



## Diversity of Edible *Cladophora* (Cladophorales, Chlorophyta) in Northern and Northeastern Thailand, Based on Morphology and Nuclear Ribosomal DNA Sequences

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### ABSTRACT

Edible *Cladophora*, known as “kai” in northern Thailand, is an economically and ecologically important green algal genus. Although *Cladophora* has been the focus of many systematic studies, there is an urgent need for taxonomic re-evaluation of kai. We collected 19 isolates of kai from 25 sites in northern and northeastern Thailand during the cool-dry season and during summer. Morphological observation revealed that kai comprised *Aegagropila linnaei* Kützing and *Cladophora glomerata* (L) Kützing. *A. linnaei* occurred only in the Golden Triangle in the northern Thai province. *C. glomerata* occurred commonly in all locations surveyed, and was so variable that we were able to distinguish 12 morphotypes. Phylogenetic analysis of SSU rDNA sequences corroborated our morphological identifications of *A. linnaei* and *C. glomerata*, and revealed the monophyly of all 12 *C. glomerata* morphotypes. However, infraspecific variation in *C. glomerata* SSU sequences was greater than reported variation among other species within the genus. This is the first systematic study of edible *Cladophora* in southeastern Asia based on morphology and molecular (SSU rDNA sequences) comparisons.

**Keywords:** Green algae, *Aegagropila linnaei*, *Cladophora glomerata*, Kai, nrDNA SSU, systematics, Thailand.

### 1. INTRODUCTION

During the cool-dry and summer seasons, many green macroalgae grow on cobbles and gravel substrata in streams and rivers of northern and northeastern Thailand. These green algae have long been used as a source of food by local

people. The algae are commonly known as “kai” and are used in salads. Peerapornpisal *et al.* [1] reported that kai comprised green algae of the genera *Cladophora* and *Microspora*.

The genus *Cladophora* (Cladophorales,

Chlorophyta) contains approximately 183 species worldwide [2]; many frequently occur in freshwater, brackish, and marine habitats in both temperate and tropical regions [3, 4]. They are often attached to substrata below flowing water where they are visible as long filaments. Free-floating masses also occur in surface water and on bottom sediments [5]. These algae have multinucleate cells, reticulate chloroplasts, thick cell walls lacking mucilaginous sheaths, and filamentous branches [3, 4].

Analysis of the small subunit of nuclear ribosomal DNA (SSU) by Zechman *et al.* [6] showed that the genus *Cladophora* is monophyletic. However, a later study of evolutionary relationships in the Siphonocladales/Cladophorales complex [7] reported that this was not the case. Based on morphological and molecular (SSU) analyses, Hanyuda *et al.* [8] proposed that *Aegagropila linnaei* Kützing is the correct name for the ball (“Marimo”) morphology previously referred to as *Cladophora aegagropila* in Japan.

In Thailand, kai is widely utilized, especially as a nutraceutical to relieve peptic ulcers, dyspepsia, rheumatoid arthritis, and hypertension [9]. However, Thai edible green algae have been little studied and few species can be identified by morphology alone as the existing database is inadequate.

In the present study, we investigated morphologies of various kai populations from northern and northeastern Thailand; we also performed corroborative molecular analyses using nuclear ribosomal DNA SSU sequences from many isolates. Our genetic assessment of kai provides a baseline for future appraisals of populations, particularly those with ecological and applied foci.

