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Deep genetic divergences and geographic distribution of the red algal genus *Caulacanthus* (Gigartinales)

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An increasing number of studies have demonstrated that genetic differentiation and cryptic diversity in the sea occur over considerably smaller spatial scales than previously comprehended, considering the wide distribution range of many morphologically defined macroalgal species. However, knowledge of the turf-forming red alga *Caulacanthus* is incomplete regarding its species diversity, as well as genetic differentiation within the genus. We analyzed *Caulacanthus* specimens from the NW Pacific, NE Pacific, Central Pacific, SW Pacific, SE Indian, NE Atlantic, and SE Atlantic Ocean using mitochondrial cytochrome oxidase subunit I (COI-5P), plastid ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L), and Rubisco spacer (*rbc*L-S). The objectives of this study were to 1) determine the number of species that exhibit the morphology of *C. ustulatus*, 2) investigate the present distribution pattern of *Caulacanthus* species, and 3) estimate the degree of genetic connectivity between the populations of *Caulacanthus* species from different regions. Our results revealed molecular evidence that the genus *Caulacanthus* comprises of at least seven species with deep genetic divergence, which is indicative of not only a strong geographical subdivision but also a relatively long temporal discontinuity. Most species exhibited limited geographic distribution, showing considerable genetic divergence in the populations isolated by distance. Our study provides evidence of a greater evolutionary independence of *Caulacanthus* populations, which have undergone a series of allopatric diversification events.

KEYWORDS

***Caulacanthus*, distribution pattern, haplotype diversity, macroalgae, species delimitation**

Introduction

The existence of widespread or cosmopolitan species has been widely accepted in marine organisms because of their high dispersal potential and the continuity of the marine environment (Avis, 1998). However, recent studies using molecular data have revealed that many supposedly widespread macroalgal species consist of cryptic species complexes (Díaz-Tapia et al., 2018; Nauer et al., 2019; Vieira et al., 2019), raising questions concerning the real distribution of such cosmopolitan species. Widespread species have frequently been found to be highly genetically differentiated across regional boundaries (Díaz-Tapia et al., 2018; Yang et al., 2020) and sometimes exhibit fine-scale endemism (Leliaert et al., 2018; Yang and Kim, 2018). Generally, regional endemism is considered to have evolved in

regions that have experienced a stable environment over a long period (Myers and Giller, 1988).

With the discovery of cryptic speciation, molecular analyses have been developed to identify and delimit species (Díaz-Tapia et al., 2020; Lagourgue et al., 2022). Among the widespread macroalgal species, molecular data have clarified the real distribution of traditionally identified species solely based on morphological characteristics (Yang et al., 2015; Boo et al., 2019; Kang et al., 2021), with deep genetic divergence seen between regional populations owing to their limited natural dispersal ability (Nauer et al., 2019; Yang et al., 2021b). Other widely distributed species showed genetic affinities among populations despite separation by large oceanic distances, suggesting other dispersal mechanisms for the present distribution pattern (Fraser et al., 2013; Sherwood et al., 2014). Such long dispersal mechanisms can be explained by the artificial distribution of non-buoyant macroalgae (Fraser et al., 2013). Although the hidden algal diversity is increasingly being discovered, our knowledge on the global diversity and distribution of red macroalgae is still incomplete.

The turf-forming red algal genus, *Caulacanthus* Kützing, is a component of intertidal ecosystems and is commonly found in temperate and tropical marine waters (Guiry and Guiry, 2023). The genus *Caulacanthus* includes three currently accepted species, *Caulacanthus okamurae* Yamada, *Caulacanthus salifugus* A. B. Cribb, and *Caulacanthus ustulatus* (Turner) Kützing (Guiry and Guiry, 2023). The type species of the genus, *C. ustulatus*, was described from Cádiz, Spain, and subsequently reported in various regions, including the Western Pacific Ocean, western North America, Mediterranean Sea, Africa, Australia, and New Zealand (Guiry and Guiry, 2023). Whether the actual distribution of this species belong to the widespread species, *Caulacanthus* was investigated by Zuccarello et al. (2002), who conducted molecular analyses using Rubisco and *cox*2-3 spacers to confirm the phylogenetic relationships between collections from the Northwest (NW) Pacific (including Korea and China), tropical Western (W) Pacific (including the Philippines), Southwest (SW) Pacific (including Australia), Northeast (NE) Pacific (including the USA), and the NE Atlantic (including France, Spain, and Portugal). They found that *C. ustulatus* included two distinct phylogenetic groups (Pacific and Atlantic) and concluded that the Pacific lineage indicated *C. okamurae*, which has long been regarded as a synonym of *C. ustulatus* (Searles, 1968; West and Calumpong, 1990; Rueness, 1997). *C. okamurae* was described based on the morphological features of vegetative plants (Yamada, 1933). Since then, taxonomic position of these species has been under debate (Searles, 1968; West and Calumpong, 1990); however, the distinctness of *C. okamurae* has been accepted based on different cystocarpic development and gemination patterns of tetraspores (Kamura, 1963), as well as molecular data (Rueness and Rueness 2000; Zuccarello et al., 2002). This suggests the need to confirm the actual distribution range of *C. ustulatus* in different regions, comparing with specimens from type locality through molecular analyses.

