

INTEGRATIVE MULTI-MARKER BARCODING AND ITS PHYLOGENY RESOLVE VIETNAMESE STRAIN CCAH013/2 AS *Mychonastes rotundus* (SPHAEROPLEALES, CHLOROPHYCEAE)

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ABSTRACT

A coccoid green microalga (strain CCAH013/2) isolated from a mangrove-edge freshwater site in Can Gio, Viet Nam was identified through an integrative workflow combining morphology with multi-marker DNA evidence. Nuclear 18S rRNA and plastid 23S rRNA sequences showed very high similarity to named *Mychonastes* taxa, supporting placement at the genus level but offering limited interspecific resolution due to locus conservatism. The ITS region (ITS1–5.8S–ITS2) provided species-level power: BLAST results reached $\geq 99\%$ identity to *Mychonastes rotundus* and dropped markedly for other congeners ($\leq \sim 95\%$). A maximum-likelihood tree inferred from ITS recovered CCAH013/2 within the *M. rotundus* clade with strong bootstrap support, mirroring the pairwise identity pattern. Morphological characters-small, spherical to slightly oval, non-motile cells occurring in minute clusters and reproducing by autospores-were congruent with the emended generic concept and the species diagnosis. Together, these data support a robust assignment of CCAH013/2 to *Mychonastes rotundus* and provide a basis for applied studies under local conditions, including CO₂-enrichment tolerance, lipid and carotenoid productivity, and small-scale pilot tests in tropical coastal freshwater niches.

Keywords: *Mychonastes rotundus*, ITS phylogeny, molecular identification, *Chlorophyceae*, Can Gio.

1. INTRODUCTION

Microalgae are fundamental components of aquatic ecosystems, performing critical ecological roles in primary production, nutrient cycling, and maintaining ecological resilience, especially within tropical mangrove ecosystems characterized by distinct salinity gradients and dynamic hydrological conditions [1]. Despite the recognized ecological significance of mangrove ecosystems, the microalgal biodiversity associated with these habitats remains inadequately studied, particularly in terms of isolation, characterization, and preservation in culture collections [2]. Mangrove ecosystems in Southeast Asia are globally recognized biodiversity hotspots due to their unique transitional nature between terrestrial and marine environments, supporting diverse biological communities and providing essential ecosystem services, including coastal protection, carbon sequestration, and nursery habitats

for various species [3]. Within these systems, microalgae exhibit diverse adaptive strategies that enable them to thrive under fluctuating environmental conditions. Nevertheless, the extent of microalgal biodiversity in these regions remains underrepresented in major international algal culture collections, such as the Culture Collection of Algae and Protozoa (CCAP, UK), the Culture Collection of Algae at the University of Texas at Austin (UTEX, USA), and the Microbial Culture Collection at the National Institute for Environmental Studies (NIES, Japan), which have focused more on temperate and freshwater taxa [2, 4].

Vietnam's Can Gio Mangrove Biosphere Reserve (CGMBR), located in southern Ho Chi Minh City, represents the largest restored mangrove system in Southeast Asia, covering approximately 75,740 hectares and designated by UNESCO as a Biosphere Reserve since 2000 [5, 6]. Although extensive floristic surveys have recorded considerable phytoplankton biodiversity, there remains a critical gap in isolation, molecular characterization, and ex situ conservation of indigenous microalgal taxa from this unique ecosystem [7]. Addressing this gap, the Culture Collection of Algae at Ho Chi Minh City (CAAH) has been recently initiated with the objective of systematically isolating, preserving, and characterizing indigenous microalgal resources from the CGMBR. Given the challenges in morphological identification, due to cryptic speciation and morphological plasticity commonly encountered within microalgae [8, 9], CCAH employs an integrated approach combining morphological methods with multi-marker molecular identification. Specifically, the nuclear-encoded 18S rRNA gene, internal transcribed spacer (ITS) region, and chloroplast-encoded 23S rRNA gene have been selected due to their demonstrated effectiveness in resolving taxonomy at various hierarchical levels within microalgae [10, 11].

