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Insight into the species identification and distribution of Grateloupiaceae (Halymeniales, Rhodophyta) having *Grateloupia filicina*-like morphology in the Northwest Pacific

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Accurately identifying species is the basis of all biological studies. There has been much confusion in the identification of Grateloupiacean species, which have finely pinnate gross morphology similar to *Grateloupia filicina* (the type species of the family). The objective of this study was to comprehensively investigate species identification and distribution of *G. filicina*-like species in the Northwest Pacific, based on the *rbcL* sequences. A total of 118 specimens from 78 sites in Korea and Japan were collected from 2001 to 2021 and analyzed for their *rbcL* sequences. Additional 341 sequences downloaded from the GenBank were included in our comprehensive dataset. Based on these sequences, we documented the nomenclatural history and geographical distribution of the species, and commented on the application of species name. *G. asiatica* was the most abundant *G. filicina*-like species in the Northwest Pacific, and its high degree of morphological variation caused many misidentifications. In particular, *G. dalianensis*, *G. serra*, and *G. variata* require reconsideration of their conspecificity with *G. asiatica* using more specimens from China. By contrast, *G. oligoclora* was presumed to be a heterotypic synonym of *G. subpectinata*. The occurrence of *G. acuminata*, *G. ramosissima*, and *G. livida* in Korea resulted from misidentifications with other species.

Keywords: distribution; Grateloupiaceae; Northwest Pacific; *rbcL*; species identification

INTRODUCTION

The red algal family Grateloupiaceae includes nine genera: *Grateloupia*, *Dermocorynus*, *Mariaramirezia*, *Neorubra*, *Pachymeniopsis*, *Kintokiocolax*, *Phyllymenia*, *Prionitis*, and *Yonagunia* (Kim et al. 2021). Species belonging to this family are a notorious red algal group that are difficult to identify based only on the gross morphology due to significant intraspecific and interspecific variations (e.g., Verlaque et al. 2005, Kim et al. 2013). In general, any *Grateloupia* species with a finely pinnate

thallus have been considered as *Grateloupia filicina* (J. V. Lamouroux) C. Agardh, the type species of the family (De Clerck et al. 2005). Identifying species in Grateloupiaceae is difficult for even algal taxonomist. Thus, many misidentifications of these species have been detected. For example, recent biochemical studies about the effect of extracts from *Grateloupia* species collected from Korea and China used the name *G. filicina* (e.g., Jung et al. 2016, Sun et al. 2017, Liu et al. 2019). Using *G. filicina* in a patent



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can be problematic because the distribution of *G. filicina* is limited to the Mediterranean basin (De Clerck et al. 2005). *G. filicina*-like species from the Northwest Pacific are different entities that share superficially similar gross morphology with *G. filicina* (Kawaguchi et al. 2001).

Three species in the Northwest Pacific have been considered as *G. filicina* because of their similar gross morphology, such as *G. asiatica* S. Kawaguchi & H. W. Wang, *G. catenata* Yendo, and *G. subpectinata* Holmes. Kawaguchi et al. (2001) concluded that *G. filicina*-like specimens from Japan and China were *Grateloupia asiatica* sp. nov., showing that they were distantly related to genuine *G. filicina* from the Mediterranean, the type locality of the species. Subsequently, the occurrence of *G. asiatica* has been confirmed in Korea using *rbcL* sequences (Lee et al. 2009, Kim et al. 2013, Yang and Kim 2015). Recently, Yang et al. (2021) showed that *G. asiatica* is common along the coasts of the Korean peninsula and Jeju Island.

Grateloupia catenata is known as *G. filicina* var. *lomentaria* Howe, *G. filicina* var. *porracea* (Mertens ex Kützinger) Okamura, and *G. filicina* var. *porracea* f. *lomentaria* (Howe) Okamura (Okamura 1936). It was reinstated as *G. catenata* based on *rbcL* sequences from Japanese specimens reported by Wang et al. (2000). The occurrence of *G. catenata* in Taean (west coast), Uljin (east coast), and Jeju Island, Korea was confirmed by *rbcL* sequences (Lee et al. 2009, Kim et al. 2013, Yang and Kim 2015). Sheng et al. (2012) demonstrated the presence of *G. catenata* in China, revising *Sinotubimorpha catenata* (Yendo) W.-X. Li & Z. -F. Ding, *S. claviformis* W.-X. Li & Z. -F., *S. guangdongensis* W.-X. Li & Z. -F., *S. qingdaoensis* W.-X. Li & Z. -F., and *S. ramosissima* W.-X. Li & Z. -F. as heterotypic synonyms with *G. catenata*.

Grateloupia subpectinata, which was originally described by Holmes in 1912, was long placed into synonymy under *G. filicina* by Okamura (1936) based on morphology. However, *G. subpectinata* has been resurrected by Faye et al. (2004) based on the *rbcL* phylogeny and the detailed morphology of six specimens from Japan. Identifying *G. subpectinata* in the field is difficult because its gross morphology is similar to that of *G. asiatica*. In addition, the distribution range of *G. subpectinata* is unclear compared to that of *G. asiatica*. Several reports of *G. subpectinata* have been made in Korea based on *rbcL* sequences, but most are limited to the northeast coast and Jeju Island (Lee et al. 2009, Kim et al. 2013, Yang and Kim 2015).

The plastid *rbcL* gene is a suitable marker for the identifying halymenialean species (De Clerck et al. 2005, Lee et al. 2009, Yang and Kim 2015). In addition, the female

reproductive structures (i.e., type of ampullae and post-fertilization events) reflecting genus delimitations are consistent with the *rbcL* phylogeny (Gargiulo et al. 2013, Calderon et al. 2014, Lee and Kim 2019). During the past two decades, many taxonomic studies have been conducted on Grateloupiacean species in the Northwest Pacific based on the *rbcL* sequences. (e.g., Kawaguchi et al. 2001, Faye et al. 2004, Kim et al. 2013, 2021). Accordingly, numerous *rbcL* sequences have been deposited in NCBI GenBank. However, there is much taxonomic confusion. For example, when blasting using the *G. asiatica* sequence in NCBI homepage, almost 100% sequence identity can be obtained under the names *G. asiatica* and also *G. subpectinata* and *G. fastigiata* W. -X. Li & Z. -F. Ding, making it difficult for non-taxonomic researchers to correctly identify their specimens. The objective of this study was to comprehensively investigate the identification and distribution of Grateloupiacean species with *G. filicina*-like morphology in the Northwest Pacific based on *rbcL* sequences.

We selected 29 species within Grateloupiaceae based on morphology, nomenclatural history, and distribution (Table 1). Among the 29 species, 8 Chinese species names (*Grateloupia didymecladia* W. -X. Li & Z. -F. Ding, *G. fastigiata*, *G. qingdaoensis* W. -X. Li & Z. -F. Ding, *Sinotubimorpha catenata*, *S. guangdongensis*, *S. qingdaoensis*, *S. claviformis*, and *S. ramosissima*) were included for a comprehensive understanding of *G. filicina*-like species, but they were not discussed in detail. These species have been described as new species based on the morphology described by Xia (2004). Subsequently, they were synonymized based on *rbcL* sequences. *G. didymecladia* was synonymized to *G. subpectinata*, and *G. fastigiata* and *G. qingdaoensis* were synonymized to *G. asiatica*, and the other five *Sinotubimorpha* species (*S. catenata*, *S. claviformis*, *S. guangdongensis*, *S. ramosissima*, and *S. qingdaoensis*) were synonymized to *G. catenata* (Sheng et al. 2012, Li et al. 2016, Liu et al. 2017, 2020).

MATERIALS AND METHODS

In total, 21 *G. filicina*-like species from the Northwest Pacific are discussed in detail in the Results and discussion section (Table 1). The *G. filicina*-like habit of four representative species (*Grateloupia asiatica*, *G. catenata*, *G. divaricata* Okamura, and *G. subpectinata*) is shown in Fig. 1.