C. okamurae is native to the NW Pacific, including Japan, Korea, China, and Taiwan, and has recently been reported in many regions, including France (Zuccarello et al., 2002; Burel et al., 2019), Washington (Zuccarello et al., 2002), California (Miller, 2012; Hartnell College Genomics Group et al., 2020), Gulf of California (Norris et al., 2017), Italy (Petrocelli et al., 2020), and Spain (Bárbara et al., 2019). Recently, after molecular examinations, the name of *C. ustulatus* has been changed to *C. okamurae* in several region (Zuccarello et al., 2002; Petrocelli et al., 2020). *C. okamurae* forms a dense turf on substrates, thereby providing shelter for other macroalgae and marine invertebrates (Petrocelli et al., 2020). Another taxonomically accepted species, *C. salifugus*, was described based on sterile material growing in a semi-marine cavern in Queensland, Australia (Cribb, 1965), and has not been recorded since then. Other species, such as *C. spinellus* (Hooker f. Harvey) Kützing from New Zealand, *C. indicus* Weber-van Bosse from Indonesia, and *C. rigidus* Kützing from Senegal, were previously accepted within the genus. However, they have been regarded as synonyms of *C. ustulatus* because of their similar morphologies (Searles, 1968; West and Calumpong, 1990; Adams, 1994).

Mitochondrial DNA sequences generally constitute a powerful tool for species delimitation, particularly in red algal groups that are difficult to resolve using morphological data (Yang et al., 2021a; Muangmai et al., 2022; Preuss et al., 2022). The advent of species delimitation methods based on DNA sequences has provided a useful method for validating previously described species and identifying new evolutionary units (Muangmai et al., 2022; Boo et al., 2022). The present study involves *Caulacanthus* samples from 14 countries and addresses species diversity within the genus. We collected the specimens from Cádiz in Spain, which is type locality of *C. ustulatus*, to determine the regional distribution to which *C. ustulatus* was distributed.

In this study, using mitochondrial COI-5P and *rbc*L data with a previously determined *rbc*L-S spacer, we addressed several questions concerning taxa regarded as being *C. ustulatus* and *C. okamurae*. Specifically, we aimed to 1) determine the number of species that exhibit the morphology of *C. ustulatus*, 2) investigate the present distribution pattern of the genus *Caulacanthus*, and 3) estimate the degree of genetic connectivity between the populations of *Caulacanthus* species from different regions.

Materials and methods

Sample collections

Samples identified as *Caulacanthus* based on morphological criteria were collected during low tide from Korea, Japan, China, Taiwan, New Zealand, and Spain. The COI-5P sequences from Canada and Australia were provided by Prof. Gary W. Saunders of the University of New Brunswick. Additionally, we obtained sequences from France, the USA, the Philippines, Italy, Spain, Portugal, and Namibia from the GenBank to construct the phylogenetic trees for mitochondrial COI-5P and plastid *rbc*L and *rbc*L-S spacer dataset. Details of the specimens and sampling details are listed in Table S1. For molecular analyses, specimens were air-dried and preserved in silica gel, and epibionts were then carefully removed under a dissecting microscope in a laboratory prior to DNA analysis. For anatomical observations, specimens were preserved in 5% formalin, and voucher specimens were deposited in the herbarium of Jeju National University (JNUB), Korea.

Morphological observations

The generitype, *C. ustulatus*, from type locality was used to analyze the detailed morphology. Specimens were sectioned using a freezing microtome (MFS no 222; Nipon Optical Works, Tokyo, Japan). Sections were stained with 1% aniline blue acidified with 1% HCl and mounted in 35% corn syrup solution. Photomicrographs were taken using a BX43 microscope (Olympus, Tokyo, Japan) using an EOS 600D digital camera (Canon, Tokyo, Japan). Digitized images were edited to produce a plate using Adobe Photoshop software v. 6.1 (Adobe System Inc., San Jose, CA, USA).

DNA extraction, amplification, sequencing, and phylogenetic analyses

Apical regions were separated from the thallus and extracted using the LaboPass Tissue Genomic DNA Isolation Kit (Cosmogenetech, Seoul, Korea), according to the manufacturer's instructions. The extracted DNA was stored at -20°C and underwent polymerases chain reaction (PCR) amplification using AccuPower PCR Premix (Bioneer, Daejeon, Korea) in final volume of 20 µL. Targeted sequences of COI-5P and *rbc*L gene were amplified and sequenced using the primers GazF2 and GazR1 for COI-5P (Saunders, 2005; Lane et al., 2007), and F7–R898 and F762–R1442 for *rbc*L (Gavio and Fredericq, 2002; Kim et al., 2010). The PCR conditions were as described in Yang et al. (2021a). The PCR products were visually checked on a 1% agarose gel, purified using ExoSAP-IT (USB, Cleveland, Ohio, USA), and then commercially sequenced (Macrogen Inc., Seoul, Korea).

All successful amplifications were sequenced in both directions. Assembly and manual editing were conducted using Geneious v.9 (http://www.geneious.com). All edited sequences were combined with the available GenBank sequences. Multiple sequence alignment was performed using the MUSCLE algorithm in Geneious v.9. *Catenella caespitosa* was used as an outgroup.

Phylogenetic trees of the COI-5P gene were constructed using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were performed using RAxML with 1,000 bootstrap replicates, and BI was performed with MrBayes (v.3.2.1) (Ronquist et al., 2012) using the Metropolis-coupled Markov Chain Monte Carlo (MCMC) with the models. Four million generations of two independent runs were performed with four chains and sampling trees for every 100 generations. The burn-in period was graphically identified by tracking the likelihoods in each generation to determine whether whey reached a plateau. Further, 25% of the saved trees was removed, and the remaining trees were used to calculate the Bayesian posterior probabilities (BPPs). For comparison with sequences from GenBank, we generated the combined phylogenetic tree using the sequences of *rbc*L and *rbc*L-S spacer including the representative samples of each lineage on the COI-5P based phylogeny.

Genetic diversity, population structure, and species delimitation analysis

To estimate the genetic diversity of *Caulacanthus* species, we calculated the number of polymorphic sites (*S*), number of haplotypes (*H*), haplotype diversity (*Hd*) and nucleotide diversity (π) for COI-5P data using Arlequin v.3.5 (Excoffier and Lischer, 2010). Haplotype networks were built using the TCS method (Clement et al., 2000) in Arlequin. Genetic distances based on COI-5P were estimated using MEGA X v. 10.2.6 (Kumar et al., 2018). Pairwise fixation indices (F_{ST}) were calculated using Arlequin, to measure genetic differentiation among biogeographic regions within the lineage to assign the species as *C. okamurae*.