This study focuses on the microalgal strain CCAH013/2, isolated from freshwater habitats within the CGMBR, to demonstrate the effectiveness and resolution power of this multi-marker approach for microalgal species identification. By combining morphological observations with molecular analyses based on multiple genetic markers, we aim to provide a robust, reproducible protocol for precise species-level identification. Furthermore, accurate taxonomic identification is essential not only for biodiversity conservation but also for unlocking the biotechnological potential of microalgae, as evidenced by recent successes in screening microalgae for high-value bioactive compounds and applications in aquaculture and bioremediation [12, 13]. Through this detailed methodological validation using strain CCAH013/2, this paper contributes to the broader goals of improving taxonomic accuracy and facilitating the sustainable use of microalgal resources, specifically from underrepresented tropical mangrove habitats.

2. MATERIALS AND METHODS

2.1. Sampling and Isolation of Microalgal Strain CCAH013/2

The microalgal strain CCAH013/2 was isolated from a freshwater site located within the Can Gio Mangrove Biosphere Reserve (CGMBR), Ho Chi Minh City, Vietnam (coordinates: 10.651512°N, 106.783268°E), on 10 March 2023. Water samples (500 mL) were collected aseptically into sterile glass bottles and immediately stored in an insulated cool box with gel ice packs to maintain a temperature approximately 4 °C to preserve the integrity of the natural microalgal community, then transported to the laboratory within 24 hours [5].

In the laboratory, samples were gently concentrated using low-speed centrifugation (1500 rpm, 10 min) to enrich microalgal cells without causing damage [5]. A single microalgae cell exhibiting typical morphology was isolated from the sample using a glass capillary pipette under an inverted microscope (GX-series, China) at magnifications of 100-400x and transferred into a drop of sterile BG-11 medium placed on a glass slide [14]. The cell was washed by sequentially transferring it through several drops of sterile BG-11 medium to minimize the possibility of co-isolating other cells. The final single cell was then inoculated into a sterile test tube containing 10 mL of liquid BG-11 medium. The purified cultures were maintained under controlled laboratory conditions at 25 ± 2 °C, illuminated at approximately $50 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a 12:12 h light–dark cycle [5]. Scale-up cultivation was performed by gradually increasing the culture volume in Erlenmeyer flask, with each growth cycle lasting 7–10 days, until stable unialgal cultures were established. During cultivation, the cellular morphology in the cultures was periodically examined to ensure that the liquid cultures remained free of contamination.

2.2. Morphological Examination

Morphological characterization of strain CCAH013/2 was carried out under a bright-field Olympus CX43 microscope (Olympus Corporation, Tokyo, Japan) equipped with an Olympus DP22 digital camera at 1000× magnification. Because this system does not provide phase-contrast or DIC capability, fine structures such as flagella may not be visible. Morphological traits, including cell shape, size, colony formation, mucilaginous envelope presence, and pigmentation, were recorded. Cell dimensions were measured using ImageJ software [15], based on a random selection of 20 cells per sample to ensure objectivity and statistical reliability. Results are presented as means ± standard deviation (μm).