## 2. MATERIALS AND METHODS

### 2.1 Collection sites and morphological observations

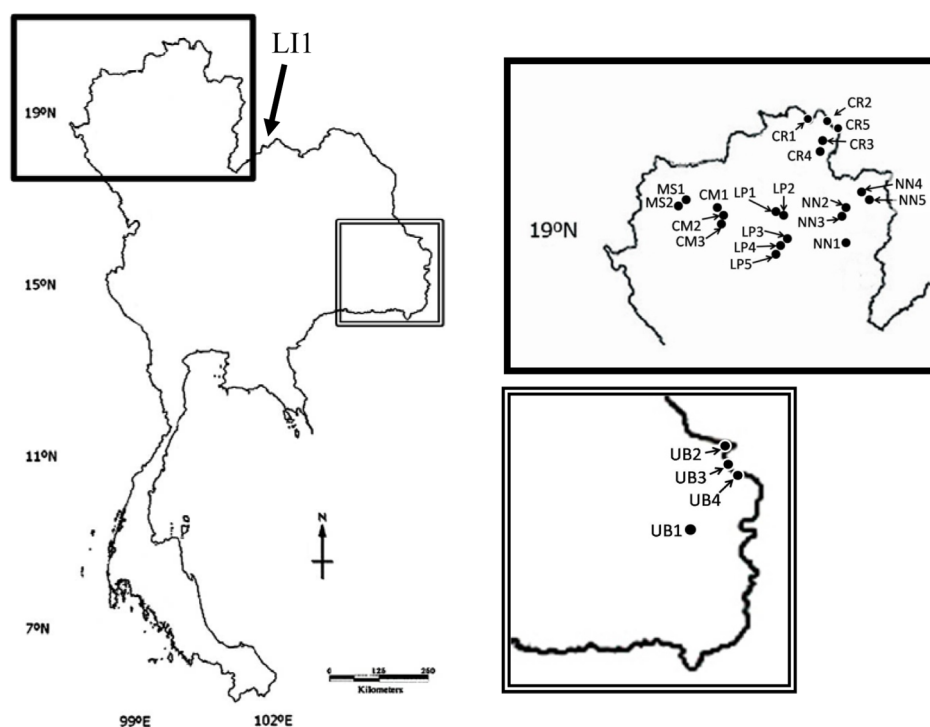
Specimens were collected from 26 locations in northern and northeastern Thailand: five locations in Chiang Rai, five in Lampang Province, three in the Mae Sa River, Chiang Mai Province, two in the Pai River, Mea Hong Son Province, five in Nan Province, one in Loei Province, and five in Ubon Ratchathani Province (Figure 1).

Single thalli or groups of thalli were collected from substrata such as rocks, cobbles, and submerged tree branches. Specimens were kept in plastic boxes at 0-4°C and transferred to the laboratory for further observations.

Materials for morphological observations were mounted on herbarium sheets or preserved in 5% formalin solution. Those for molecular analysis were desiccated in silica gel. For our morphological study of variation within *Cladophora glomerata*, we measured widths and lengths of apical cells, ultimate branches, and main axial cells. We examined thalli for qualitative characters, such as organization of branches, cell-shape, characteristics of chloroplasts, and formation of branches. The morphological terminology followed freshwater algal floras and guide books [3, 4]. Photographs were taken with a camera attached to a light microscope with Nomarski optics (Olympus, Tokyo, Japan). Voucher specimens were deposited in the Chiang Mai University herbarium in Chiang Mai, Thailand.

### 2.2 DNA sequencing and phylogenetic analysis

Total genomic DNA was extracted with a Labo Pass™ Blood/Tissue Mini Kit, (Seoul, Korea) from approximately 5 mg of



**Figure 1.** Map of sampling sites in northern and northeastern regions of Thailand. Abbreviations are as follows. In the northern region: Chiang Mai, CM1 (Mae Sa River), CM2 (Mae Sa River2), CM3 (Mae Sa River3), Chiang Rai, CR1 (Golden Triangle), CR2 (Had Krai Village), CR3 (Ngao River1), CR4 (Ngao River2), CR5 (Jam Pong Village); Lampang, LP1 (Chae Son Waterfall), LP2 (Chae Son Resort), LP3 (Kewlorn Dam), LP4 (Kewlorn Dam2), LP5 (Wang River); Mae Hong Son, MS1 (Chinese Village), MS2 (Punna Resort); Nan, NN1 (Had Pha Khon), NN2 (Tha Khum Village), NN3 (Na Tao Village), NN4 (Yao River1), NN5 (Yao River2); Loei, LI1 (Kang Kud KU). In the northeastern region: Ubon Ratchatani, UB1 (Kang Sa Peau), UB2 (Na Wang Village), UB3 (Lad Charearn Village), UB4 (Sam Pan Boke).

dried thalli ground in liquid nitrogen. Extracted DNA was stored in a freezer (-20°C) and used for SSU region amplification.