A total of 118 specimens of *G. filicina*-like species were collected from Korea and Japan (78 localities) between

2001 and 2021 (Supplementary Table S1). Specimens were pressed on herbarium sheets for habit observation, and most specimens were dehydrated in each plastic bag with silica gel for molecular study. Total genomic DNA was extracted from the silica gel-dried specimens or herbarium materials using the LaboPass Tissue Mini kit (Cosmo Genetech, Seoul, Korea) following the manufacturer's instructions. The *rbcl* gene was amplified using the F7-R898 and F7-R1442 primer pairs (Freshwater and Rueness 1994, Kim et al. 2010). Polymerase chain reaction (PCR) amplification was performed using AccuPower PCR PreMix (Bioneer, Daejeon, Korea) or TaKaRa Ex Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). The reaction was run with an initial denaturation at 94°C for 4 min, followed by 35 cycles of amplification (denaturation at 94°C for 30 s, an-

nealing at 50°C for 30 s, and extension at 72°C for 1 min), and a final extension at 72°C for 6 min. The PCR products were purified using the LaboPass PCR purification kit (Cosmo Genetech) and then sequenced commercially by Macrogen Co. (Daejeon, Korea). The sequence reads were edited and assembled into a consensus using Chromas v. 1.45 (<http://www.technelysium.com.au/chromas.html>) or Geneious v. 11.1.5 (<http://www.geneious.com>) (Kearse et al. 2012), and aligned by manually.

In total, 459 *rbcl* sequences were used to infer the phylogeny and confirm the distribution of each species in the Northwest Pacific. The *rbcl* sequences of the *G. flicina*-like species were searched by name and downloaded from GenBank. We used a nucleotide BLAST search to include available GenBank sequences whether they were

Table 1. List of 29 *Grateloupia flicina*-like species in the Northwest Pacific

Species	Taxonomic note (reference)	Clade	Distribution (based on <i>rbcl</i>)
<i>G. didymecladia</i>	Synonymized to <i>G. subpectinata</i> (Liu et al. 2017 in Chinese)	<i>G. subpectinata</i> clade	CH
<i>G. fastigiata</i>	Synonymized to <i>G. asiatica</i> (Li et al. 2016 in Chinese), sequence problem	<i>Prionitis</i> clade	CH
<i>G. qingdaoensis</i>	Synonymized to <i>G. asiatica</i> (Liu et al. 2020)	<i>Prionitis</i> clade	CH
<i>Sinotubimorpha catenata</i>	Synonymized to <i>G. catenata</i> (Sheng et al. 2012)	<i>Grateloupia sensu stricto</i>	CH
<i>S. claviformis</i>	Synonymized to <i>G. catenata</i> (Sheng et al. 2012)	<i>Grateloupia sensu stricto</i>	CH
<i>S. guangdongensis</i>	Synonymized to <i>G. catenata</i> (Sheng et al. 2012)	<i>Grateloupia sensu stricto</i>	CH
<i>S. ramosissima</i>	Synonymized to <i>G. catenata</i> (Sheng et al. 2012)	<i>Grateloupia sensu stricto</i>	CH
<i>S. qingdaoensis</i>	Synonymized to <i>G. catenata</i> (Sheng et al. 2012)	<i>Grateloupia sensu stricto</i>	CH
<i>G. sorocarpus</i> ^a	Unpublished, same sequence with <i>G. asiatica</i>	<i>Prionitis</i> clade	CH
<i>G. constricta</i> ^a	Unpublished, same sequence with <i>G. asiatica</i>	<i>Prionitis</i> clade	CH
<i>G. corymbcladia</i> ^a	Unpublished, same sequence with <i>G. subpectinata</i>	<i>G. subpectinata</i> clade	CH
<i>G. acuminata</i> ^b		<i>Prionitis</i> clade	JP (South)
<i>G. asiatica</i> ^b		<i>Prionitis</i> clade	KR, JP, CH
<i>G. catenata</i> ^b	Resurrected (Wang et al. 2000)	<i>Grateloupia sensu stricto</i>	KR, JP, CH
<i>G. dalianensis</i> ^a	Sequence problem, <i>G. asiatica</i> complex	<i>Prionitis</i> clade	CH
<i>G. divaricata</i> ^b		<i>Prionitis</i> clade	KR (East), JP
<i>G. huanghaiensis</i>	Sequence problem	<i>Grateloupia sensu stricto</i>	CH
<i>G. livida</i> ^b		<i>Prionitis</i> clade	JP, CH
<i>G. luxurians</i>	Synonymized to <i>G. subpectinata</i> (Verlaque et al. 2005), AY435175-6: not monophyletic with <i>G. subpectinata</i>	<i>G. subpectinata</i> clade	UK, AUS
<i>G. oligoclora</i>	Synonym with <i>G. subpectinata</i> (this study)	<i>G. subpectinata</i> clade	CH (South)
<i>G. orientalis</i>		<i>Grateloupia sensu stricto</i>	CH (South)
<i>G. ramosa</i>	JF810597: sequence problem	<i>Grateloupia sensu stricto</i>	CH (South)
<i>G. ramosissima</i>		<i>Grateloupia sensu stricto</i>	JP (South), CH (South), VN
<i>G. serra</i> ^a	Sequence problem, <i>G. asiatica</i> complex	<i>Prionitis</i> clade	CH
<i>G. subpectinata</i> ^b	Resurrected (Faye et al. 2004)	<i>G. subpectinata</i> clade	KR, JP, CH
<i>G. tenuis</i>	Sequence problem	<i>Grateloupia sensu stricto</i>	CH (South)
<i>G. variata</i> ^a	Sequence problem, <i>G. asiatica</i> complex	<i>Prionitis</i> clade	CH
<i>G. yangjiangensis</i> ^a	Only 3 bp difference with <i>G. hawaiiensis</i> : AY772030	<i>Grateloupia sensu stricto</i>	CH (South), ML, USA (Hawaii)
<i>G. yinggehaiensis</i>	Sequence problem	<i>Grateloupia sensu stricto</i>	CH (South), SL

Bold indicates the 21 species discussed in the Results and Discussion section.

CH, China; JP, Japan; KR, Korea; UK, United Kingdom; AUS, Australia; VN, Vietnam; ML, Malaysia; SL, Sri Lanka.

^aSpecies that have to be confirmed.

^bA species that was sequenced in this study.