Species delimitation in the genus *Caulacanthus* was studied *via* the single and multiple-threshold generalized mixed Yule coalescent methods (GMYC; Pons et al., 2006; Fujisawa and Barraclough, 2013), automatic barcode gap discovery analysis (ABGD; Puillandre et al., 2012), and a Bayesian implementation of the Poisson tree processes (bPTP; Zhang et al., 2013). The GMYC method requires an ultrametric tree, which was obtained by Bayesian analyses in BEAST v.1.10.4 (Suchard et al., 2018), with divergence times estimated under an uncorrelated lognormal relaxed molecular clock model (Drummond et al., 2012) and the Yule-process as the prior tree. The Bayesian Markov chain Monte Carlo (MCMC) was run for 20 million generations, with trees and parameters were sampled every 100 generations. The output was checked for convergence using Tracer v.1.7.1 (Rambaut et al., 2018). After removing 25% of the trees as burn-in, the remaining trees were used to generate a single summarized tree in TreeAnnotator v.1.10.4 (part of the BEAST v.1.10.4 package) as an input file for GMYC analyses. GMYC analyses with a single threshold model were performed in R software (R Development Core Team, http://www.R-project.org) under the "splits" package using the "gmyc" function (R-Forge, http://r-forge.r-project.org/projects/splits/). The ABGD method was conducted *via* a webserver (ABGD web, https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) under the default parameters, except for the related gap width (X) of 1.3, using the Kimura-2-parameter (K2P) distance matrix as the input file, generated in MEGA7. The bPTP analysis was performed using a bifurcated phylogenetic input tree, as implemented in the bPTP web server (https://species.h-its.org). The parameters were set as follows: 500,000 MCMC generations, thinning by a factor of 100, and a 10% burn-in.

Results

Molecular analyses

The analysis of the mitochondrial COI-5P gene (552 bp) across 94 *Caulacanthus* sequences revealed 171 polymorphic sites, of which 139 were parsimony-informative sites. In total, 26 haplotypes were identified, of which 14 were by at least two samples and 12 were singletons. The average nucleotide compositions were as follows: A, 27.2%; C, 15.4%; G, 25.2%; and T, 32.2%. The total genetic diversity within a genus was 0.8518 ± 0.0296 for haplotype diversity and 0.05962 ± 0.0290 for nucleotide diversity.

Both ML and BI analyses for COI-5P data produced an identical phylogenetic topology (Figure 1) that was divided into six distinct lineages (Lineages 1-6), which were strongly supported. Three different species delimitation methods suggested the existence of multiple cryptic *Caulacanthus* species, and these results were highly congruent with phylogenetic analyses (Figure 1). Both the GMYC and ABGD

