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A new record of epiphytic red alga *Madagascaria erythrocladioides* (Erythropeltidales, Rhodophyta) in Korea

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Abstract: The Erythropeltidales are a common group of small, mostly epiphytic, marine red algae. However, they are little known in Korea. Many of the described species of Erythropeltidales differ subtly in morphology, and often the morphological differences are due to the substrate or environmental changes. Integration of molecular data with standardized culture conditions has been recommended to account for these algae. A *Madagascaria* species was first collected from the western coast of Korea and was identified as *Madagascaria erythrocladioides* based on the morphological and molecular characteristics. Morphological characteristics conformed well with its original description, and the phylogenetic analysis based on *rbcL* sequence showed Korean *M. erythrocladioides* nests in the same clade with the original species described in Japan with a genetic distance of 0.0–0.1%. This species was isolated from a red alga, *Pterocladiella capillacea*, in laboratory culture. The thallus ontogeny and host preference were examined by a co-culture with 13 different species of algae. Results showed a relatively broad host preference in mono-spore attachment and epiphyte development of *Madagascaria erythrocladioides*. Mono-spores of *M. erythrocladioides* attached to most of the red algal hosts' surfaces but no crustose thalli developed on some of the algal hosts even after one month of co-culture.

Keywords: *Madagascaria erythrocladioides*, red alga, epiphytic, host preference

INTRODUCTION

The Erythropeltidales is placed in the class Compsopogonophyceae, which consists mostly of small, ubiquitous marine red algae (Soares *et al.* 2019). The most common and widely reported genera in the order are *Erythrotrichia*, *Erythrocladia*, *Sahlingia* and *Madagascaria* (e.g., Silva *et al.* 1996; Brodie and Irvine 2003; Zuccarello *et al.* 2010). Morphologically, they are quite simple, consisting of filaments, crusts, or simple blades, rarely exceeding a few centimeters in size, but more often less than 0.5 cm (Zuccarello *et al.* 2010). The order has few defining features, and their small size has made this order along with many of the other Bangiophyceaen orders difficult to characterize by simple characters (Zuccarello *et al.* 2010). The advent of molecular analysis revealed a wide range of divergent lineages in Bangiophyceae including Erythropeltidales (Yoon *et al.* 2006).

There are few defining features of the Erythropeltidales although the order is well supported by molecular data (Zuccarello *et al.* 2010). Most species in the order have a central plastid with a pyrenoid and all species studied do not have pit plugs, but these characters are also found commonly in the other orders (e.g., Stylonematales, Zuccarello *et al.* 2008). A more defining feature is the production of monospores (archeospores) often by the unequal division of vegetative cells, producing what is called a lenticular cell,

and the release of this cell as a spore. This characteristic would distinguish it from the Stylonematales in which the entire vegetative cell is liberated as a spore (Zuccarello *et al.* 2010). Laboratory culture is essential to confirm these defining features but few Korean species of Erythropeltidales has been studied using laboratory culture.

Madagascaria is a crustose monotypic genus originally described from specimen collected in Madagascar and Japan, characterized by more or less circular, free end crusts without bifurcate marginal cells and plastids with circular (Zuccarello *et al.* 2010). *Madagascaria* has been reported in many localities of Indo-Pacific region and even in Atlantic Sea (Soares *et al.* 2019), but not in Korea so far. In this study, we isolated culture strain of *Madagascaria erythrocladioides* West & Kikuchi from *Pterocladiella capillacea* which collected from Jeokbyeokgang in the western coast of Korea. We observed morphological development of the algae using laboratory culture and analyzed its phylogeny using *rbcL* sequences.