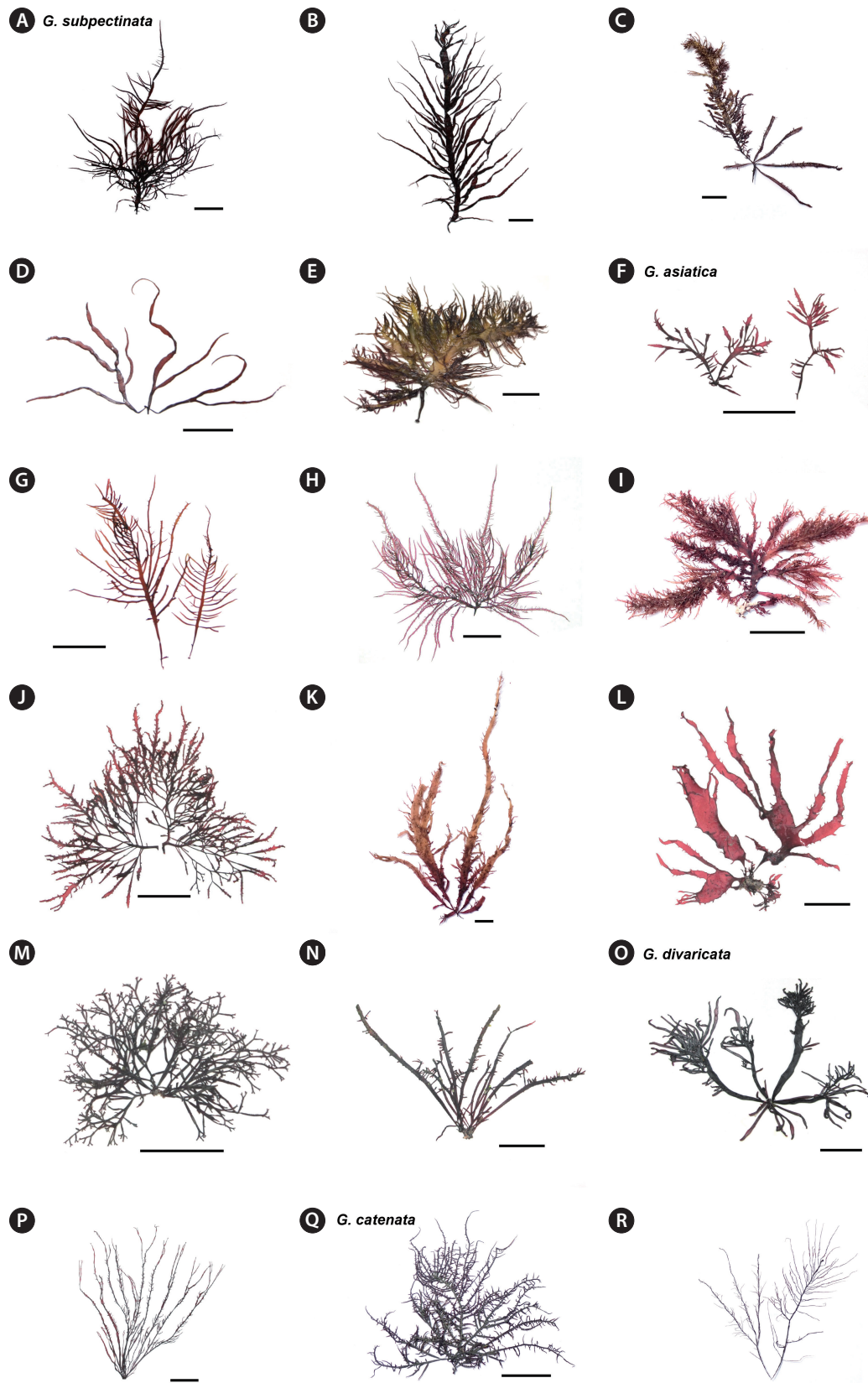


Fig. 1. Habits of *Grateloupia filicina*-like species in the Northwest Pacific. (A–E) *G. subpectinata*. (F–N) *G. asiatica*. (O & P) *G. divaricata*. (Q & R) *G. catenata*. Scale bars represent: A–R, 3 cm.

correctly identified or not. (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Based on $\geq 99\%$ identity with target species, all sequences were downloaded and aligned with our sequence data. The collection information found in each study for the downloaded sequences is listed in Supplementary Table S2.

Phylogenetic analyses were performed using the maximum likelihood (ML) method and the RAxML program (Stamatakis 2006) with the GTR + G evolutionary model. We performed 200 independent tree inferences using the `-#` option with default `-I` (automatically optimized SPR rearrangement) and `-c` (25 distinct rate categories) option in the program to identify the best tree. We used 1,000 replications under the same model settings to generate the bootstrap values. Bayesian inference was performed with MrBayes (v3.2.2) software (Ronquist et al. 2012). Two independent searches were done under the default settings, such as four chains of the Metropolis-coupled Markov Chain Monte Carlo, every 100th trees sampling, for 20 million generations, and the same evolution model (GTR + G) used in the ML search. Twenty-five percent of saved trees were discarded (as a burn-in point) for the Bayesian posterior probability calculations.

RESULTS AND DISCUSSION

Phylogeny of the *Grateloupia filicina*-like species within the family Grateloupiaceae

Despite their highly similar gross morphology, the specimens were grouped into three different clades within the Grateloupiaceae, including the *G. subpectinata* clade, the *Prionitis* clade, and the *Grateloupia sensu stricto* clade (Fig. 2). These three clades have been suggested to be separate genera by Gargiulo et al. (2013). Although Rodríguez-Prieto et al. (2022) combined the *G. subpectinata* clade with the genus *Phyllymenia*, we do not agree because *Neorubra* species were not included in the combined tree. *Neorubra* species should be included to discuss relationships among *Phyllymenia*, the *G. subpectinata* clade, and *Neorubra*. As shown in Fig. 2, the monophyly of those three genera has been moderately supported (88% Bootstrap support [BTS]) (Fig. 2), while the monophyly of *Phyllymenia* and *G. subpectinata* clade is not supported (53% BTS not shown). We considered that the *G. subpectinata* clade could be a distinct new genus separate from *Grateloupia sensu stricto*. In addition, *Pachymeniopsis*, *Prionitis*, and *Neorubra* were suggested as the *Phyllymenia* / *Prionitis* complex by Rodríguez-Pri-

eto et al. (2022), are also considered to be three distinct genera within Grateloupiaceae. A more comprehensive study on this issue will be our next publication. In this study, we suggest a detailed discussion of the 21 *G. filicina*-like species for each of the three separate clades, following the view of Gargiulo et al. 2013, including the *G. subpectinata* clade (= *G. subpectinata* group in Gargiulo et al. 2013), the *Prionitis* clade (= *G. americana* group in Gargiulo et al. 2013), and the *Grateloupia sensu stricto* clade (= *G. filicina* group in Gargiulo et al. 2013).

Grateloupia subpectinata clade

The *G. subpectinata* clade was fully supported, including *G. subpectinata* and other western Pacific species such as *G. huangiae* Show M. Lin & H. -Y. Ling, *G. phuquocensis* Tanaka & Pham-Hoàng Hô, *G. sparsa* (Okamura) Chiang, *G. taiwanensis* Show M. Lin & H. -Y. Ling, and *G. turuturu* Y. Yamada (100% BTS) (Figs 2 & 3). Although *Grateloupia luxurians* (A. Gepp & E. S. Gepp) R. J. Wilkes, L. M. McIvor & Guiry has been placed in synonymy with *G. subpectinata* (Verlaque et al. 2005), two *rbcL* sequences were designated *G. luxurians* from Australia (AY435175) and the United Kingdom (AY435176), which are distinct from *G. subpectinata* (Fig. 2). These specimens need to be confirmed as reported by Nelson et al. (2013). With the exception of these two specimens, and 12 specimens reported as introduced cases (marked with * in Fig. 3), *G. subpectinata* has been confirmed from Korea, Japan, and China. Liu et al. (2017) collected and analyzed 11 specimens known in China as *G. didymecladia* from Dalian, Wenzhou, and Shantou (KY047357-67) and synonymized them with *G. subpectinata*. Of the 11 specimens, the two from Shantou (Guangdong province) represented the southernmost point of the species distribution to date. The latitudinal distribution of *G. subpectinata* ranges from Shantou in the southern part of China (not shown in the map of Fig. 3), to Hakodate in the northern part of Japan (marked as “9” in Fig. 3). Collectively, this species is not abundant compared to other Grateloupiaceae species, but appears to be widely distributed in the Northwest Pacific, including the southern part of China.

Among the published *G. subpectinata* sequences, it is noteworthy to mention about two recently published plastid genomes (i.e., MG598531 and AP018129). The former (MG598531) was published as *G. filicina* by Zhang et al. (2018), who collected specimens from Xiangshan Harbor, Zhejiang Province, China, which is outside the distribution range of genuine *G. filicina*. The latter (AP018129) was published as *G. asiatica* from Hokkaido, Japan by