2.3. DNA Extraction and PCR Amplification

Genomic DNA was extracted from actively growing cultures of strain CCAH013/2 using a CTAB protocol [16]. DNA integrity was verified on 1–1.5% agarose gels and quantity/purity were measured on a NanoDrop 2000 (Thermo Fisher Scientific). Three loci were targeted for barcoding: nuclear 18S rRNA (SSU) with universal eukaryotic primers EukA (5'-AACCTGGTTGATCCTGCCAGT-3') and EukB (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Medlin et al., 1988); the ITS region (ITS1–5.8S–ITS2) with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [17]; and plastid 23S rRNA (LSU) with primers p23S-F (5'-GGACAGAAAGACCCTATGAA-3') and p23S-R (5'-TCAGCCTGTTATCCCTAGAG-3') [11]. PCRs (25 μL) contained 12.5 μL GoTaq Green Master Mix (2×; Promega), 0.5 μM of each primer, 50–100 ng template DNA, and nuclease-free water; no-template controls were included in each run. Thermocycling used an initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, locus-specific annealing, and 72 °C extension; and a final extension at 72 °C for 10 min. Locus-specific programs were as follows: 18S (expected amplicon ~1.7 kb), annealing 57 °C for 30 s and extension 72 °C for 90 s; ITS (amplicon ~600–750 bp), annealing 55 °C for 30 s and extension 72 °C for 60 s; plastid 23S (amplicon ~350–450 bp for the p23S region), annealing 55 °C for 30 s and extension 72 °C for 60 s. Amplicons were checked on agarose gel (1.5% for ITS/23S; 1% for 18S), purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific), and bidirectionally Sanger sequenced using the amplification primers by automated sequencing at Macrogen Inc. (Seoul, South Korea).

2.4. DNA Sequence Analysis and Molecular Identification

Raw DNA sequences obtained from Sanger sequencing were manually edited, assembled, and quality-checked using BioEdit v7.2 [18]. The final consensus sequences were compared against reference sequences in GenBank (NCBI) using the BLASTn algorithm. Identification thresholds followed current practice in algal barcoding (≥ 97% for genus-level and ≥ 99% for species-level assignments) [19, 20]. Reference sequences selected from GenBank were carefully chosen based on reliability, completeness, and clear taxonomic designation.

2.5. Phylogenetic Analysis

The ITS region (ITS1–5.8S–ITS2) of strain CCAH013/2 and reference taxa of Mychonastes plus closely related chlorophytes were retrieved from GenBank (accession numbers are shown next to taxon names in Figure 1). Sequences were aligned with MAFFT v7 [21] using the Auto strategy (--auto), then visually inspected and trimmed to retain homologous blocks; ambiguously aligned positions were excluded and gaps were treated as missing data. Maximum-likelihood inference was performed in MEGA11 [22] under the best-fit nucleotide substitution model selected by the Bayesian Information Criterion using the “Find Best DNA/Protein Models (ML)” function. Node support was assessed with 1000 nonparametric bootstrap replicates. Trees were rooted with *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Pseudomuriella engadinensis* as outgroups, and branch lengths represent substitutions per site.

3. RESULTS

3.1. Morphological characterization of strain CCAH013/2

Cells of strain CCAH013/2 appeared predominantly spherical to round, clearly delineated, and measured approximately 5–10 μm in diameter (mean ± SD: 7.3 ± 1.5 μm, n = 20 cells), based on precise measurements directly derived from calibrated microscopic images (Figure 1). Flagella were not

detected under the applied culture conditions. Cells were predominantly observed forming small clusters of 6-8 cells, consistent with recent cellular division events. Solitary cells were rarely observed. No large organized colonies or extended mucilaginous sheaths were detected under the applied magnification, and no gelatinous matrices connecting individual cells were observed, indicating a non-mucilaginous phenotype. Internally, the cells contained clearly discernible chloroplasts uniformly distributed within the cytoplasm, a feature characteristic of coccoid green microalgae of the order Sphaeropleales (family Mychonastaceae, class Chlorophyceae). Chloroplast structure appeared parietal, clearly visible, and typical for members within the family Mychonastaceae. The clear delineation and absence of mucilage or gelatinous structures strongly suggest affinity with certain species within the genus *Mychonastes*.