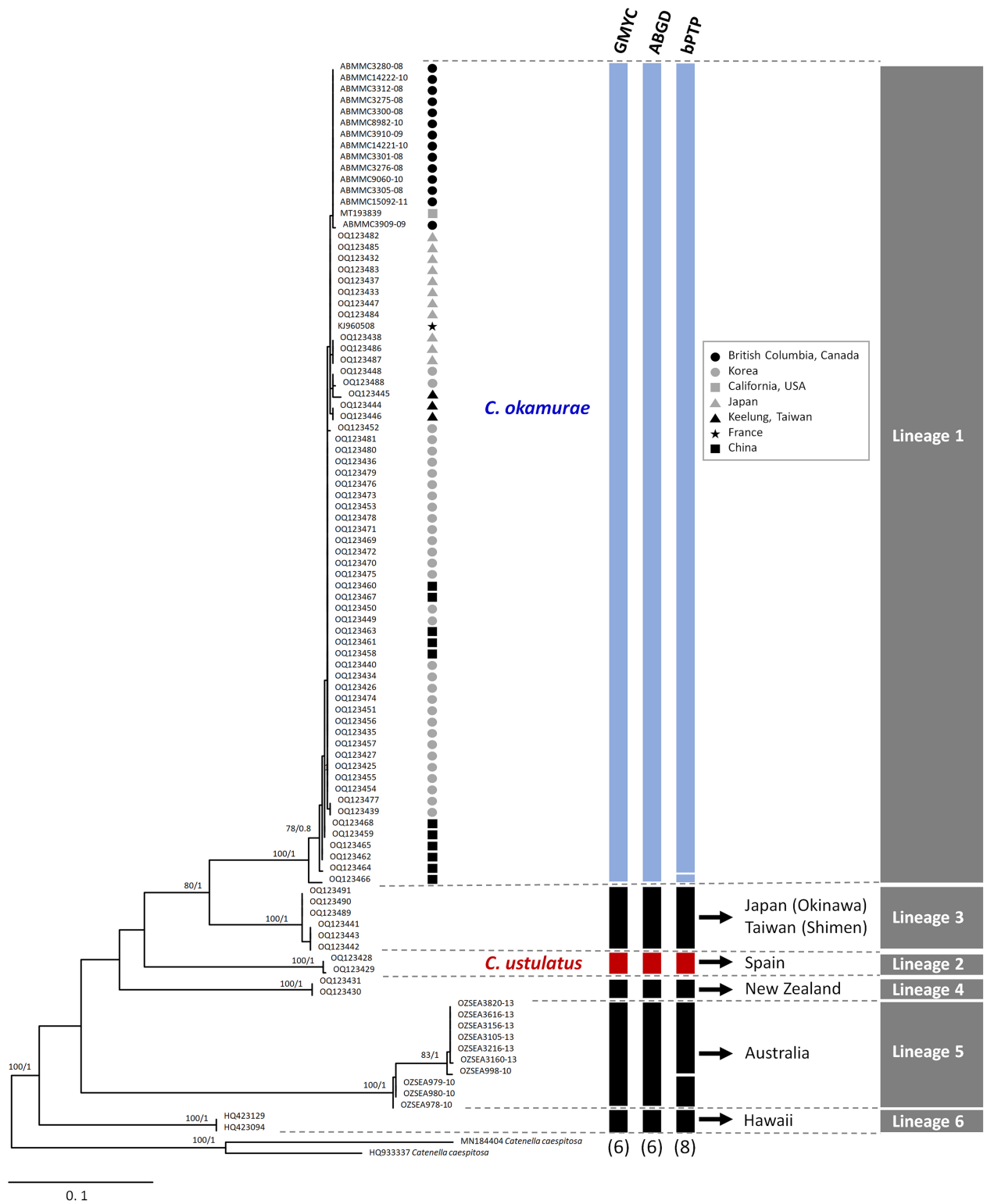

FIGURE 1
Maximum likelihood phylogeny of the genus *Caulacanthus* based on the COI-5P gene. ML bootstrap values (left) and Bayesian posterior probabilities (right) are indicated at the nodes. Values of <50% ML bootstrap and <0.8 posterior probabilities are not shown. The results of three species delimitation methods (GMYC, ABGD, and bPTP) for COI-5P gene are shown in the columns at the edge of the tree, with the number of putative species depicted at the bottom. Blue and red columns indicate *Caulacanthus okamurae* and *C. ustulatus*, respectively. Black columns indicate other lineages of *Caulacanthus*. Gray columns on right edge summarize the results for the tested species delimitation methods.

models defined six operational taxonomic units (OTUs), whereas bPTP estimated eight OTUs within the genus *Caulacanthus* (Figure 1). We recognized *C. okamurae* for Lineage 1 according to the phylogenetic lineage, which included samples from its native region, Japan, Korea, and China. The name *C. ustulatus* was assigned to the Lineage 2 based on the inclusion of the specimens from Cádiz in Spain, which is the type locality.

The molecular phylogenetic analysis using the *rbc*L and *rbc*L-S spacer included overlapping portion (60–82 bp) for 24 *Caulacanthus* sequences, and excluded the Lineages 5–6, which were with only COI-5P sequences. The plastid phylogenetic tree was resolved into five lineages (Figure 2), four of which corresponded with the previously determined lineages in the study using *rbc*L-S spacer (Zuccarello et al., 2002). *C. okamurae* (Lineage 1) included sequences from the NW Pacific (Korea, Japan, China, and Taiwan), NE Pacific (the USA) and NE Atlantic (France and Spain) oceans, and Mediterranean Sea (Italy). The sequences from Cádiz (OQ136657 and OQ136658) generated in this study, referred as *C. ustulatus* (Lineage 2), were grouped with sequences from Portugal, Spain, and Namibia. Additionally, we unexpectedly found that Lineage 3 matched the two sequences (AF453721 and AF453720) from Queensland, Australia. Lineage 4 was placed at the most basal position in this phylogenetic tree. The independent Lineage 7 from the Philippines was a sister group to the Lineage 1, *C. okamurae*.

The TCS haplotype network analysis based on the COI-5P data (Figure 3A) revealed six major groups corresponding to the six COI-5P based phylogenetic lineages and the GMYC/ABGD based OTUs. Each lineage was separated from the others by 52–86 missing steps. Among them, *C. okamurae* (Lineage 1) showed the highest diversity (accounting for 78% of the total sample diversity), and relatively complex haplotype network with 15 haplotypes. Within *C. okamurae*, the most abundant haplotype H04 and its connected haplotypes, H05–H08, occurred in Korea and China. Haplotypes H01–H02 were only observed in the populations of the NE Pacific Ocean (British Columbia and California; Table S1). The central haplotype H03 and H13 comprised specimens from Japan and France. The singletons of H10–H11 and H14–H15 occurred in Korea and China, respectively, whereas H09 and H12 were observed only from Taiwan. *C. ustulatus* (Lineage 2) had two haplotypes from Cádiz and was connected to Lineage 3, which comprised two haplotypes that represented Japan (Okinawa) and Taiwan (Shimen) by 71 mutational steps. The other three lineages were confined to a single country, such as New Zealand (Lineage 4; one haplotype), Australia (Lineage 5; five haplotypes), and Hawaii (Lineage 6; one haplotype). A clear gap between the intra-lineage and inter-lineage *p*-distances was detected for the COI-5P gene (Figure 3B). The minimum inter-lineage divergence of 9.4% (between Lineages 1 and 3) was considerably higher than the maximum intra-lineage divergence of 3.62% (Lineage 5; Table 1), indicating that each lineage was genetically distinct.

Pairwise F_{ST} values were calculated only for *C. okamurae* based on the COI-5P data (Table 2), as the other lineages had only up to ten samples collected from one or two locations. For *C. okamurae*, we divided into the following five populations, excluding the French sample, which contained only one specimen; (1) Pop1 (n = 16) included W Korea and Dailan, (2) Pop2 (n = 20) from southern (S) Korea and eastern (E) Korea, (3) Pop3 (n = 8) from the coasts from Nanji Island and Taiwan, (4) Pop4 (n = 11) from the coast of mainland Japan, and (5) Pop5 (n = 15) from the NE Pacific coast. Among the five populations, low F_{ST} value (F_{ST} range = 0.0349) was observed within the populations from Korea and Dailan (Pop1/Pop2) populations. The mainland Japan population (Pop4) showed genetic affinity (F_{ST} range = 0.1412) to those along the East China Sea (Pop3) rather than to the populations of Korea and Dailan (Pop1/Pop2; F_{ST} range = 0.6140–0.6887). Moderate to high F_{ST} values (F_{ST} range = 0.4655–0.8646) were detected between populations from the NE Pacific Ocean (Pop5) and others (Pop1/Pop2/Pop3/Pop4).