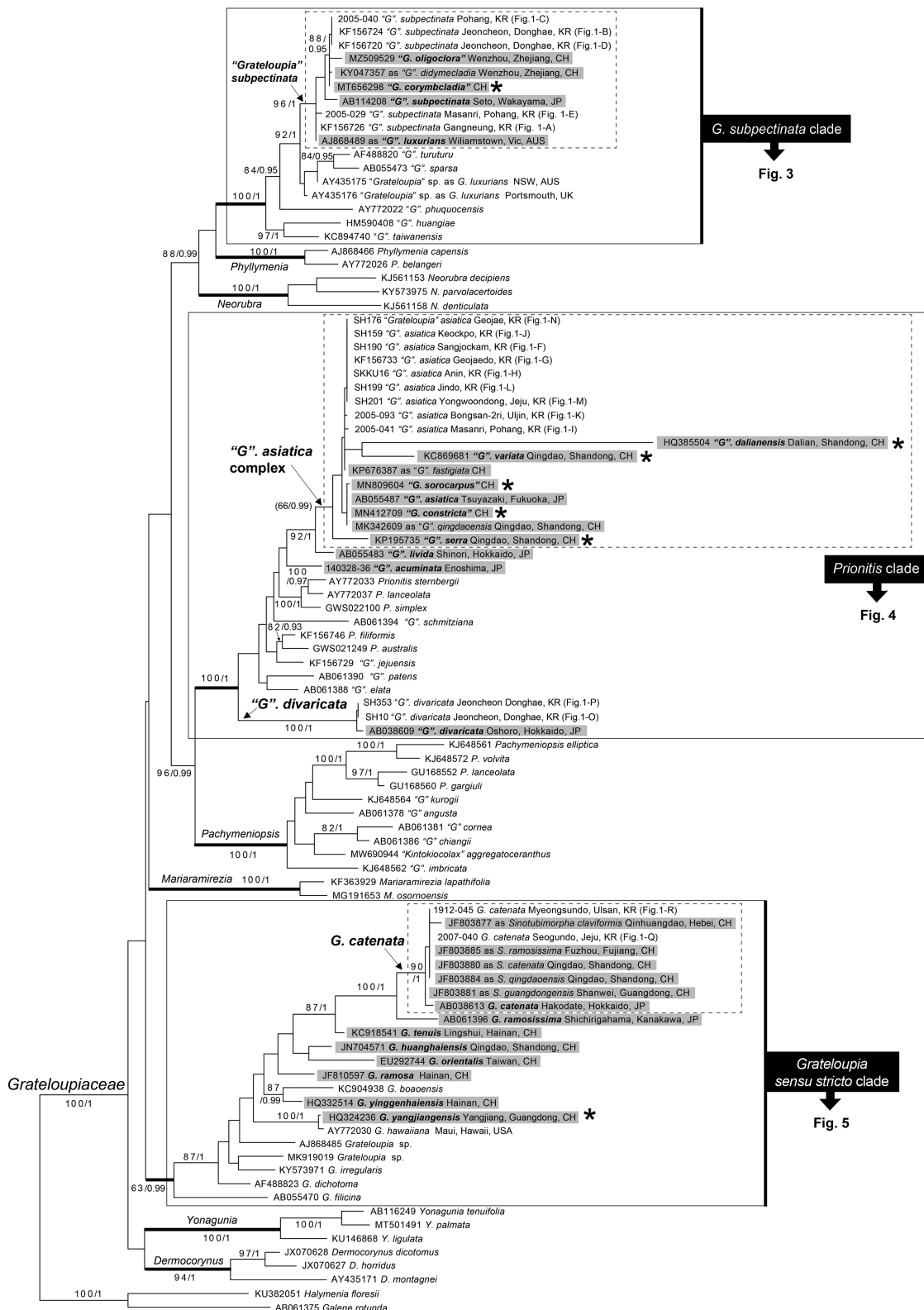


Fig. 2. Maximum likelihood phylogenetic tree of *Grateloupia filicina*-like species in the Northwest Pacific using *rbcL* sequences. Maximum likelihood bootstrap value and Bayesian posterior probability are shown near the branches. Gray boxes indicate the 29 *G. filicina*-like species names in the Northwest Pacific. The 21 species included in the Result and discussion section are in bold. The species that need confirmation are marked with an asterisk (*). The species or genus names in quotation marks indicate that the name should be changed.

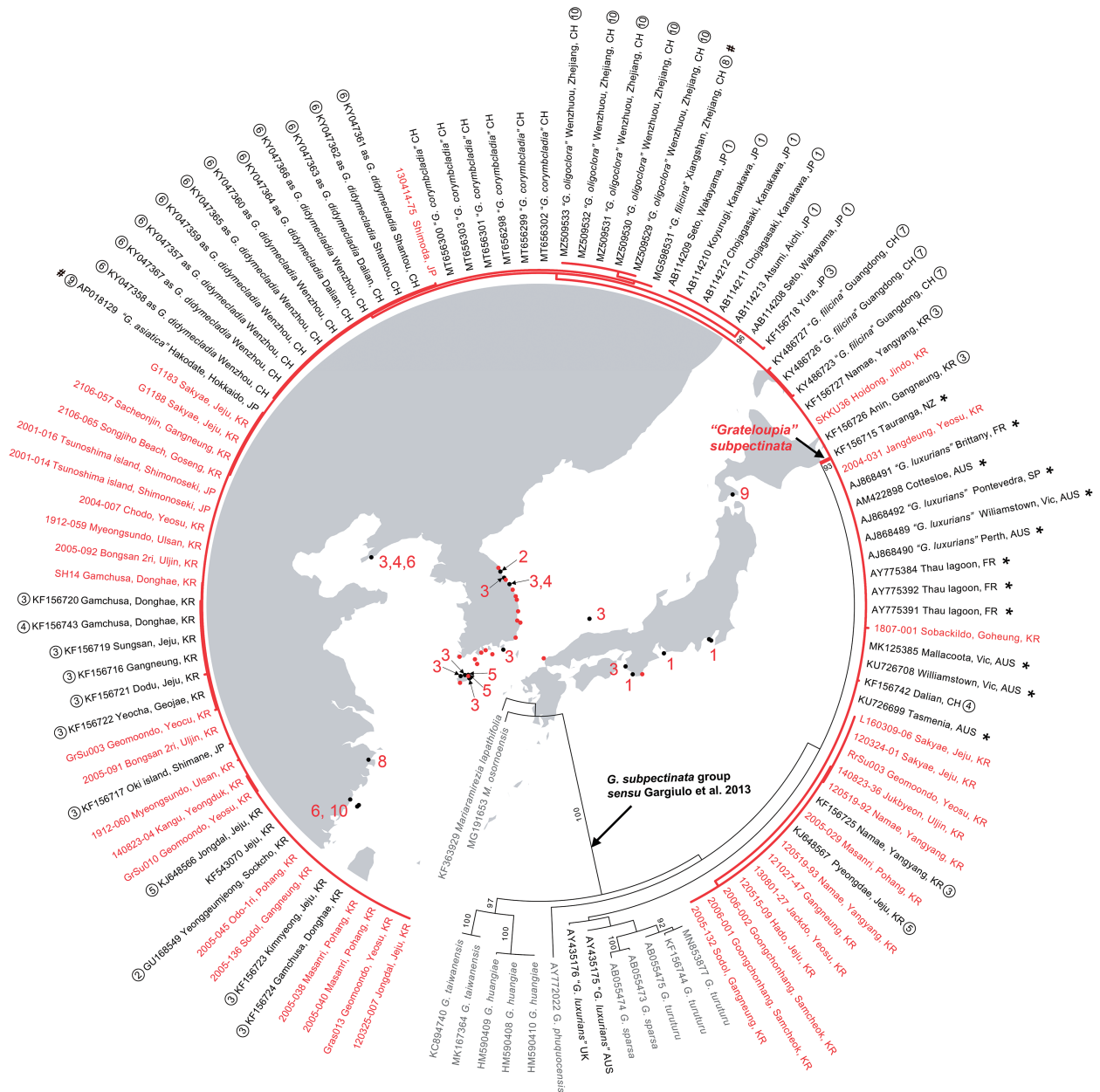


Fig. 3. Maximum likelihood phylogeny of the *Grateloupia subpectinata* clade with a distribution map of *G. subpectinata* based on *rbcl* sequences. Asterisk (*) indicates sequences known to have been introduced to outside of the Northwest Pacific. Sharp (#) indicates sequences extracted from published plastid genomes. Red dots on the map indicate collection locations of new sequences and black dots indicate collection locations of published sequences with the following reference number: 1, Faye et al. (2004); 2, Lee et al. (2009); 3, Nelson et al. (2013); 4, Kim et al. (2013); 5, Yang and Kim (2015); 6, Liu et al. (2017); 7, Peng et al. (2018); 8, Zhang et al. (2018); 9, Sumikawa et al. (2020); 10, Duan et al. (2022).

Sumikawa et al. (2020). However, their genuine identity was *G. subpectinata* based on the *rbcl* sequence (marked with # in Fig. 3). This is one of the examples of the prevailing misidentification of *G. filicina*-like species in the Northwest Pacific region. This finding is significant because it could affect comparative plastid genomic studies of Halymeniales. The genomic differences between

MG598531 and AP018129 should be treated as intra-specific differences rather than interspecific differences between distantly related species (i.e., *G. filicina* and *G. asiatica*).