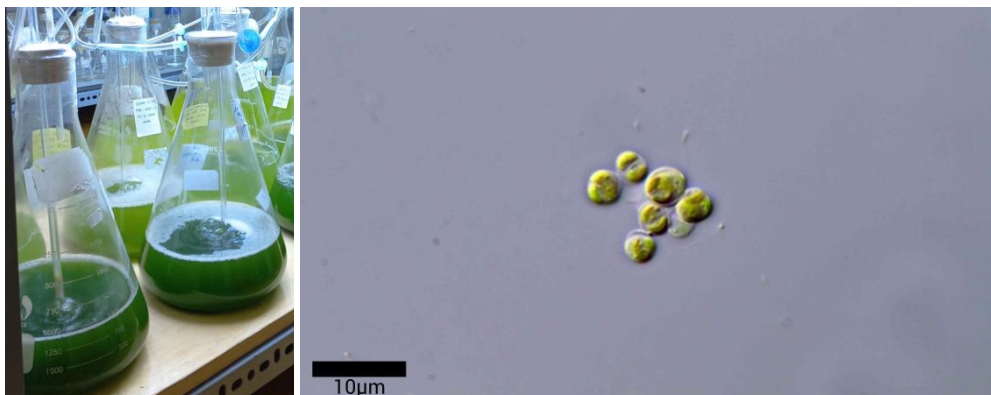


Figure 1. Culture of strain CCAH013/2 (left) and microscopic image of its cells (right).

3.2. Molecular identification based on multi-marker regions

To support molecular identification, the ~1,700 bp 18S rRNA gene of strain CCAH013/2 was queried against GenBank using BLASTN. The highest-scoring matches are summarized in Table 1.

Table 1. BLAST results for the 18S rRNA sequence of strain CCAH013/2 (top matches)

Closest match	Query cover (%)	Identity (%)	GenBank accession
<i>Pseudomuriella</i> sp. Itas 9/21 14-1d	100	99.72	AY195974.1
<i>Nephrocytium agardhianum</i> SAG 34.81	98	98.28	KY013477.1
<i>Nephrocytium agardhianum</i> FACHB:2124	97	98.19	KY094107.1

These 18S results indicate high similarity to chlorophyte genera outside *Mychonastes* (i.e., *Pseudomuriella* and *Nephrocytium*).

BLAST results for the plastid 23S rRNA region

The plastid 23S rRNA (LSU) region of the strain was next queried to further inform the assignment. Key BLAST matches are listed in Table 2.

Table 2. BLAST results for the plastid 23S rRNA sequence of strain CCAH013/2 (top matches)

Closest match	Query cover (%)	Identity (%)	GenBank accession
<i>Mychonastes homosphaera</i> (isolate “grn”)	96	100.0	MN533912.1
<i>Mychonastes jurisii</i>	99	98.8	NC_028579.1
<i>Mychonastes homosphaera</i> (strain GA11)	100	98.5	MW363967.1

The 23S results also place the strain within *Mychonastes* (again clearing a $\geq 97\%$ genus-level criterion). As with 18S, however, the plastid 23S locus is relatively conserved across *Mychonastes*, as reflected by a 100% match to one *M. homosphaera* record alongside ~98.5–98.8% identity to *M. jurisii*.

BLAST results for the ITS region (ITS1-5.8S-ITS2)

The internal transcribed spacer (ITS) region—well known for higher variability and species-level resolving power—was then examined. The principal BLAST outcomes are shown in Table 3.

Table 3. BLAST results for the ITS region of strain CCAH013/2 (top matches)

Closest match	Query cover (%)	Identity (%)	GenBank accession
<i>Mychonastes</i> sp. XG2H4 (unidentified)	100	100.0	JX289897.1
<i>Mychonastes rotundus</i> (CCAP 260/14)	100	99.28	GQ477053.1
<i>Mychonastes pusillus</i> (NIES-2896)	97	94.5	LC853074.1

The ITS sequence of CCAH013/2 shows species-level concordance with *Mychonastes rotundus*, including a perfect match to an unnamed *Mychonastes* entry (XG2H4) and 99.28% identity to the reference strain CCAP 260/14. By contrast, identities to other congeners decline sharply (e.g., ~94-95% for *M. pusillus*, ~88% for *M. ovahimbae*, ~85% for *M. homosphaera*).