In the global distribution of the genus *Caulacanthus* based on the combined result of mitochondrial and plastid data (Figure 4), *C. okamurae* was most widespread species, found in the NW Pacific (Korea, Japan, China, and Taiwan), NE Pacific (Canada and the USA), and NE Atlantic (Spain and France) oceans and Mediterranean Sea (Italy). In contrast to this result, *C. ustulatus* was distributed only in the NE Atlantic Ocean (Spain and Portugal) and Namibia. In the NW Pacific, two *Caulacanthus* species (*C. okamurae* and Lineage 3) co-occurred with partial geographic division, indicating that the Lineage 3 was distributed from subtropical to tropical region, including Okinawa, Keelung, Cooktown, and Magnetic Island (Figure 4). Another Lineages 4–7 were found only in a single region, whereas Lineage 5 occurred in the SE Indian and SW Pacific oceans. On examining the detailed distribution of *C. okamurae* within the NW Pacific Ocean based on the COI-5P haplotype (Figure 5), the haplotypes from mainland Japan were distinct to those from Korea/China; however, a part of haplotype from Nanji Island were connected to those from Korea.

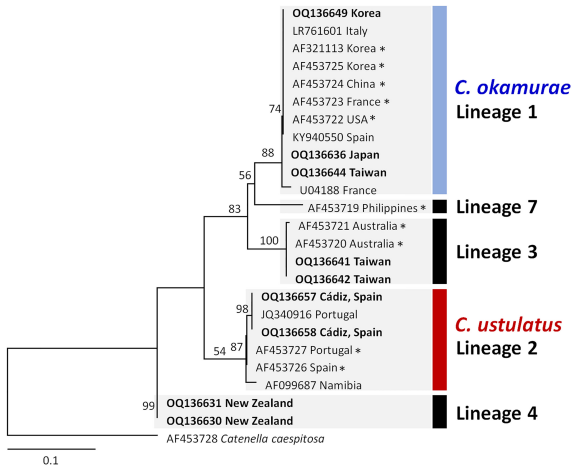

FIGURE 2
Maximum likelihood phylogeny of the genus *Caulacanthus* based on the combined sequences of *rbc*L and *rbc*L-S spacer. ML bootstrap values are indicated at the nodes. Numbering of lineages was applied as in Figure 1. Asterisk (*) behind the sequence name indicates the *rbc*L-S sequences downloaded from the GenBank. Bold type indicates the sequences generated in this study.

Morphological observations

The detailed morphology of specimens collected from Cádiz, where is the type locality of the generitype, *C. ustulatus*, was

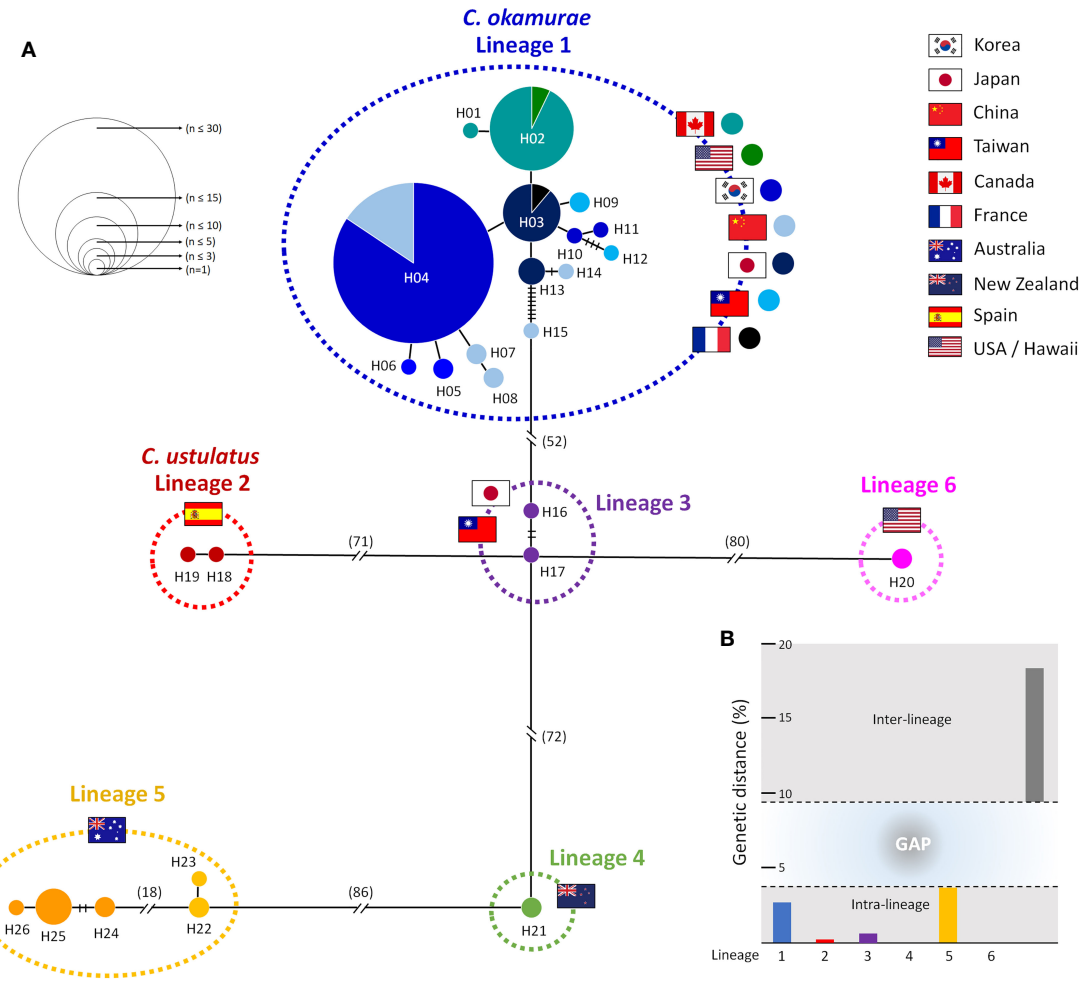

FIGURE 3
Haplotype network and genetic distance for six lineages of *Caulacanthus* using the COI-5P sequences. In the network **(A)**, each circle denotes a single haplotype, and each connecting line indicates one mutation step between haplotypes. The size of the circles is proportional to haplotype frequency. The pie chart colors within Lineage 1 correspond to the matching colors next to the flag. The cross bars represent the number of mutational steps between two haplotypes when the mutational steps are >1. Flags indicate the country of origin of specimens for each lineage. Minimum and maximum inter-lineage (gray), and intra-lineage (colors refer to lineages defined in the haplotype network) genetic distances (*p*-distance) in percent are shown in **(B)**.