Duan et al. (2022) reported that five specimens collected from Wenzhou, Zhejiang, China were *G. oligoclora* H. W. Wang & Y. Bian sp. nov. They suggested that this new

species was the closest sister species to *G. subpectinata* with 1% sequence divergence. However, they compared the new sequences (five specimens were 100% identical to each other) with only one *G. subpectinata* sequence from Japan (AB114213) and did not consider genetic variation within *G. subpectinata*. Intraspecific variation of *G. subpectinata* ranged from 0 to 12 bp (0–1%) in our analyses. The *G. oligoclora* sequences were different from MG598531 at only 3 bp (0.27%) after including 81 *G. subpectinata* sequences from various regions. In our phylogenetic analysis, *G. oligoclora* was consistently monophyletic with other *G. subpectinata* sequences with high support (96% BTS in Fig. 2 & 93% BTS in Fig. 3). Duan et al. (2022) suggested that lateral branches and the branching patterns were the most obvious differences between *G. oligoclora* and *G. subpectinata* (*G. oligoclora*: unbranched, dichotomously branched or irregularly split; *G. subpectinata*: pinnately branched). However, we observed several specimens that dichotomously branched similar to *G. oligoclora*, as shown in Fig. 1D. Therefore, dichotomous branching cannot be used as a key to distinguish *G. subpectinata* from *G. oligoclora*. We consider that *G. oligoclora* should be synonymous with *G. subpectinata* based on available data (monophyletic in ML phylogeny, very low genetic divergence, and no distinct in gross morphology).

Among the sequences downloaded from GenBank, the sequences under the name *G. corymbcladia* W. -X. Li & Z. -F. Ding (MT656298-303) had the same *rbcl* sequences as *G. subpectinata*, in spite of no accessible references or specimens could be obtained. Considering the previous publications (e.g., Sheng et al. 2012, Li et al. 2016, Liu et al. 2017, 2020), this species may be synonymous with *G. subpectinata*.

Prionitis clade

The *Prionitis* clade was fully supported (100% BTS in Figs 2 & 4) and it includes following 7 selected *G. filicina*-like species; *Grateloupia acuminata* Holmes, *G. asiatica*, *G. dalianensis* H. W. Wang & D. Zhao, *G. divaricata*, *G. livida* (Harvey) Yamada, *G. serra* H. W. Wang & Y. Lou, and *G. variata* Cao, C. C., Li, Y. Z. & H. W. Wang. The monophyly of *Grateloupia asiatica*, *G. variata*, *G. serra*, and *G. livida* was fully supported within the *Prionitis* clade (Fig. 4). Although *G. dalianensis* also appears to be included in this clade (92% BTS) (Fig. 2), the discussion of this species is in the last section of the Results and discussion. Based on our sequences from collected specimens and the downloaded sequences, *G. asiatica* was considered

the most abundant species not only within the *Prionitis* clade but also among *G. filicina*-like species in the Northwest Pacific. However, their latitudinal distribution range (from Hirado, Nagasaki, Japan to Oshoro, Hokkaido, Japan; the southernmost and northernmost points were marked with red stars in Fig. 4 because there were too many collection sites in Korea) was narrower than that of *G. subpectinata*. It may be different when more specimens are added from southern China, but so far, the southern distribution limit of this species is north of that of *G. subpectinata*.

Kawaguchi et al. (2001) suggested that *G. livida* is one of the few western Pacific *Grateloupia* species with a similar habit as *G. asiatica*. In this study, we collected four *G. livida* specimens from three different collection sites in Japan (Shimoda, Oshoro, and Nagasaki) (Supplementary Table S1), but *G. livida* has never been found in Korea. These four specimens from Japan formed a monophyletic group together with the previously published *G. livida* sequences from Weihai, China (marked with “8” in Fig. 4) and several regions on the Pacific side of Japan (marked with “1”, “6”, “7” in Fig. 4). Although *G. livida* has been reported by ecological studies in Korea (i.e., Lee and Kang 1986, Kim and Kim 2000), the correct species identification should be re-examined according to our collections.

G. serra and *G. variata* were monophyletic with all *G. asiatica* specimens (marked as “*G. asiatica* complex”) (Figs 2 & 4). *G. variata* was described based on the specimens collected from one site (four specimens; Luxun Park, Qingdao, China) on May 3, 2011 and *G. serra* has been described based on four specimens collected from the same site as *G. variata* on May 25, 2013 (Cao et al. 2016, Lou et al. 2019). We assigned these three species (*G. asiatica*, *G. serra*, and *G. variata*) to the “*G. asiatica* complex” because further analysis with more individuals is necessary.

Lou et al. (2019) suggested that the minimum interspecific divergence between *G. asiatica* and *G. serra* (3.72%) was evidence of a new species. However, they reported that the genetic difference between *G. asiatica* and *G. serra* was 15 bp, and the alignment comparison was 1,186 bp. The genetic divergence between *G. asiatica* and *G. serra* was 1.2–1.9% in our analyses (Supplementary Table S3). The highest p-distance value within the *G. asiatica* specimens (1.34% between MZ753751 and MZ 753752) was higher than the p-distance value between *G. asiatica* and *G. serra* (1.25% between AB055489 and KP195735). In other words, the lowest p-distance value between *G. asiatica* and *G. serra* was included in the intraspecific variation range of *G. asiatica*. The numerous proliferous

