indicated that both TD18 and TD22 belong 189 to Batrachospermaceae. For 18S rDNA, the sequences 190 covered more than 99% of the gene fragment, show-191 ing homology of more than 98%. Similarly, for trpA, 192 the figures were approximately 81.5% for gene cover-193 age and 77.5% for pairwise similarity. 194

Separate ML phylogenetic trees for trpA and 18S 195 rDNA were constructed based on the sequences of 196 the samples and Batrachospermaceae taxa to identify 197 the taxonomy of TD18 and TD22 (Figures 2 and 3). 198 For the concatenated phylogenetic tree, as not all taxa had corresponding sequences for 18S rDNA and trpA, 200 the concatenated alignment was assembled for genera 201 based on sequences of 18S rDNA and trpA extracted 202 from species in the same genus. Following trimming 203 and concatenation, the final alignment used for ex-204 amination comprised 2363 bases and was analyzed to 205 determine the relationship between TD18 and TD22 ²⁰⁷ and other genera in Batrachospermaceae (Figure 4).

DISCUSSION

The morphological characteristics of the two Tam Dao209samples revealed that these samples were closely re-210lated to species of *Batrachospermum* sensu stricto.211These features include large, multibranched filaments212in a mucilaginous or gelatinous mass; undifferenti-213ated straight carpogonial branches arising from both214fascicle and pericentral cells; carpogonia with club-215to urn-shaped trichogynes; and small, globose, pedi-216cellate carposporophytes at various distances from the217whorl axis.218

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In recent years, the taxonomy of the genus Batra- 219 chospermum has undergone significant changes. Sev- 220 eral sections have been elevated to the genus level, 221 such as Kumanoa (formerly sections Contorta and 222 Hybrida) with the type species is K. virgatodecais- 223 neana (Basionym: Batrachospermum virgatodecais- 224 neanum), Sheathia (formerly section Helminthoidea) 225 with the type species is S. boryana (Basionym: Ba- 226 trachospermum boryanum), Torularia (synonym At- 227 rophycus, formerly section Setacea) with the type 228 species is T. dillenii (Basionym: Batrachospermum puiggarianum), Virescentia (formerly a sec- 230 tion with the same name) with the type species 231 is Virescentia helminthosa (Basionym: Batrachosper- 232 mum helminthosum), Acarposporophycos (formerly 233 section Acarposporophytum) with the type species 234 is Acarposporophycos brasiliensis (Basionym: Batra- 235 chospermum brasiliense), Visia (formerly section Aris-236 tata) with the type species is Visia cavennensis (Ba- 237 sionym: Batrachospermum cayennense), Montagnia 238 (formerly section Macrospora) with the type species 239 is Montagnia macrospora (Basionym: Batrachosper- 240 mum macrosporum), and Paludicola (formerly section 241 Turfosa) with the type species is Paludicola turfosa 242 (Basionym: *Batrachospermum turfosum*)¹. 243 There was only one species of freshwater red algae 244 recorded in Vietnam, and this species was published 245

in the Checklist of Plant Species was published 245 in the Checklist of Plant Species of Vietnam (2001): 246 *Batrachospermum moniliforme*, which is now considered a synonym of *B. gelatinosum*. The characteristics 248 of the carpogonial branches, carposporophytes, and 249 other features of the Tam Dao specimens indicate that 250 their morphological phenotypes are relatively similar 251 to those of the genus *Batrachospermum*. However, the 252 trichomes of these specimens had an obovoidal shape, 253 which means that they differ from the description of 254 the trichogyne of *B. gelatinosum*, which is clavate or 255 lanceolate in shape¹. 256

In terms of the taxonomy of *B. moniliforme*, only *B.* ²⁵⁷ *moniliforme* var. *pilosissimum* has been identified as ²⁵⁸ the true *B. moniliforme*. Other varieties of this species ²⁵⁹



Figure 1: Morphological characteristics of fresh water algal samples from Tam Dao. a. Habitat of the specimen; b. Whorls of thallus; c. The whorls of a female gametophyte showing carposporophytes within the whorls and exerted (arrowheads); d. Carpogonium-bearing; e. Carposporophytes; f. Spermatangia (arrowheads) produced terminally on the fascicle; g. The trichogyne with a spermatium. Scale bars: 50 μ m.

260 are now regarded as synonyms of different species. 261 For example, the species *B. moniliforme* and most of its varieties are currently classified as B. gelatinosum, 262 the type species of Batrachospermum. B. moniliforme 263 var. stagnale has been reclassified as Sheathia bo-264 ryana, whereas B. moniliforme var. confusum and 265 B. moniliforme var. condensatum are now known as 266 267 Sheathia confusa. Similarly, B. moniliforme var. nod-268 iflorum has been redirected to Kumanoa nodiflora, 269 and both B. moniliforme var. dillenii and B. monili-270 forme var. detersum have been changed to Torularia ²⁷¹ atra¹³. The taxonomy of the genus Batrachospermum 272 has changed significantly, and Vietnamese freshwater ²⁷³ red algae have also been subjected to these changes.