observed. Thalli of *C. ustulatus* were erect or creeping, entangled, cartilaginous, 10–35 mm in height, and dark brown to dark red (Figures 6A–C). Branches were irregular to sub-dichotomous with short branchlets, divided at right angles or slightly curved toward the apex, and tapering to points (Figures 6B, C). Small projections were attached to the substratum or adjacent branches (Figure 6C). On a surface view, cortical cells of a thallus exhibited various from circular to polygonal (Figure 6D). The thallus was uniaxial (Figure 6E) with siphonous axis articulated and divided dichotomously toward the thallus surface (Figure 6F). The main axes were cylindrical and 350–390 µm in diameter (Figure 6G). In the cross-sectional view, the central core of axial filament was surrounded by 3–4 large and polygonal cells with 50–85 µm in diameter (Figures 6G, H). The cortex comprised mainly two layered ovoid cells (Figure 6H).

TABLE 1 Intra- (gray) and inter-lineage *p*-distances of *Caulacanthus* calculated using the COI-5P gene.

	Lineage 1	Lineage 2	Lineage 3	Lineage 4	Lineage 5	Lineage 6
Lineage 1	2.53%					
Lineage 2	13.4–14.3%	0.18%				
Lineage 3	9.4–10.7%	12.9–15.6%	0.54%			
Lineage 4	14.1–15.4%	14.8%	13.0–13.6%	0%		
Lineage 5	16.5–17.6%	16.3–17.0%	15.9–16.1%	15.6–16.7%	3.62%	
Lineage 6	14.7–15.4%	15.0–15.2%	14.5%	17.3%	17.4–18.3%	0%

TABLE 2 Pairwise estimates of F_{ST} between five groups in *C. okamurae* based on COI-5P data.

	Pop1	Pop2	Pop3	Pop4	Pop5
Pop1 (n = 16)					
Pop2 (n = 20)	0.0349				
Pop3 (n = 8)	0.2594*	0.3004*			
Pop4 (n = 11)	0.6887*	0.6140*	0.1412*		
Pop5 (n = 15)	0.8646*	0.8131*	0.4655*	0.8030*	

P values were obtained after 10,000 replicates. Populations: (1) W Korea/Dailan (Pop1), (2) S Korea/E Korea (Pop2), (3) Nanji Island/Taiwan (Pop3), Japan (Pop4), and British Columbia/California (Pop5). *P < 0.01.

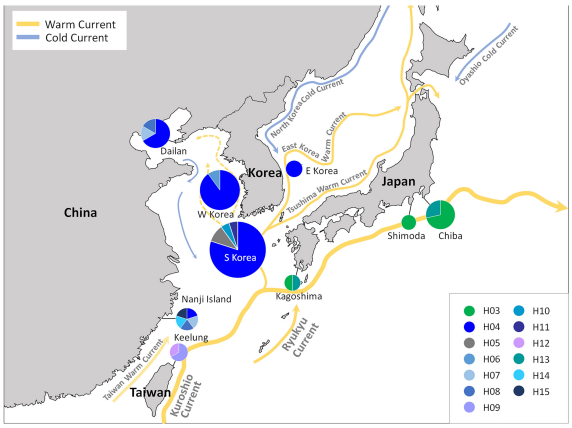

FIGURE 5
COI-5P haplotype distribution of *Caulacanthus* distributed in the NW Pacific Ocean. *C. okamurae* occurs in Korea, Japan, China, and Taiwan, and Lineage 3 is distributed only in Shimen and Okinawa in this region. Each pie chart represents the proportion of the haplotype presented within the site.

Tetrasporangia formed in the basal to mid part of the branchlets (Figure 6I). Mature zonately divided tetrasporangia were 22–28 µm in diameter and 48–56 µm in length (Figure 6J).

Discussion

We suggest at least seven OTUs within the genus *Caulacanthus*, through a combination of phylogenetic analyses using the mitochondrial and plastid sequence data, molecular species delimitation methods, and haplotype network, although bPTP resolved two additional OTUs within the Lineages 1 and 6 (Figures 1, 2). The finding of cryptic species diversity was similar to that for other red algal taxa, particularly turf algae, which has typically been overlooked because of their small size, structural simplicity, and densely entangled nature (Díaz-Tapia et al., 2020). Together with a detailed morphological description, our study provides molecular evidence that the generitype *C. ustulatus* is not a species with a widespread distribution but rather confined to the E Atlantic Ocean (Figures 5, 6). Another previously recognized species, *C. okamurae*, was found in the Northern Hemisphere, including nine countries. This finding was consistent in terms of previous studies that reported that *C. okamurae* was introduced from several countries (Petrocelli et al., 2020). The other five lineages were distributed in Japan/Taiwan/Australia (Lineage 3), New Zealand (Lineage 4), Australia (Lineage 5), Hawaii (Lineage 6), and the Philippines (Lineage 7) (Figure 4).