3.3. ITS phylogeny: placement of strain CCAH013/2

On the maximum-likelihood tree inferred from ITS1-5.8S-ITS2 (837 bp) (Figure 2), CCAH013/2 is resolved in the *Mychonastes rotundus* clade. The strain forms a tight bipartition with the reference strain *M. rotundus* CCAP 260/14, supported by a bootstrap of 100, and this pair is joined by *Mychonastes* sp. XG2H4 in a well-supported triad (bootstrap ≈ 99). Internal branch lengths within this triad are minimal, indicating negligible ITS divergence among the three sequences, whereas substantially longer internal branches separate the triad from neighboring clades (*M. pusillus*, *M. homosphaera*, etc.), all with high bootstrap support across those nodes.

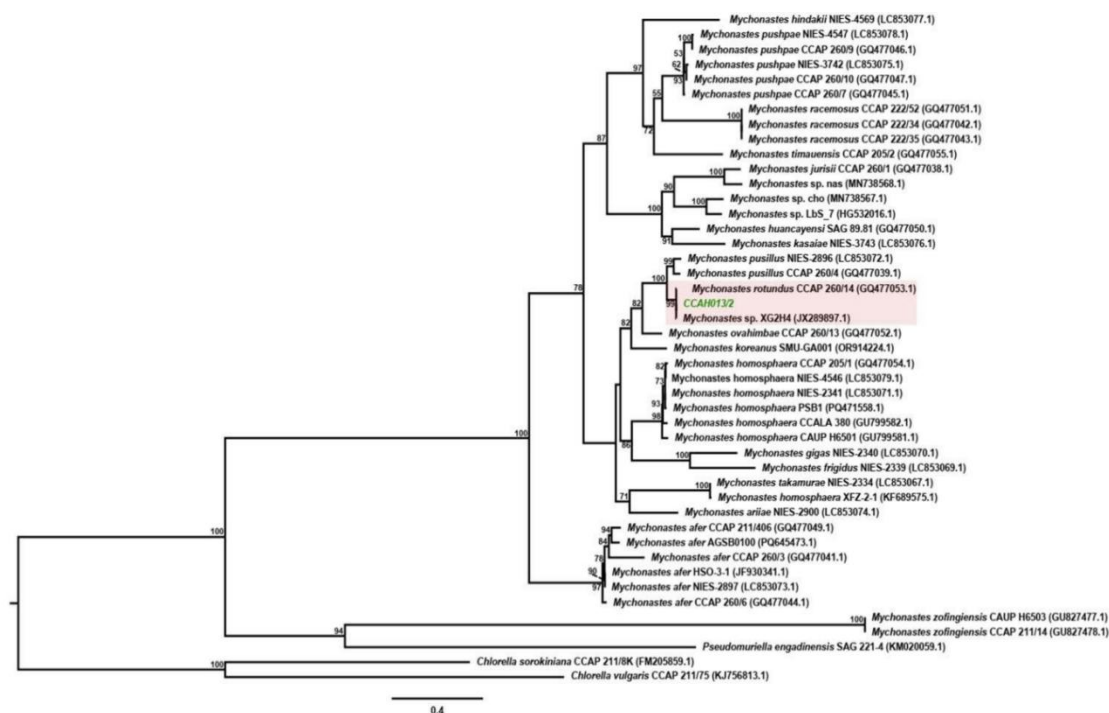


Figure 2. Maximum-likelihood phylogeny of the genus *Mychonastes* based on ITS1–5.8S–ITS2 sequences, showing the placement of strain CCAH013/2.

Topological context further supports the assignment. The *M. rotundus* triad is recovered as a distinct, strongly supported lineage that is sister to, but clearly separated from, non-rotundus congeners. No conflicting placements are observed for CCAH013/2 elsewhere on the tree. The scale bar shows that inter-clade distances exceed the within-clade distance by an order of magnitude, a pattern consistent with species-level cohesion in ITS. Considering (i) the monophyletic grouping of CCAH013/2 with the type-linked reference *M. rotundus* CCAP 260/14 at bootstrap 100, (ii) the short internal branches within that clade and strong separation from adjacent *Mychonastes* clades, and (iii) the concordant BLAST

evidence (ITS identities $\geq 99\%$ to *M. rotundus* and $\ll 99\%$ to other congeners), the strain CCAH013/2 is identified as *Mychonastes rotundus*.