Previously published primer pairs [10] were used for amplification and sequencing of the nuclear DNA SSU gene. We also designed a new primer set for amplifying and sequencing (Table 1). All PCR amplifications were performed with a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Irvine, CA, USA) using an Ex Taq reaction kit (Takara Shuzo, Shiga,

Japan). Total reaction volumes of 25 µl consisted of 2.5 µl 10× Ex Taq buffer, 2.0 µl 25 mM MgCl<sub>2</sub>, 1.0 µM dNTP mixture, 0.15 µM each primer, 0.625 units Ex Taq, and 3.0 µM DNA solution (containing 0.5-1.0 µg DNA). PCR was performed with an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, with a final 6 min extension cycle at 72°C. PCR products were purified with a High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany)

**Table 1.** Amplification and sequencing primers of 18S rDNA used in the present study.

Primer names	Sequences (5'-3')	Reference
SB1F	GAT TAA GCC ATG CAT GTS TAR G	In this study
SB1R	CGT YCG TTA CCG GAA TCA ACC TGA C	In this study
SR4	AGC CGC GGT AAT TCC AGC T	Nakayama <i>et al.</i> [10]
SR12	CCT TCC GCA GGT TCA CCT AC	Nakayama <i>et al.</i> [10]

according to the manufacturer's instructions. Sequences of the forward and reverse strands of purified PCR products were determined for each specimen using an ABI 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Both electropherogram outputs from each sample were edited with Chromas version 1.45 software, and rechecked manually for consistency.

Multiple alignments were created with referenced sequences from GenBank using the Molecular Evolutionary Genetics Analysis (MEGA) version 4.02 software package. Gaps or ambiguous positions were omitted. Maximum-parsimony (MP) analyses of nrDNA SSU sequences were conducted using PAUP v. 4.0b10 software [11]. All heuristic searches were performed with 1000 replicates, employed random addition of taxa, retained only the best tree, held 10 trees at each step, used tree bisection-reconnection (TBR) branch swapping, collapsed zero-length branches, and used MULTREES. Bootstrap analyses were conducted using 1000 replicates with the following options selected: heuristic search, TBR branch swapping, collapse of zero-length branches, and random sequence-addition with one replicate.

Maximum likelihood (ML) analyses were performed using RAxML version 7.2.6 software [12]. Tree likelihoods were estimated using 200 independent replications, each with a random starting point. The GTRGAMMA model was used for SSU

rDNA data, and automatically optimized SPR branch rearrangements were used during the rapid hill climbing tree search for each replication. Bootstrap analyses were conducted using 1000 replications with evolution model settings used for the best topology search.