The COI-5P haplotype network of *Caulacanthus* clearly showed deep genetic divergence with considerable mutational steps between the lineages (Figure 3). The pairwise level of sequence divergence reported herein provided a clear gap between maximum intra-lineage

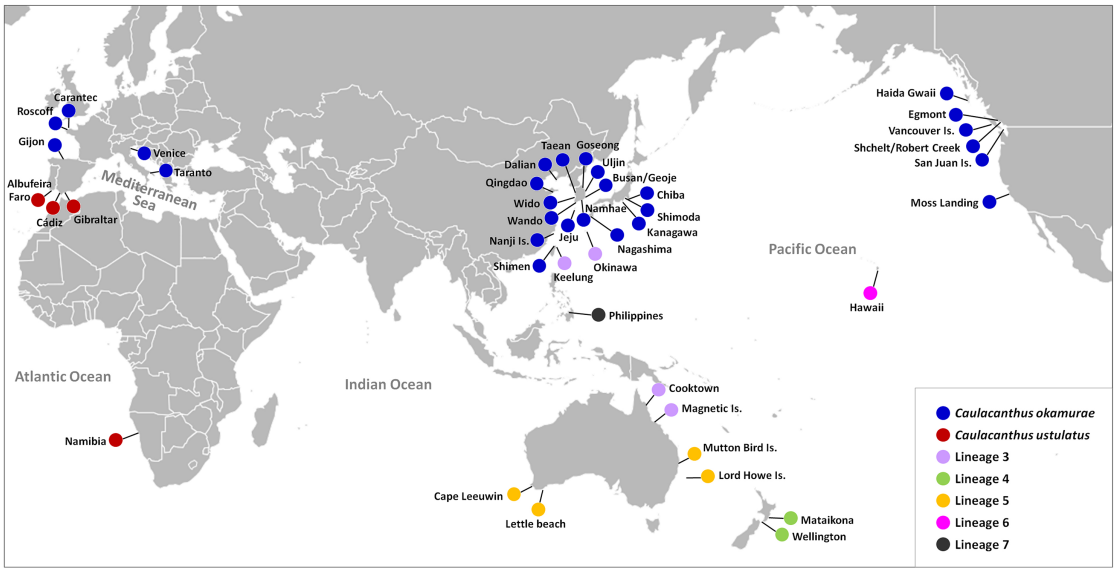

FIGURE 4
Map showing updated global distribution of seven *Caulacanthus* lineages in the world according to the combined result of mitochondrial and plastid sequence data.

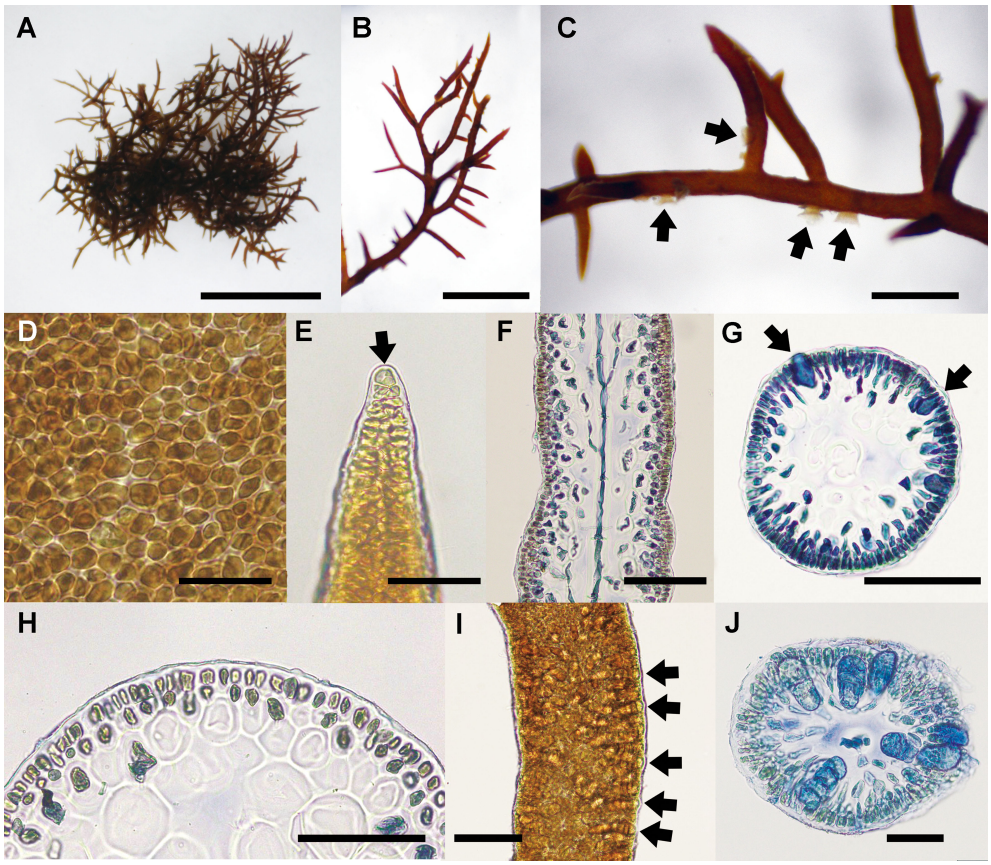

FIGURE 6
Caulacanthus ustulatus from Cádiz, Spain. **(A)** Habit of thallus collected at San Sebastian, Cádiz, on April 28, 2014. Scale bar = 300 µm. **(B)** Apical part of branches with numerous short branchlets. Scale bar = 200 µm. **(C)** Several projections (arrows) on a branch. Scale bar = 100 µm. **(D)** Surface view of a thallus showing circular to polygonal cortical cells. Scale bar = 30 µm. **(E)** Apical cell (arrow) of a thallus tip. Scale bar = 50 µm. **(F)** Longitudinal section of a thallus median part showing an axial filament. Scale bar = 100 µm. **(G)** Cross section of distal portion of tetrasporophytic thallus showing immature tetrasporangia (arrows). Scale bar = 100 µm. **(H)** Layer of ovoid cortical cells. Scale bar = 30 µm. **(I)** Tetrasporangia of a branchlet. Scale bar = 50 µm. **(J)** Zonately divided tetrasporangia (arrows) in the cortex. Scale bar = 50 µm.