The alignment was generated with MAFFT v7 using the Auto strategy (--auto) and the tree was inferred in MEGA11 under the best-fit nucleotide-substitution model selected by BIC; node labels indicate bootstrap support from 1,000 replicates. Taxon names are followed by strain codes and GenBank accessions as shown in the Figure 2; the position of CCAH013/2 is highlighted. The tree is rooted with *Chlorella sorokiniana* CCAP 211/8K, *Chlorella vulgaris* CCAP 211/75, and *Pseudomuriella engadinensis* SAG 21.84. Branch lengths represent substitutions per site; the scale bar corresponds to 0.4 substitutions per site. CCAH013/2 clusters within the *M. rotundus* lineage, clearly separated from other *Mychonastes* clades.

4. DISCUSSION

4.1. Morphological characterization of strain CCAH013/2

Morphologically, strain CCAH013/2 aligns with descriptions provided for species such as *Mychonastes rotundus* [8], characterized by solitary to loosely clustered spherical cells within a similar size range (3-5 μm , occasionally up to $\sim 8 \mu\text{m}$ in mature cells). In addition, members of the genus *Mychonastes* are widely distributed across the globe, inhabiting brackish and marine waters, freshwater, soils, and aerial habitats [23]. Overall, the morphological and ecological characteristics documented here clearly confirm genus-level affiliation to *Mychonastes*. However, recent studies documented high levels of phenotypic plasticity and cryptic diversity of the genus *Mychonastes* [23]. Morphology was congruent with the molecular signal. The strain exhibited the hallmark coccoid, non-motile habit with small, spherical to subspherical cells occurring singly or in minute groups and reproducing by autospores-traits that fall within the emended generic concept of *Mychonastes* and the circumscription of *M. rotundus* [8]. Minor size differences among cultures are well known for coccoid chlorophytes and are commonly attributed to growth conditions rather than species boundaries, reinforcing the need for DNA evidence in this group [20].

Similarly, Huiyin et al. (2015) emphasized that in the green algae, the morphological forms are often very similar, making identification difficult and only DNA sequencing can solve it [24]. Likewise, Martynenko et al. (2022) analyzed the diversity in the genus *Mychonastes* and showed that culture conditions caused substantial morphological variation, while only molecular markers allow clear species differentiation [23]. Coesel et al. (2008) also analyzed the “Chlorella-like” genera (including *Mychonastes*) and demonstrated the phenomenon of morphological convergence even between unrelated strains, and emphasized the importance of molecular data for correct classification [25]. Based on our results and comparison with published studies, strain CCAH013/2 can be morphologically and ecologically assigned to *Mychonastes*. DNA sequencing is essential for species-level confirmation. Thus, additional molecular evidence, particularly based on robust genetic markers such as 18S rRNA and ITS region, is required to provide reliable species confirmation and to accurately resolve the taxonomic placement of strain CCAH013/2 at the species level.

4.2. Molecular identification based on multi-marker regions

The 18S results indicate high similarity to chlorophyte genera but are not specific to *Mychonastes*, consistent with the limited resolving power of this conserved locus among coccoid green algae. Accordingly, the 18S marker is used here only to provide broad taxonomic context; genus- and species-level assignment is established with more variable loci, in particular the ITS region. At the same time, 23S alone is not sufficient for species delimitation, and perfect matches should be interpreted cautiously given the conserved nature of the marker and potential database inconsistencies. Following commonly applied thresholds for microalgae $\geq 99\%$ identity in ITS (or ITS2/rbcL) for species-level naming, 98–<99% for “cf.”/“aff.” [19], the ITS data meet the criterion for conspecific assignment. Taken together, the low-variation markers (18S, 23S) anchor the strain within *Mychonastes* but do not separate closely related species, which is expected for these loci. In contrast, the ITS region provides $\geq 99\%$ identity to *M. rotundus* with a pronounced drop to other congeners. On this evidence, strain CCAH013/2 is assigned to *Mychonastes rotundus*.