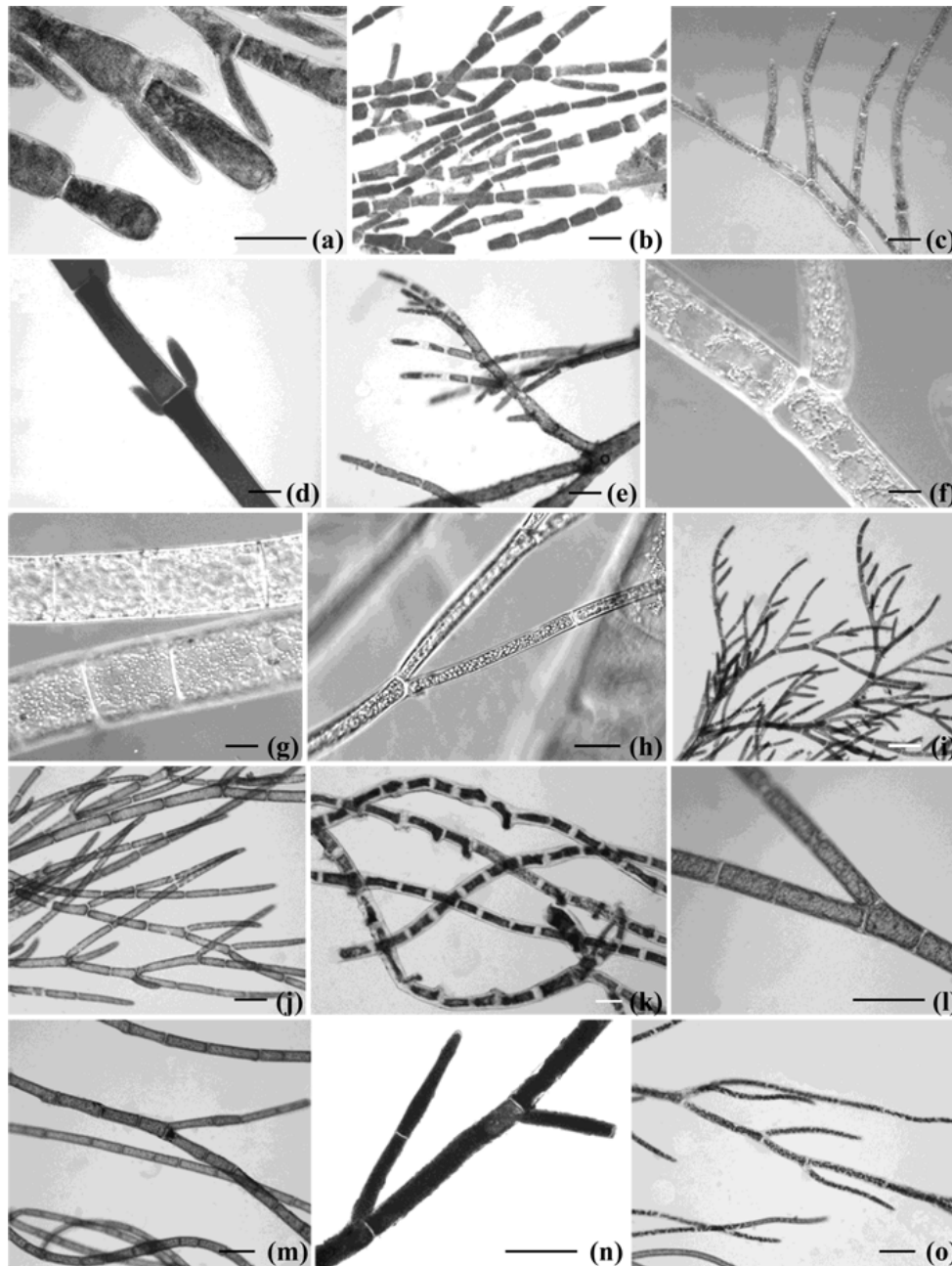
### 3. RESULTS AND DISCUSSION

We identified kai from northern and northeastern Thailand as *Aegagropila linnaei* Kützing and *Cladophora glomerata* (L.) Kützing using both morphology and nrDNA SSU sequences (Figures 2, 3). *Aegagropila linnaei* occurred only in the northern region known as the Golden Triangle, while *Cladophora glomerata* was found in many northern and northeastern sites. Both species occurred mostly in clean (oligotrophic) to moderate (mesotrophic) waters [13, 14]. *Cladophora glomerata* was morphologically very variable and we were able to recognize 12 different morphotypes (Table 2). All 12 morphotypes of *C. glomerata*, including a published Japanese sequence, were monophyletic. Intraspecific variation of SSU sequences within *C. glomerata* was similar to or higher than variation among other species of the genus as discussed later.