MATERIALS AND METHODS

1. Sampling, culture, and microscopy

The samples deposited in National Marine Biodiversity Institute of Korea (MABIK) (KNU cluture No. KNU 000499) were used. The samples were isolated from *Pterocladiella capillacea* collected in Jeokbyeokgang (35° 38′20.7″N, 126°27′46.09″E), Gyeokpo, Korea, 2013. In the laboratory, samples were cultured in medium composed of sterilized seawater with IMR medium described in Kim *et al.* (2006). Culture conditions were as follows: temperature of 15°C, irradiance of 10 μmol photons m^{-2} s^{-1}, 16 : 8 h light: dark cycle, renewal IMR medium every two weeks. To examine if there are any host preferences, *M. erythrocladioides* was co-cultured with 13 different algae for a month and monospore attachment to the host and thallus development were observed (Table 2). Samples was observed under microscope (BX50; Olympus, Japan). Photomicrographs were taken with camera (DP72; Olympus, Japan) and CellSens program (Olympus, Japan). Fluorescent and histochemical stains used in this study are as follows: Calcofluor White stain (Sigma-Aldrich, USA), DAPI (Sigma-Aldrich, USA), Eosin Y (Sigma-Aldrich, USA), Methylene blue (Showa, Chemical, Japan). Cellulose in cell walls were stained with Calcofluor White stain for 5 minutes in the culture medium and observed under DAPI filter (Klaus and Andreas 1969). Nuclei were stained with 1 μg mL^{-1} DAPI for 5 minutes in the culture medium and observed under DAPI filter (Kim *et al.* 2001). Cytoplasmic proteins were stained with 1 mg mL^{-1} eosin Y for one hour and observed under bright filter (Suvarna *et al.* 2013). Acidic polysaccharides were stained with 0.23 mg mL^{-1} methylene blue for one hour under bright filter (Levine and Black 1949).

2. Molecular analysis

Total DNA was extracted from fresh or dried samples using Wizprep Plant DNA mini kit (Wizbiosolution, Korea). Partial sequence of plastid encoded ribulose-1; 5-bisphosphate carboxylase large subunit regions (*rbcL*) was amplified using the primers (Mada_rbcL_F 5′-GGCCAAAATAAATTGCCTTGAAC-3′ and Mada_rbcL_R 5′-CGTGCTCCTATTATGCTAAAGTC-3′), which were designed in this study based on the chloroplast genome data of *M. erythrocladioides* (MW675672). The PCR conditions consisted of an initial denaturation at 95°C for 4 min, followed by 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and 30 s, final extension at 72°C for 7 min, was followed by 35 cycles. Multiple alignments of nucleotide sequences were performed among *M. erythrocladioides* and *rbcL* of other species, from the NCBI database, using MAFFT in Geneious Prime v2019.0.4 (Biomatters Ltd., New Zealand). A phylogenetic analysis was performed using MrBayes 3.2.6 and RAxML 8.2.11 software program for Bayesian phylogenetic analysis and Maximum likelihood analysis. The following settings were used in MrBayes 3.2.6: generations = 3,000,000; subsampling frequency = 600, burn-in length = 300,000; model = GTR GAMMA. The following settings were used in RAxML 8.2.11: Bootstrap support values (%) = 1,000; model = GTR GAMMA (Lee and Kim 2017). We used the *Porphyridium aerugineum* as the outgroup for phylogenetic tree. IQ-TREE ModelFinder was used to find the best model (Kalyaanamoorthy *et al.* 2017).

RESULTS

Genus *Madagascaria* 점붉꽃 (신칭)

***Madagascaria erythrocladioides* J.A. West & N. Kikuchi**
점붉꽃 (신칭)

Type locality. Intertidal, epiphytic on *Gelidium* sp. Ampasi Pohry, Nossi Be, Madagascar.