Based on multilocus evidence, strain CCAH013/2 is assigned to the genus *Mychonastes* (Mychonastaceae, Sphaeropleales, Chlorophyceae) and, at the species rank, to *Mychonastes rotundus*.

BLAST searches of the nuclear 18S rRNA and plastid 23S rRNA loci returned high sequence identities ($\geq 97\%$) to several Chlorophyceae lineages, including named *Mychonastes* strains. The 18S marker was used here solely to provide broad taxonomic context, within which strain CCAH013/2 showed high similarity to coccoid green algae. Support at the genus level was subsequently provided by the plastid 23S rRNA marker, which yielded high BLAST identities ($\geq 97\%$) to named *Mychonastes* sequences. As expected for conserved loci, these markers did not resolve closely related congeners: a perfect 23S match to a record annotated as *M. homosphaera* co-occurred with ~ 98.5 – 98.8% identities to *M. jurisii* - a pattern consistent with the low interspecific divergence typically observed in plastid ribosomal regions across this clade [8].

These results are consistent with the publication of Liu et al. (2020), who performed a phylogenetic analysis using 18S rRNA to verify the phylogenetic position of *M. homosphaera* [26]. Similarly, the study by Lee et al. (2025) demonstrated that the combination of 18S and ITS2 data allowed accurate identification to the species level in the description of the new species *Mychonastes koreanus* [27]. Yuan et al. (2011) used 18S rRNA sequences for genus-level identification and ITS sequences to distinguish among species [28].

4.3. ITS phylogeny: placement of strain CCAH013/2

In green algae, 18S rDNA is considered the standard molecular marker for genus-level identification. Many distinct species share identical 18S sequences or differ by only one to two nucleotides. It makes 18S rDNA insufficient for species delimitation or for detecting transitional lineages. The 23S rDNA region serves mainly as a supplementary marker, useful for validating higher-level phylogenetic placements (family or order) and for supporting the recognition of divergent or potentially misassigned lineages. Nevertheless, the availability of specific primers for cp23S remains limited, which poses challenges when investigating rare taxa or deep clades. In addition, the ITS phylogeny obtained in this study provided clear resolution at the species level, strongly supporting the assignment to *M. rotundus*.

Species-level resolution was achieved with the ITS region (ITS1-5.8S-ITS2). The query sequence showed an exact match to an unnamed *Mychonastes* record (GenBank JX289897) and $\geq 99\%$ identity to the reference strain of *M. rotundus* (CCAP 260/14), whereas identities to other congeners declined sharply (typically $\leq 95\%$). Under commonly applied DNA-barcode practices in green microalgae-where ITS identities around or above $\sim 99\%$ are taken as strong evidence of conspecificity-these values support a species-level identification [19,20]. The maximum-likelihood ITS tree corroborated this assignment by placing CCAH013/2 in a strongly supported *M. rotundus* clade, with negligible internal branch lengths relative to the distances separating it from the *M. pusillus* and *M. homosphaera* lineages. Pairwise identity and phylogenetic topology provide convergent evidence for *M. rotundus*. The ITS identity of 100% to the GenBank record JX289897 also provides a plausible phylogeographic link. That accession derives from the tropical East Asian coastal belt (Hainan), a climate regime that mirrors the mangrove-fringing freshwater-brackish mosaic at Can Gio. Although identical ITS sequences do not by themselves prove recent exchange, long-distance aerial transport of viable green microalgae has been demonstrated, making regional connectivity a reasonable working hypothesis for *Mychonastes* populations in tropical East Asia [29].