Identifying closely related species in *Batrachosper-* ²⁷⁴ *mum* sensu stricto based on morphology has proven ²⁷⁵ difficult and remains challenging due to morphological similarities. For instance, the separation of *Ba-* ²⁷⁷ *trachospermum* and *Sheathia* has relied primarily on ²⁷⁸ the occurrence of heterocortication, which is unique ²⁷⁹ to the majority of species in *Sheathia*; however, this ²⁸⁰ feature is absent in *S. arcuata* ¹. Therefore, in this situation, only DNA sequence data provide a feasible approach. ²⁸³

Both ML phylogenies depicting the relationships of
TD18 and TD22 with other genera of Batrachosper-
maceae, based on 18S rDNA and trpA, revealed that
the Tam Dao specimens formed a separate clade with285285286



Figure 2: ML phylogeny depicting the relationship of TD18 and TD22 with other genera of Batrachospermaceae based on *18S rDNA* using PHYML in the Geneious Prime^{*} 2023 plugins with 1000 bootstrap support replicates.

288 their nearest neighbors, Psilosiphon (Figure 2) and Kumanoa (Figure 3). In Figure 2, the TD samples plot 289 on the same branch as the PsiloSiphon samples; how-290 ²⁹¹ ever, the morphological features of the PsiloSiphon ²⁹² samples are completely different from those of the TD 293 samples. TD specimens possessed Batrachospermum sensu stricto characteristics, similar to Kumanoa or 294 295 Sheathia, whereas Psilosiphon species have cartilagi-296 nous thalli, no division into node and internode re-297 gions; they are normally unbranched with putative 298 spermatangia, no carpogonia observed, cortex com-299 posed of ellipsoidal to obovoidal photosynthetic cells of uniform size in distinct outward radiating rows 300 or filaments; dense medullary filaments composed of 301 cylindrical cells, colorless, in between the outer cortex and the uniaxial central filament. Reproduction 303 is based on putative monosporangia that develop in 304 chains from cortical filaments on the outside of the thallus or adventitious plantlets¹. 306

In recent years, many new species have been recorded ³⁰⁷ in neighboring countries of Vietnam, including ³⁰⁸ China, Thailand, Japan, and India¹. These species are ³⁰⁹ mainly found in Kumanoa and Sheathia. Therefore, ³¹⁰ freshwater algae are likely related to these species. ³¹¹







Figure 4: trpA, 18S rDNA and concatenated trpA-18S rDNA ML phylogeny depicting the relationships of TD18 and TD22 with seven genera of Batrachospermaceae

312 However, the phylogenetic trees in Figure 2 and Fig-313 ure 3 revealed that the Tam Dao samples, Batrachospermum, Kumanoa and Sheathia were separated 314 ³¹⁵ into different clades. Similarly, the concatenated ML phylogeny representing the association of TD18 and 316 TD22 with several genera of Batrachospermaceae re-317 vealed that Tam Dao specimens were more closely 318 related to Kumanoa and Montagnia than to Batra-319 chospermum and Sheathia. Although there are lim-320 ited data on the 18S rDNA (nuclear DNA) and trpA 321 (chloroplast DNA) genes in freshwater algae, the re-322 sults of morphological and molecular analysis suggest 323 that Tam Dao specimens form a new clade in Batra-324 chospermum sensu stricto. Additional evidence from 325 DNA sequences, such as rbcl, may be instrumental in 326 327 fully characterizing the freshwater red algae of Viet-328 nam.

CONCLUSION 329

330 Recently, taxonomic research based on the combination of DNA sequence data and morphological char-331 acteristics has revealed the species diversity of fresh-332 water red algae, especially within the family Batra-333 chospermaceae. These studies shed light on the phy-334 logenetic analysis of the family Batrachospermaceae 335 and simplified the taxonomic categories. By provid-336 337 ing both morphological and molecular data in the first ³³⁸ report on freshwater rhodophytes in Vietnam, this research contributes information about rhodophyte di-339 versity in South Asia and may provide new genus data 340 within the family Batrachospermaceae. 341

ABBREVIATIONS

Please add this section 343

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AUTHOR CONTRIBUTIONS

348 Please add this section

349 FUNDING

350 Please add this section

AVAILABILITY OF DATA AND 352 MATERIALS

- 353 The data and materials used and/or analyzed during
- 354 the current study are available from the correspond-
- 355 ing author upon reasonable request.

ETHICS APPROVAL AND CONSENT 357 TO PARTICIPATE

358 Not applicable.

CONSENT FOR PUBLICATION

Not

No	t applicable.	360
COMPETING INTERESTS		361
The authors declare that they have no competing in-		362
tere	terests.	
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