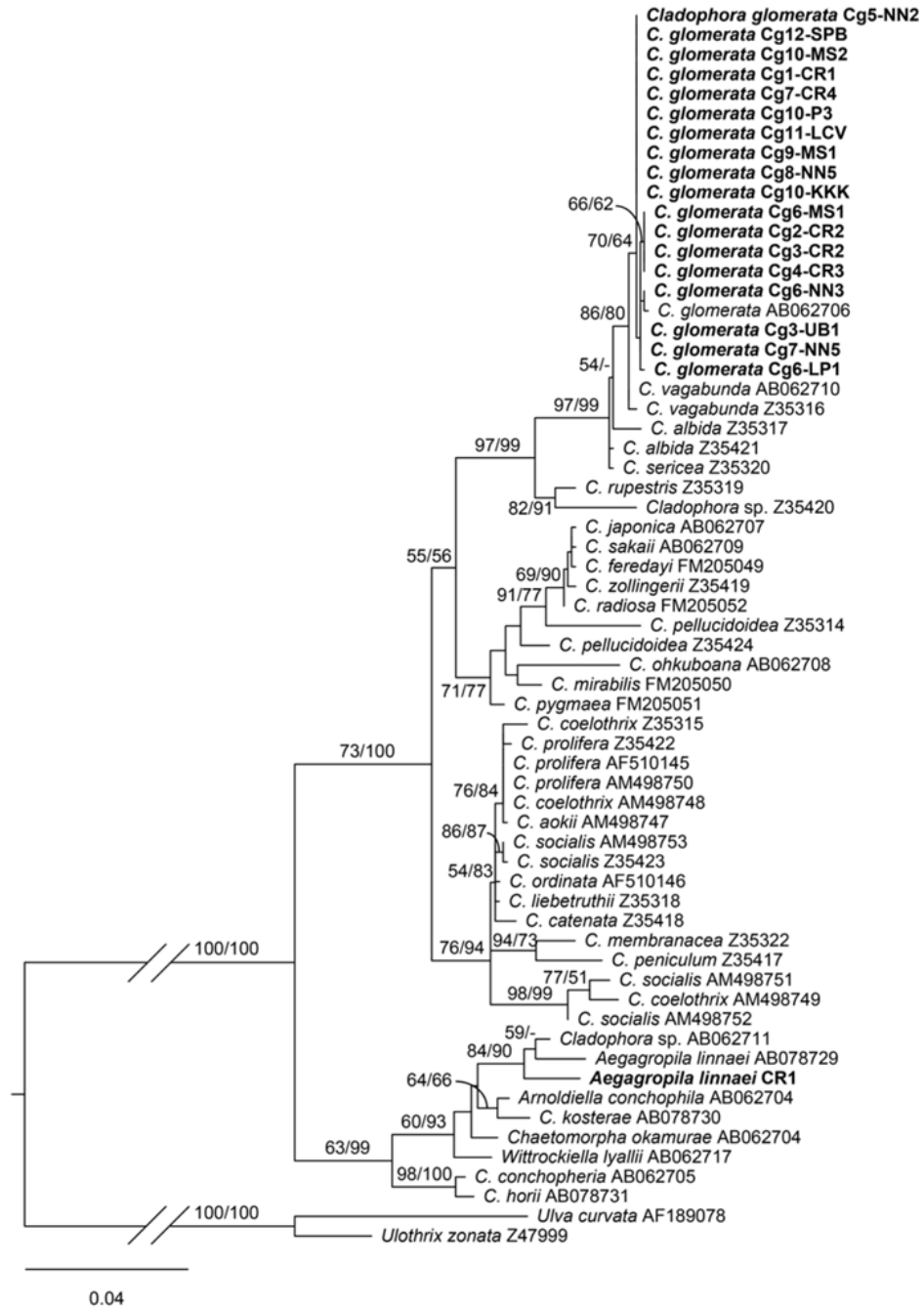
#### 3.1 Morphology

##### *Aegagropila linnaei* Kützing

Thalli densely branched, irregularly organized, and increasingly club-shaped; occurring in an irregular tangle of creeping filaments. Cell walls rather thick, darkgreen in