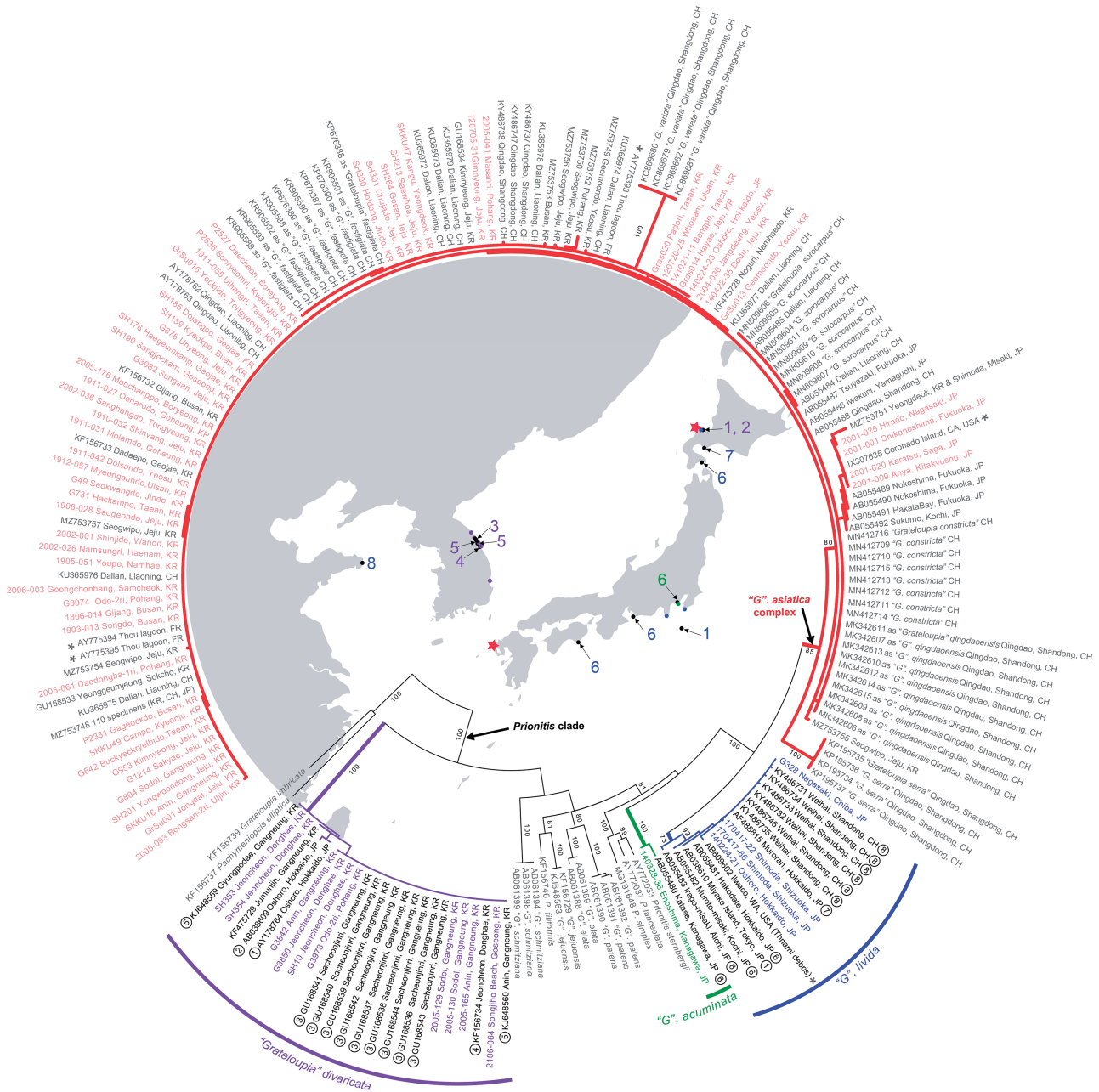


Fig. 4. Maximum likelihood phylogeny of the *Prionitis* clade with distribution map of *Grateloupia acuminata*, *G. divaricata*, and *G. livida* based on *rbcl* sequences. Pale red taxa indicate newly analyzed *G. asiatica* sequences. The southernmost and northernmost *G. asiatica* collection sites are marked with red stars due to too many collection sites. Asterisk (*) indicates the sequences known to be introduced outside of the Northwest Pacific. Purple, blue, and green dots indicate collection locations for new sequences of each species, and black dots indicate collection locations for published sequences with the following reference number: 1, Wang et al. (2000); 2, Mateo-Cid et al. (2005); 3, Lee et al. (2009); 4, Kim et al. (2013); 5, Yang and Kim (2015); 6, Kawaguchi et al. (2001); 7, Gavio and Frederiq (2002); 8, Peng et al. (2018).

branchlets on the surface of the main axes have been suggested as a key distinguishing character between *G. serra* and *G. asiatica* by Liu et al. (2019). Although Kawaguchi et al. (2001) reported “a few” proliferations on the surface of *G. asiatica*, several individuals (not common) had numerous proliferations on the surface of axes (Supplementary Fig. S1A–C).

The p-distance value between *G. asiatica* and *G. variata* was 2.3–3.2%, which is acceptable compared to other species of Halymeniales (Kim et al. 2014, Yang and Kim 2015). However, the *rbcl* sequence of this species was also monophyletic with all *G. asiatica* and *G. serra*

sequences (Figs 2 & 4). The characteristics that Cao et al. (2016) proposed to distinguish *G. variata* from *G. asiatica* (i.e., branching pattern and texture) appeared to fall within the range of intraspecific polymorphisms in *G. asiatica* (Fig. 1). For example, Kawaguchi et al. (2001) specified the texture of this species as mucilaginous, but we had specimens in our collection that were somewhat cartilaginous (Fig. 1I & K, Supplementary Fig. S1C). Therefore, characteristics, such as branching pattern and texture, cannot be used as key characters to distinguish *G. variata* from *G. asiatica*.

On the other hand, the sequences downloaded from GenBank under the name *G. sorocarpus* W. -X. Li & Z. -F. Ding (MN809604-11), and *G. constricta* W. -X. Li & Z. -F. Ding (MN412709-16) are same with *rbcL* sequences as *G. asiatica*, postulating on synonymous with *G. asiatica*. Since the possibility of simple misidentification for several species from China cannot be ruled out either so far, we look forward to further studies in China to shed light on this problem. In this study, therefore, the taxonomic treatment of synonyms is deferred to Chinese phycologists.

G. acuminata has been reported from Korea by several phycologists (Lee 1987, Kim and Park 2006, Nam and Kang 2013) and axial width has been used as a key character to distinguish *G. acuminata* from *G. asiatica* (less than 10 mm for *G. asiatica*, over 10 mm for *G. acuminata*) (Nam and Kang 2013). That key may have been based on the first description of *G. asiatica* (specified an axial width range as 2–5 mm for *G. asiatica*) (Kawaguchi et al. 2001). However, our exhaustive collection revealed several *G. asiatica* individuals with a wider axis (~20 mm) (Fig. 1K & L). In other words, regardless of whether the width of the axis is narrow (less than 10 mm) or wide (over 10 mm), they were monophyletic as in *G. asiatica* and distinguished from *G. acuminata*, which was collected from the type locality (140328-36 Enoshima, Kanagawa, Japan) (Figs 2 & 4). In addition, *G. acuminata* has a restricted distribution along the Pacific coast of central Japan and is not abundant (Kawaguchi 1991, Iima et al. 1995). Despite our efforts to find *G. acuminata* in Korea, it has never been observed. Therefore, the previous records of *G. acuminata* from Korea were actually the morphological variants of *G. asiatica* with a wide axis.

The typical gross morphology of *G. divaricata* was somewhat different from that of *G. subpectinata* and *G. asiatica*, but the morphological characteristics overlapped (Fig. 1O & P). This morphological ambiguity between *G. asiatica* and *G. divaricata* was also noted by Verlaque et al. (2005). *G. divaricata* has not been report-

ed since the first description, except some sequences as a part of another subject (i.e., Lee et al. 2009, Yang and Kim 2015). In this study, the occurrence of *G. divaricata* was confirmed, as a result of collecting and analyzing 10 specimens from the east coast of Korea (Supplementary Table S1). Interestingly, the distribution of this species was very limited to the northeast coast of Korea (only one exception is in Pohang) (Fig. 4). The collection sites of previously published sequences also limited this species to Gangneung, Sokcho (north-east coast of Korea), and Hokkaido, Japan (Fig. 4). Although we could not obtain any of the *rbcL* sequences or the actual study, this species was reported from Vladivostok, Russia (Guiry and Guiry 2022). This species is considered to be restricted to the East Sea surrounded by Korea, Japan, and Russia, while the others are broader in distribution (Figs 3–5). We suppose that *G. divaricata* may have adjusted to the cold water rather than warm water based on our collection and the published *rbcL* sequences (Fig. 4). Therefore, the reports of *G. divaricata* from Southeast Asia, such as the Philippines (Ang et al. 2014) and Vietnam (Nguyen et al. 2013), should be re-confirmed.

***Grateloupia sensu stricto* clade**

The *Grateloupia sensu stricto* clade was consistently monophyletic but the bootstrap values varied depending on the taxa included. This clade includes *Grateloupia catenata*, *G. ramosissima* Okamura, *G. tenuis* L. Yu, H. W. Wang & R. X. Luan, *G. orientalis* S. M. Lin & H. Y. Liang, *G. huanghaiensis* Wang, H. W., Guan, Y., Zhao, F. Q. & Zhao, D., *G. ramosa* H. W. Wang & R. X. Luan, *G. yinggehaiensis* H. W. Wang et R. X. Luan, *G. yangjiangensis* W. -X. Li & Z. -F. Ding, and many specimens formerly known as *G. filicina* from various regions (refer to De Clerck et al. 2005 for the detail).