divergence of 3.62% and minimum inter-lineage divergence of 9.4%, which provided the evidence for an independent genetic lineage. The high level of divergence within the *Caulacanthus* complex is indicative of not only a strong geographical subdivision but also a long temporal discontinuity (Fraser et al., 2010). Deep genetic divergences detected among the highly divergent lineage requires taxonomic validation and formal description through sufficient observations of regional specimens. In the case of Australia and New Zealand, some species names have previously been misattributed owing to morphological similarity within the region (Cribb, 1965; Searles, 1968; Rueness, 1997). *C. salifugus* was first reported in Paradise Cave in the SE Queensland (Cribb, 1965), in the middle of distribution between the Lineages 3 and 5, therefore, molecular evidence is acquired to confirm its accurate taxonomic position. *C. spinellus* was originally described in New Zealand, after it was reduced to synonymy with *C. ustulatus*. In addition, further studies are needed in other regions, such as Indonesia and Senegal, where synonyms of *C. ustulatus* have been reported (Searles, 1968; West and Calumpong, 1990; Rueness, 1997).

Based on molecular evidence, *C. okamurae* showed broad trans-oceanic distribution in the Northern Hemisphere (including Korea, Japan, China, Taiwan, France, Spain, Italy, Canada, and the USA) (Figure 4). *C. okamurae* has been typically misidentified as *C. ustulatus* because of its resemblance to the gross morphology (Petrocelli et al., 2020). As the distribution of two different species, *C. ustulatus* and *C. okamurae*, has been identified in Europe, molecular screening of *Caulacanthus* specimens is required to assess their detail distribution. Moreover, our study provides the first molecular evidence of *C. okamurae* in British Columbia, Canada, where two haplotypes (H01–H02) were found (Figure 2). Populations from the NE Pacific Ocean with two haplotypes could serve as evidence that this population had been introduced from the NW Pacific Ocean earlier than the population from the NE Atlantic Ocean. The lack of shared haplotypes between the NW and NE Pacific oceans also supported this hypothesis. Non-native species have been increasingly reported in many regions, facilitated by both natural and anthropogenic stressors (Nelson et al., 2021). Climate change can be predicted to affect species range by encouraging the settlement of temperate non-native species (Miller et al., 2011). Drastic temperature shifts, including the series of El Niño/La Niña-Southern Oscillation events, along with increased sea temperature could have favored the settlement and spreading of *Caulacanthus* in several areas (Miller et al., 2011). Moreover, the spread of non-native seaweed *C. okamurae* could have facilitated increased abundance and diversity of other

seaweeds and invertebrates by forming novel turf in intertidal zones (Smith et al., 2014; Petrocelli et al., 2020).

In the NW Pacific, two *Caulacanthus* species were found, namely *C. okamurae* and Lineage 3, of which Lineage 3 was found from tropical to subtropical regions, including Shimen and Okinawa, Cooktown, and Magnetic Island (Figure 4). Populations in the NW Pacific Ocean might be geographically separated by restricted gene flow. For example, the haplotypes from Korea/China were not shared with that from the Pacific coast of mainland Japan affected by Kuroshio Current, and this genetic division was supported by high F_{ST} values (Table 2). The distribution of the NW Pacific haplotypes is associated with ocean currents, which might mediate gene flow between populations. The gene flow by ocean currents was also shown in the distribution of haplotype H04, which occurred from Nanji Island to the coasts of Korea and Dailan (Figure 5). The occurrence of H04 in the entire coast of Korea may be the result of high gene flow between the Korean populations due to complex oceanic currents, which has also been observed in red algal species of the genus *Chondrus* Stackhouse (Yang and Kim, 2022). The influence of ocean currents affects population distribution may be confirmed in future studies including populations along the coasts where the Tsushima Warm Current passes.

The distribution of the seven *Caulacanthus* species provides information about the processes that have shaped the evolutionary history of this group, which were previously obscured by treating it as a single species. All lineages included a well-supported group with a deep split from each other, indicating that speciation occurred on an ocean basin scale. Therefore, our study provides evidence of a greater evolutionary independence among the populations of *Caulacanthus*, as measured by mtDNA divergence. Based on our phylogenetic reconstructions, we interpreted that the *Caulacanthus* species complex have undergone a series of allopatric diversification events (Derycke et al., 2008), showing considerable genetic divergence in the resulting population isolated by distance (Díaz-Tapia et al., 2020). The case of two subclades with genetic divergence of 3.62% within Lineage 5, that is, populations between the western and east coast of Australia, establishes genetic divergence by distance.

Accurately recognizing species boundaries is key to biodiversity research and conservation, as species are operational units for biodiversity quantification and management (Boo et al., 2019; Yang et al., 2020; Preuss et al., 2022). Our results revealed seven *Caulacanthus* lineages, most of which had limited geographic distribution. The lack of gene flow among lineages suggests that they have attained geographic isolation and may deserve to be assigned as species (Fraser et al., 2010). Five lineages would require validation and formal description. Additional studies should involve an integrative approach that considers other characteristics, such as morphology and ecology. Although the sampling in this study may not be sufficiently large to conclusively rule out the presence of lineages in an area, which was not sampled, the pattern emerging from our data showed seven lineages with regional distributions rather than a single cosmopolitan species. Further studies on the evolutionary relationships and genetic variation of regional populations, along with population collection from other countries, are essential to understand and effectively conserve and manage species of the genus *Caulacanthus*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

MYY and MSK conceived and designed the study and performed the collections. MYY performed laboratory work and the data analyses. MYY and MSK participated in the interpretation of the data and writing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1087507/full#supplementary-material

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