**Figure 2.** Morphology of *Aegagropila linnaei* Kützing (a) and diverse morphotypes of *Cladophora glomerata* (L) Kützing (b–o). (a) Apical branches of *A. linnaei*. (b) *C. glomerata* Morphotype 1 (Cg1), (c) Morphotype 2 (Cg2), (d) Morphotype 3 (Cg3), (e) Morphotype 4 (Cg4), (f) Morphotype 5 (Cg5), (g) Cg5 showing main axes of thallus, (h) Morphotype 6 (Cg6), (i) Morphotype 7 (Cg7), (j) Morphotype 8 (Cg8) with falcate secondary branches, (k) Morphotype 9 (Cg9), (l) Morphotype 10 (Cg10), (m) Morphotype 10 (Cg10), (n) Morphotype 11 (Cg11), (o) Morphotype 12 (Cg12) having refracto-falcate branches. (Scale bars = 50  $\mu$ m).



**Figure 3.** Maximum likelihood tree of 18S rDNA data for *Cladophora*. Numbers above and below the nodes are ML and MP bootstrap values. Only bootstrap values  $\geq 50\%$  are shown. See Figure 1 and Table 2 for abbreviations. All bold collections are from this study.

Table 2. Morphological features and collection sites for *Cladophora glomerata* morphotypes.

Morphotype	Dimensions ( $\mu\text{m}$ )			Description and collection site
	Apical cell (width/length)	Ultimate branch (width/length)	Main axes (width/length)	
Cg1	15.15/60	15.15/54.54	30.3/57.57	Dark green, up to 5 cm high, acropetally organized, club-shaped cells, densely packed chloroplasts (shaded area); Golden Triangle (CR1)
Cg2	12.12/166.65	12.12/136.35	57.57/321.18	Grass green, up to 4 cm high, comprised mainly of apical branch system, densely branched, pseudodichotomous, cylindrical cells, densely packed chloroplasts; Had Krai Village (CR2)
Cg3	27.27/166.65	24.24/136.35	54.54-57.57/ 266.58-324.21	Dark green, up to 8 cm high, dominant intercalary growth, thick walled, cylindrical cells, densely packed chloroplasts; Had Krai Village (CR2), Khang Sa Peaur (UB1)
Cg4	12.12/ 30.3-78.78	12.12/48.48	51.5-63.63/ 224.22-236.34	Grass green, up to 12 cm high, acropetally organized, falcate, cylindrical cells, densely packed chloroplasts, Ngao River (CR3)
Cg5	30.76/299.91	30.76/330.67	20.30-266.64/ 227	Grass green, mainly intercalary growth, reduced to chaetomorphoid in main axes, very wide meshes of reticulate chloroplasts formed under very strong sunlight; Tha Khan Village (NN2)
Cg6	12.16/124.64	7.69/369.12	31/407.57	Grass green, up to 11 cm high, pseudodichotomous branches, cylindrical cells, thick walled, densely packed chloroplasts; Mae Sa River (CR3), Natao Village (NN3), Chae Son Waterfall (LP1)
Cg7	15.15-18.18/ 66.66-90.9	15.15-18.18/ 42.42-75.75	27.36-30.4/ 118.56-127.68	Dark green, pseudodichotomous, acropetally organized with falcate branching, cylindrical cells, densely packed chloroplasts throughout entire cell; Ngao River (CR4), Yao River (NN5)
Cg8	30.76/ 215.32-261.46	23.07/353.74	53.83-61.52/ 253.77-276.84	Dark green, densely branched, light refracting, acropetally organized, secondary and tertiary branches formed close to apices, cylindrical cells, chloroplasts densely packed in a parietal protoplasmic layer; Yao River (NN5)
Cg9	23.07-30.76/ 60.6	38.45/84.59	46.14-69.21/ 99.97-192.25	Pale green, up to 30 cm high, sparsely branched with short lateral branching, thick walled, chloroplasts densely packed in parietal protoplasmic layer; Mae Sa River (MS1)
Cg10	23.07/123.04	38.45/84.59	46.14-69.21/ 99.97-192.25	Grass green, up to 23 cm high, branches at apical region less pseudodichotomously branched, chaetomorphoid filaments arise from main axes, densely packed chloroplasts; Kew Lon Dam (P3), 12 Punna Risort (MS2), Kang Kud Koo (KKK)
Cg11	30.3/190.89	30.3/192.25	33.33-42.42/ 251.49-321.18	Dark green, up to 20 cm high, reduced to chaetomorphoid main axes, less acropetally organized, cylindrical cells, densely packed chloroplasts throughout entire cell; Lad Charearn Village (LCV)
Cg12	9.09/130.29	9.09/178.77	23.07/ 223.91-261.46	Grass green, up to 8 cm high, cylindrical cell, densely branched, acropetally organized with curved branches, chloroplasts densely packed in parietal protoplasmic layer; Sam pan Boke (SPB)