These findings are consistent with previous reports that the 18S and 23S loci are not of sufficient resolution for species identification in chlorophytes (Krienitz et al. 2011; Martynenko et al. 2022) [8, 23]. Huiyin et al. (2015) emphasized that ITS sequences consistently provide strong separation between *Mychonastes* species [24]. An independent study of the chlorophyte genus *Monoraphidium* also demonstrated that ITS outperformed other nuclear and chloroplast markers in species discrimination by Lee et al. (2025) [30]. The ITS identity threshold of $\geq 99\%$ applied here is consistent with commonly applied standards in chlorophyte taxonomy, including the recent description of *M. koreanus* [27].

In conclusion, the ITS data of this study supported the classification of strain CCAH013/2 into the genus *Mychonastes*. However, Nozaki et al. (2025) demonstrated that combining ITS sequence analysis with four chloroplast genes and secondary ITS structure for accurate distinction of closely related species, particularly within the genus *Mychonastes* [31]. Compared with this multi-gene method, the results of the study were limited to ITS sequences, but the results of the study were consistent with previous reports by Krienitz et al. (2011) and Huiyin et al. (2015) [8, 24], which

showed that ITS sequences alone still provide strong discriminatory. Nevertheless, integrating chloroplast markers and secondary structure analysis, as in Nozaki et al. (2025), will further strengthen the classification ability in future studies.

Ecologically, the verified presence of *M. rotundus* in the Can Gio biosphere reserve indicates that small freshwater trebouxiophytes persist in low-salinity niches embedded within mangrove systems. Microalgae may persist in habitats influenced by seawater, suggesting that the distribution of *Mychonastes* is not necessarily restricted to inland waters [8]. The identification of CCAH013/2 as *Mychonastes rotundus* also raises questions about its potential applications. The species shows efficient CO₂ capture (~20% inlet) with lipid accumulation, supporting its use in photobioreactors for simultaneous emission mitigation and biomass generation [32, 33]. Carotenoid production is another possible research direction. In *Mychonastes* sp. 247, changes in light and salinity conditions increased lutein and zeaxanthin production [34]. *M. afer* accumulates nervonic acid (~3–4% of FAs) [35]. This result has not yet been reported for *M. rotundus*. Lipidomic screening of CCAH013/2 may reveal co-valorization potential. Field deployment with microalgae–bacteria consortia could enhance nutrient removal and productivity, and testing CCAH013/2 in Can Gio will allow for an assessment of its adaptability to real-world conditions regarding salinity, light, and CO₂ concentrations [36].

5. CONCLUSIONS

Using an integrative, multi-marker workflow, strain CCAH013/2 was resolved as *Mychonastes rotundus*. The nuclear 18S rRNA marker provided only broad taxonomic context, confirming affinity with coccoid green algae in the Chlorophyceae. The conserved loci plastid 23S rRNA supported placement within *Mychonastes* at the genus level, whereas the ITS region (ITS1-5.8S-ITS2) provided species-level resolution-showing ≥ 99% identity to *M. rotundus* (reference strain CCAP 260/14) and clustering with that taxon in the ITS maximum-likelihood tree with maximal bootstrap support. Morphological characters (small, coccoid, non-motile cells forming minute clusters and reproducing by autospores) were congruent with the molecular assignment, yielding a coherent and reproducible identification.

Ecologically, the record documents *M. rotundus* in a mangrove-edge freshwater setting at Can Gio, indicating that small trebouxiophyte algae can persist in low-salinity refugia embedded within coastal systems. Further studies could examine whether CCAH013/2 shares the biotechnological potential reported for *M. rotundus* and related species. Relevant aspects include growth under CO₂ enrichment, lipid and carotenoid production, and the fatty acid composition, particularly very-long-chain fatty acids. Initial experiments should establish growth responses to local light and temperature conditions, followed by tests of CO₂ tolerance at 10–20%. Pigment production could then be compared under different cultivation conditions, including a two-stage cultivation strategy.

The results support the taxonomic identification of CCAH013/2 and provide a basis for further studies of its ecological characteristics and biotechnological potential.

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Conflict of interest

The authors declare that they have no conflict of interest and no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript. No AI tools were used in the preparation of this manuscript.

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