The latitudinal distribution range of *G. catenata* was similar to that of *G. subpectinata* and wider (from Zhelang Island, Guangdong, China to Hakodate, Hokkaido, Japan) than that of *G. asiatica* (Figs 3 & 5). However, it was still restricted to Korea, Japan, and China except the introduced cases (Fig. 5). Chellamanimegalai et al. (2020) recently reported *G. catenata*, *G. filicina*, and *G. orientalis* from India. However, all of their sequences designated as *G. catenata*, *G. filicina*, *G. orientalis*, and *G. lithophila* (refer to the group named as “*Grateloupia* spp. from India” in Fig. 5) were monophyletic with AJ868485 (“*G. filicina*” from Papua New Guinea) in the *rbcL* tree (97% BTS) (Fig. 5) and distantly related from the original sequences of each species (*G. catenata*, *G. filicina*, and *G. orientalis*). As

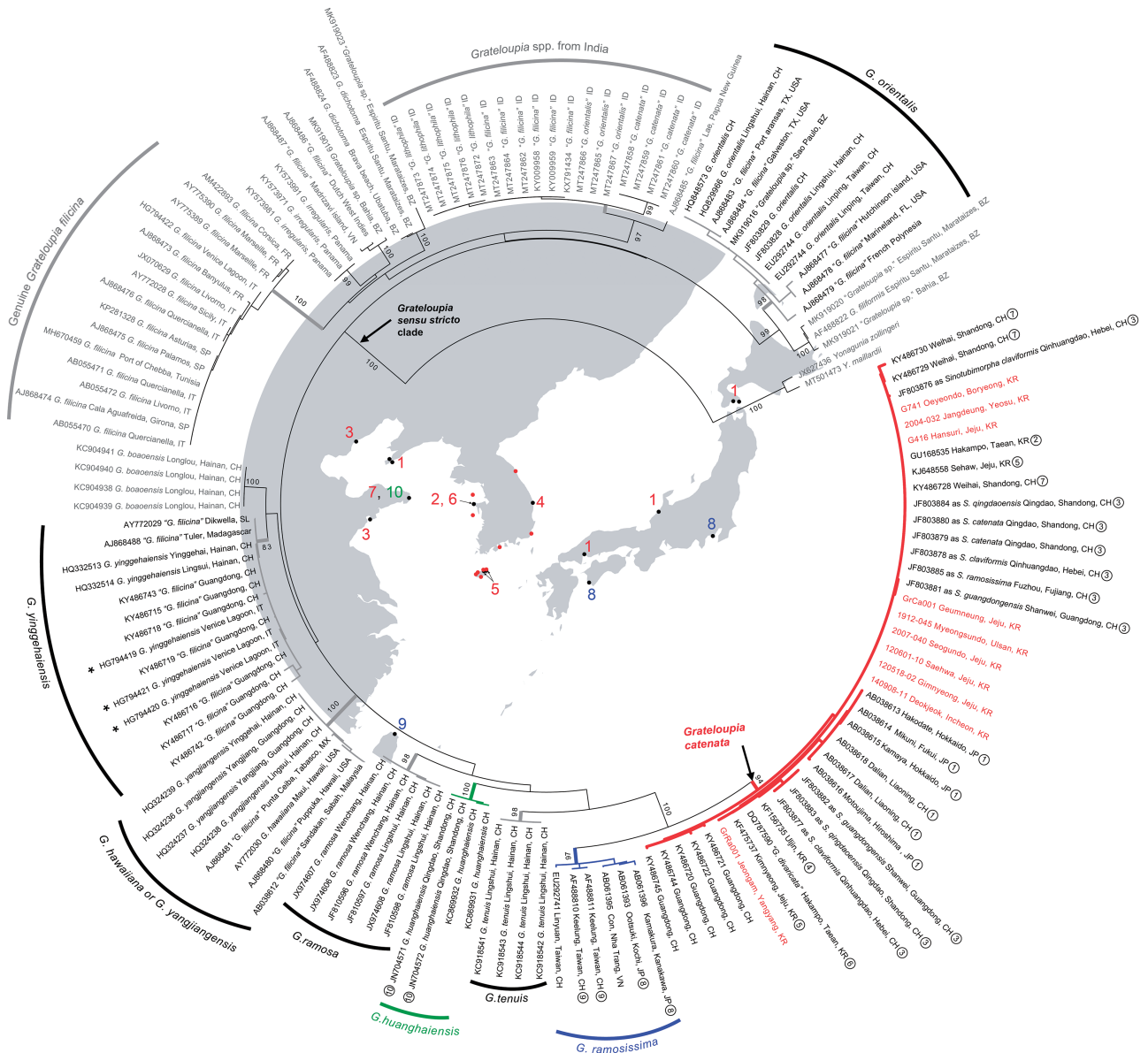


Fig. 5. Maximum likelihood phylogeny of the *Grateloupia sensu stricto* clade with a distribution map of *Grateloupia catenata*, *G. huanghaiensis*, and *G. ramosissima* based on *rbcL* sequences. Asterisk (*) indicates the sequences known to be introduced to outside of the Northwest Pacific. Red dots indicate collection locations of new sequences and black dots indicate collection locations of published sequences with the following reference number: 1, Wang et al. (2000); 2, Lee et al. (2009); 3, Sheng et al. (2012); 4, Kim et al. (2013); 5, Yang and Kim (2015); 6, Yang et al. (2016); 7, Peng et al. (2018); 8, Wang et al. (2001); 9, Gavio and Frederiq (2002); 10, Wang et al. (2015).

the type locality of *G. lithophila* is India and some of the specimens were identified as *G. lithophila*, their genuine identity is likely to be *G. lithophila*. Nevertheless, the new records of *G. catenata*, *G. filicina*, and *G. orientalis* from India should be rejected.

Grateloupia ramosissima is a sister species to *G. catenata* and also similar in morphology. *G. ramosissima* specimens was found in southern Japan (Pacific side), China (Taiwan), and Vietnam (located on the south side

of the map) based on the *rbcL* sequence (Fig. 5). In contrast to *G. divaricata*, this species has adapted to tropical rather than cold water. Therefore, the records of this species in Korea (Lee and Kang 1986) are considered as misidentifications of *G. catenata*.

Wang et al. (2014) described *Grateloupia huanghaiensis* sp. nov. as having a folios thallus after comparing this species to *G. filicina*, *G. yinggehaiensis*, and *G. orientalis*, which have *G. filicina*-like morphology. The phylogenetic

relationships of this species have not been resolved within this clade based on *rbcL* sequences (Figs 2 & 5). Given the genetic distance of this species from several species within this clade, it is an independent species (2–2.1% with *G. ramosa*, 2.6% with *G. tenuis*, 2.8–3.3% *G. orientalis*) (Supplementary Table S3). However, it is clear that the characteristics, such as proliferation on the thallus, body size, and leaf width proposed by Wang et al. (2014) to distinguish *G. huanghaiensis* from other *Grateloupia* species, cannot be used for a species delimitation key, as shown above. In addition, this species is also known only from Luxun Park (Qingdao, China), like *G. serra* and *variata*. Analyzing more specimens of this species and related species from various sites will improve our knowledge of the speciation and evolution of this clade.

The *Grateloupia sensu stricto* clade also includes five *G. filicina*-like species mainly distributed in southern China (*G. orientalis*, *G. ramosa*, *G. tenuis*, *G. yangjiangensis*, and *G. yinggehaiensis*; distribution sites were not shown in Fig. 5). *G. ramosa* and *G. tenuis* are only distributed on Hainan Island, China, whereas *G. yinggehaiensis* and *G. orientalis* are distributed not only in southern China but also geographically far away. *G. yinggehaiensis* has been described based on the samples collected from Yinggehai (Hainan, China) (Zhao et al. 2012). Subsequently, this species was confirmed from Guangdong Province, China by Peng et al. (2018) as “*G. filicina*”. In addition, two more sequences fell within the same species from Dikwella (Sri Lanka), Tuler (Madagascar) which was identified as *G. filicina* before (Fig. 5). Therefore, the distribution range of this species is from Madagascar in the south to Guangdong, China in the north. The distribution report of this species in Italy, has been known as introduced case by Wolf et al. (2014).

G. orientalis was described based on the samples collected from Taiwan with a comment that this species possesses *G. filicina*-like thalli (Lin et al. 2008). We considered that several specimens known as “*G. filicina*” from various regions (not only from China but United States, Brazil, and French Polynesia as well) (Fig. 5) are con-specific with *G. orientalis* as mentioned by Wolf et al. (2014). Considering the generally limited distribution of the *G. filicina*-like species in the Northwest Pacific (refer to Figs 3 & 4) and the many species introduction reports from the Northwest Pacific to worldwide (e.g., Verlaque et al. 2005, Bolton et al. 2016), it is highly probable that this species was also introduced to the USA and Brazil from the Northwest Pacific as a cryptic introduction.