colour. Apical cells 33-38  $\mu\text{m}$  wide and 14-136  $\mu\text{m}$  in length, ultimate branches 15-18  $\mu\text{m}$  and 84-92  $\mu\text{m}$ , cells of main axes up to 45  $\mu\text{m}$  wide and 122-137  $\mu\text{m}$  long [Figure 2 (a)].

Common names of *Aegagropila linnaei* are 'Cladophora balls' and 'Marimo' (*Cladophora aegagropila* (Linnaeus) Trevisan). However, a recent nr SSU analysis transferred *Cladophora aegagropila* back to the genus *Aegagropila* [8]. Although dimensions of apical cells and branches of *A. linnaei* were variable, thalli from northern Thailand were in agreement with descriptions of specimens from Japan [15]. This filamentous species is either attached or unattached as mats or rarely in the enigmatic lake ball form. Wave-induced rolling motion on sandy sediments often results in ball-shaped thalli [15, 16].

#### *Cladophora glomerata* (L) Kützing

Thalli profusely to very sparsely branched, growth mainly apical where intercalary divisions are some distance from apex. Pseudodichotomous branching, generally with oblique or horizontal side laterals, formed in turbulent water. Chaetomorphoid filaments often found in stagnant waters. Cells cylindrical or club-shaped, 9-266  $\mu\text{m}$  wide and 60-324  $\mu\text{m}$  long. Chloroplasts net-like or granular in the parietal network or through entire cell.

*Cladophora glomerata* is probably the most variable freshwater macroalga and the most widely distributed through the planet's freshwater ecosystems [2, 8]. Diagnostic characters often overlap among different species of the genus depending on age of thalli and habitats [3, 4, 5]. We found 12 morphotypes (Cg1-12) within *C. glomerata*; each morphotype was distinguished by its densely arranged branches, shapes, sizes, colours of cells, and dimensions of apical cells and branches (Figure 2 [b-o]). Descriptions

and sample locations of morphotypes are summarized in Table 2. Our observations of morphological variation within *C. glomerata* agree with those of Sheath and Cole [17]; both studies indicate that cell dimension is dependent on branch abundance. For example, thalli with dense branches have cells 3-6 times longer than wide, and thalli with sparse branches have cells up to 20-30 times longer than wide. However, morphotype Cg6 with pseudodichotomous branches had cells 10 times longer than wide. Morphotypes Cg5 and Cg10, both with a chaetomorphoid form, had cells less than 5 times longer than broad. Club-shaped cells in morphotype Cg1 were quite unique within *C. glomerata*. The transformation of morphological features may be triggered by environmental factors, including low temperatures or short photoperiod. However, there are many previously reported varieties in the species [2], and further study is necessary to determine whether some of these morphotypes are in fact varieties or subspecies of *C. glomerata*.