Last, it is necessary to confirm whether *G. yangjiangensis* and *G. hawaiiiana* are conspecifics. *G. hawaiiiana*

was described as a folioid *Grateloupia* species from the Hawaiian Islands by Dawson in 1958. *G. yangjiangensis* was described as a new species with *G. filicina*-type morphology from China (Xia 2004). Subsequently, this species was re-examined by Wang et al. (2014) using the *rbcL* sequences of four specimens collected from Yangjiang (the type locality of *G. yangjiangensis*), and Hainan Island, China. The base difference between the two species was only 0.2% (3 bp out of 1,286 bp), which is reasonable to consider as an intraspecific not interspecific variation (Wang et al. 2001). However, there was no information or discussion about *G. hawaiiiana* or *G. yangjiangensis* in the study by Wang et al. (2014).

Numerous misidentifications of *Grateloupia filicina*-like species based on gross morphology

As shown by the many examples described above, many misidentifications have been made of *G. filicina*-like species in the Northwest Pacific. The most likely reason is a lack of understanding of the ranges in the gross morphological variations of each species. In particular, the overall range of gross morphological variations was much wider in *G. asiatica* than was known or expected (Fig. 1). Typical *G. asiatica* has flat, narrow axes, with finely pinnate lateral branches and a mucilaginous texture (Kawaguchi et al. 2001). However, we observed many gross morphological variations within this species, such as width of the axis, the branching pattern, proliferation, and texture (Fig. 1). The overall thallus shape of *G. asiatica* has numerous pinnate proliferations along the margin that taper upwards (Kawaguchi et al. 2001), as shown in Fig. 1H, but we found many individuals that may not be pinnate (Fig. 1F, J, L, M & N). The branching patterns are hard to define in one category. In addition, Kawaguchi et al. (2001) specified the texture of *G. asiatica* as mucilaginous, soft, and gelatinous, but we observed several individuals with a somewhat cartilaginous texture (Fig. 1I & K, Supplementary Fig. S1C). These high-level morphological variations in *G. asiatica* have been observed based on individuals introduced to France (Verlaque et al. 2005). Our study demonstrated that this high level of morphological variation is the nature of this species and could not be the result of inbreeding or interspecific hybridization in the introduced habitats as suggested by a previous study (Verlaque et al. 2005).

The gross morphological similarities between phylogenetically distant species have led to many misidentifications. The gross morphology of *G. subpectinata* (Fig. 1A–E) is similar to that of *G. asiatica* (Fig. 1F–N). Based

on our observations, we considered that distinguishing *G. subpectinata* from *G. asiatica* based solely on gross morphology is almost impossible in the field. Although Faye et al. (2004) suggested several morphological differences between *G. subpectinata* and *G. asiatica* (i.e., fleshy texture, wider axes, generally longer proliferations that are constricted at the base), they were difficult to apply.

Several issues with the *rbcl* sequences in some Grateloupiaceae species

We found sequences from several species (i.e., *Grateloupia dalianensis*, *G. huanghaiensis*, *G. ramosa*, *G. serra*, *G. tenuis*, *G. variata*, and *G. yinggehaiensis*) with some issues. First, the middle part of the *G. dalianensis* (HQ385503–385506) sequence encoded completely different amino acids compared to other red algae *rbcl* sequences extracted from published plastid genomes of various red algal lineages (Supplementary Fig. S2). Although Zhao et al. (2012) reported no insertions or deletions, there were two insertions at positions 661 (1 adenine-insertion) and 1,165 (2 adenine-insertion) of the *rbcl* gene. These two sites were polyadenine sites. When the additional adenines were deleted, assuming a sequencing error, the amino acid sequence changed to be similar to other halymenialean species sequences, but two stop codons appeared in the middle of the gene (refer to the sequence named as “HQ385504 “*Grateloupia*” *dalianensis* (corrected)” in the Supplementary Fig. S2). In addition, particularly high genetic divergence was detected between *G. dalianensis* and other the *Prionitis* species (7.3–10.4%) (Supplementary Table S3) and it appeared as a long branch in the ML tree compared to the other species (Fig. 2). Zhao et al. (2012) mentioned that there is a great difference in size between *G. dalianensis* and *G. asiatica*. They suggested the range of the length of the thallus was 14–24 cm for *G. asiatica* and 20–30 cm for *G. dalianensis*. However, the range was different in a table and the text (Zhao et al. 2012, Table 2: 7–75 cm for *G. asiatica* and 15–30 cm for *G. dalianensis*). Therefore, confirmation is required because the peculiar range of length (7–75 cm) was also assigned to *G. acuminata* and *G. livida*. However, whether it is 15–30 cm or 20–30 cm, the range of *G. dalianensis* length overlaps with that of *G. asiatica* (10–30 cm) (Kawaguchi et al. 2001), and is not a large difference. The thallus length of *G. asiatica* based on our collection (87 specimens) was 5–32 cm, which was almost consistent with the first description (10–30 cm) by Kawaguchi et al. (2001). No additional sequences have been published since the first description of *G. dalianen-*

sis using four specimens collected from Dalian, China. Therefore, confirmation is necessary to clarify the true status of this species.

Some peculiar nucleotide sequences were detected at the beginning and end of the several species (*Grateloupia huanghaiensis*, *G. ramosa*, *G. serra*, *G. tenuis*, *G. variata*, and *G. yinggehaiensis*) based on the amino acid comparison with various red algal lineages (Supplementary Fig. S3). As shown in Supplementary Figs S2 & S3, the *rbcl* gene is very conserved among all red algal lineages, even Cyanidiales, which are known as the most primitive red algal lineage (Yang et al. 2016). Therefore, the *rbcl* sequences of these species should be checked. As the peculiar nucleotide sequences were located only at the beginning and end of the sequences, it seems that they have not been sufficiently excluded to avoid ambiguous base pairs at both ends of the electropherograms. Fortunately, those sequences, even if excluded, would not affect their taxonomic status or phylogenetic position within the Grateloupiaceae. Nevertheless, these sequences should be verified again using additional samples because most of the species have been based on only a few specimens.

CONCLUSION

In this study, *G. filicina*-like species from the Northwest Pacific were comprehensively collected and analyzed based on *rbcl* sequences. *G. asiatica* was the most common species in the Northwest Pacific. We unexpectedly found various gross morphological variations causing many misidentifications with other species. In particular, *G. dalianensis*, *G. serra*, and *G. variata* should be reconsidered as conspecifics with *G. asiatica* using more specimens from China. By contrast, *G. oligoclora* should be considered a synonym of *G. subpectinata* based on our extended sampling. In addition, *G. constricta*, *G. sorocarpus*, *G. corymbcladia*, and *G. yangjiangensis* need to be confirmed. The distribution of *G. divaricata* and *G. ramosissima* were noteworthy. The former was restricted to the Donghae (East Sea) affected by the cold current, and the latter was found on the Pacific side of Japan, Taiwan, and Vietnam affected by the warm current. We believe that the occurrence of *G. ramosissima*, *G. acuminata*, and *G. livida* in Korea should be considered misidentifications with other similar species.

This study contributes to a better understanding of the identification and distribution of *G. filicina*-like species in the Northwest Pacific, which is likely a center of species diversity for Grateloupiaceae. Comparison of *rbcl*

sequences based on comprehensive sampling demonstrated that even species described by the *rbcL* sequences and / or morphology can still have nomenclature problems. Our study sends a message of warning on the blind faith for the names deposited in GenBank. In addition, a detailed investigation of the published *rbcL* sequences compared to other red algal lineages revealed issues with the sequences itself. Thus, a more thorough investigation of related species is essential when considering the identities of these species.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. Voucher number and collection information of newly analyzed sequences (<https://www.e-algae.org>).

Supplementary Table S2. Sequence information of downloaded from GenBank (<https://www.e-algae.org>).

Supplementary Table S3. Genetic distance of several species within each clade using *rbcL* gene (<https://www.e-algae.org>).

Supplementary Fig. S1. Specimens that have numerous surface proliferations on the thallus (<https://www.e-algae.org>).

Supplementary Fig. S2. Amino acid sequence alignment of *rbcL* gene. *Grateloupia dalianensis* show totally different amino acid sequence compared to representatives of all red algal orders (<https://www.e-algae.org>).

Supplementary Fig. S3. Amino acid sequence alignment of *rbcL* gene. Several species show peculiar amino acid compared to representatives of all red algal orders at the beginning and end of the sequence (<https://www.e-algae.org>).

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