#### 3.2 Molecular systematics

We analyzed 1248 bp of nrDNA SSU from 19 isolates of *Aegagropila linnaei* and *Cladophora glomerata*. Nineteen newly generated sequences were registered in GenBank (JQ071987- JQ072005). The topology of SSU rDNA phylogeny in the present study was congruent with that of a previous study [8]. Our specimens of *A. linnaei* from Thailand were very similar to Japanese specimens in having predominantly apical growth, limited enlargement of cells with age, a strong propensity toward polarity inversion, thick walls and a consequently stiff texture, and dense dark-green cell contents [15, 18]. However, a difference of 18 bp (1.3% pairwise divergence) between specimens from Thailand and Japan, and a difference

of 28 bp (2.1% pairwise divergence) between a Thai specimen and *Cladophora* sp. (AB062711) suggest that specimens from Thailand and Japan may belong to different species. Analysis of material collected in Sweden, the type locality of *A. linnaei* [2], is absolutely required to identify specimens that are close to European *A. linnaei*. *Aegagropila linnaei* was found only in the Golden Triangle sites (CR1) of Chiang Rai Province, Thailand where the water is mesotrophic [13, 14]. Because of its rarity in the Netherlands, *A. linnaei* has been proposed for inclusion as an endangered species in the national red list [19].

In the present study, we generated 18 new sequences of the SSU gene from samples of *Cladophora glomerata* collected in Thailand. However, in eight samples the SSU gene was not amplified for unknown reasons. To date, only two sequences (AB062707 and GQ337527) of the nrDNA SSU gene from *Cladophora glomerata* have been registered in GenBank, but GQ337527 was not included in our analysis because only a partial sequence (525 bp) was available.

All nineteen sequences of *C. glomerata*, including AB062706 from Japan, were monophyletic (Figure 3). The clade containing *C. glomerata*, *C. vagabunda*, *C. albida*, and *C. seriacea* was well supported with bootstrap values (97% for ML and 99% for MP), as found in a previous study [8]. The SSU sequences differed by pairwise divergences of 0.14-0.22% among Japanese (AB062707) and Thai specimens of *C. glomerata*. Because this degree of pairwise divergence is slightly higher than that (0.14%) between *C. feredayi* Harvey and *C. japonica* Yamada, and that (0.15%) between *C. coelothrix* Kützing (AM498748) and *C. prolifera* (Roth) Kützing, further morphological comparisons of Thai and Japanese specimens may well reveal taxon differentiation at the species level.

In addition, because of its cosmopolitan distribution, it is also absolutely necessary to analyze sequences of *C. glomerata* material from geographically isolated regions in addition to material from the type locality in Europe [20].

Although the northern and northeastern Thai provinces are about 350 km distant (Figure 1), analysis of SSU data revealed no geographical structure within Thailand. Close phylogenetic relationships among Cg2, Cg3, Cg4, and Cg6 are reflected in the similar dense packing of chloroplasts (other than in Cg3). However, although Cg1 had unique club-shaped cells, this was not reflected in the SSU tree, and other morphological groupings, such as similarly curved branching in Cg4 and Cg7, were not reflected in our SSU phylogeny. Fast-evolving genes such as the nrDNA internal transcribed spacer (ITS) may be more suitable for resolving infraspecific morphological variation in *C. glomerata* [21].

#### 4. CONCLUSIONS

Although freshwater green macroalgae are mostly viewed as nuisance bloom species in temperate regions [20], the abundant growth of kai in northern and northeastern Thailand is a valuable resource for local people. Our resolution of the species composition of kai through morphological and molecular assessment provides a baseline for future studies, particularly those dealing with food resources. In other parts of Thailand, these algae grow abundantly and are often seen as nuisance weeds, but they may well be a valuable resource for future exploitation.

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