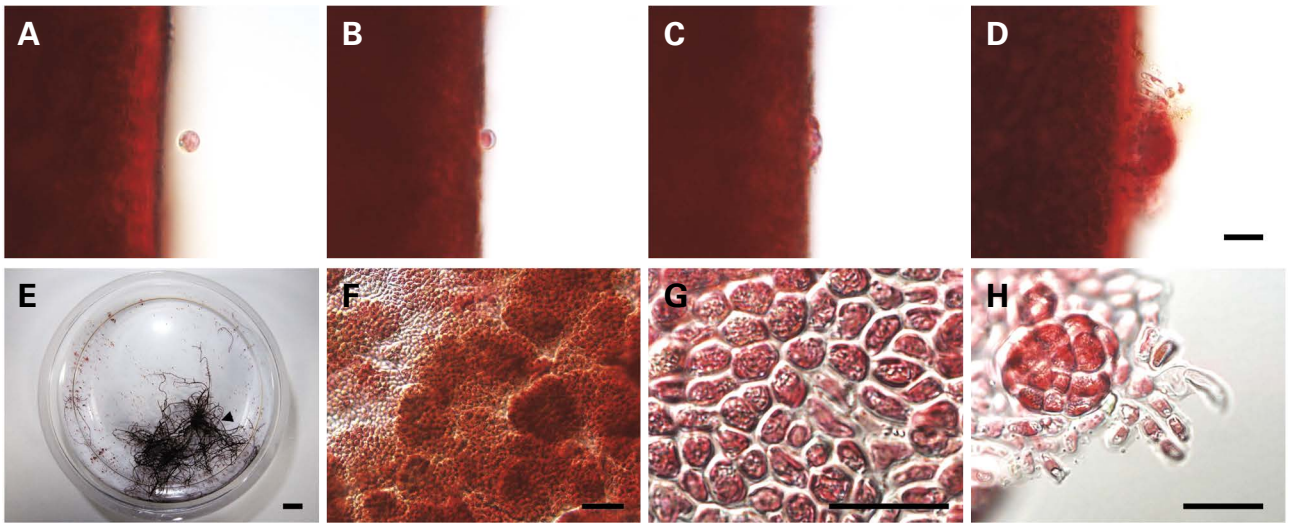

Fig. 1. Laboratory culture of *Madagascaria erythrocladioides*. A–D. Thallus ontogeny on the surface of the host, *Pterocladiella capillacea*. A. The initial stage of monospore attachment. B. Tight binding occurred between the host and epiphyte. C. Epiphytic cells dive first along the surface of the host. D. Mature epiphyte showing free-end crusts. E. Co-culture of *M. erythrocladioides* with *Pterocladiella capillacea*. F. *M. erythrocladioides* culture without a host. G. Enlarged view of the culture in the culture dish. H. Mature thallus showing free end. Scale bars: A–D = 10 μm; E = 1 cm; F = 50 μm; G, H = 20 μm.

Table 1. Morphological characters of *Madagascaria* species

Epiphyte species	Isolate	Distribution	Monospore diameter	Reference
Madagascaria erythrocladioides	*Pterocladiella capillacea*	Korea	4.9–7.0 μm	This study
	Gelidium sp.	Madagascar	4.9–7.8 μm	Zuccarello *et al.* (2010)
	–	Japan	5.3–6.5 μm	Zuccarello *et al.* (2010)
Madagascaria atlantica	–	Brazil	5.0–7.5 μm	Soares *et al.* (2019)
Erythrocladia irregularis	*Gigartina acicularis*	Italy	7–8 μm	Gargiulo *et al.* (1987)

Collection site in Korea. Epiphytic on *Pterocladiella capillacea* collected from Jeokbyeokgang (35°38′20.7″N, 126°27′46.09″E); collected 30 March 2013.
Specimens examined. KNU000499H1- KNU000499H6 (Jeokbyeokgang (35°38′20.7″N, 126°27′46.09″E), Gyeokpo, Korea, 30 March 2013, Ga Hun Boo and Se Yong Kim).
Habitat. Intertidal, epiphytic on other algae.
GenBank accession number. MZ702790 (*rbcL*).
Morphology. Plants were rose red to intense red, forming irregular overlapping crusts (20–200 μm) with free filament ends. Marginal cells are not bifurcated. Central crust cells 4.0–8.7 μm long and 3.7–6.0 μm wide. Monospores formed in the center of the crust and 4.9–7.0 μm in size. Monospore germinated by elongation and oblique divisions forming rhizoidal extensions. Central crust cells 4.9–8.9 μm long and 3.7–7.3 μm wide. Cells contain a laminate or parietal plastid with a pyrenoid.

Morphological characters of *M. erythrocladioides* from Korea are in good agreement with the original description except that Korean strain was isolated from *Pterocladiella capillacea* while the type specimen was isolated from *Gelidium* sp. (Table 1). Thallus ontogeny on the surface of the host, *Pterocladiella capillacea*, was observed (Fig. 1A–D). Monospores of *M. erythrocladioides* were initially loosely attached to the host (Fig. 1A), and 2–3 days after attachment, tight binding occurred between the host and epiphyte, then the epiphytic cell began dividing (Fig. 1B). Cell division initially occurred horizontally on the host surface (Fig. 1C), but the thallus eventually developed into crustose form with free ends (Fig. 1D). Monospores often attached to the bottom of culture dish and formed a membrane covering the surface (Fig. 1E–H). Although the

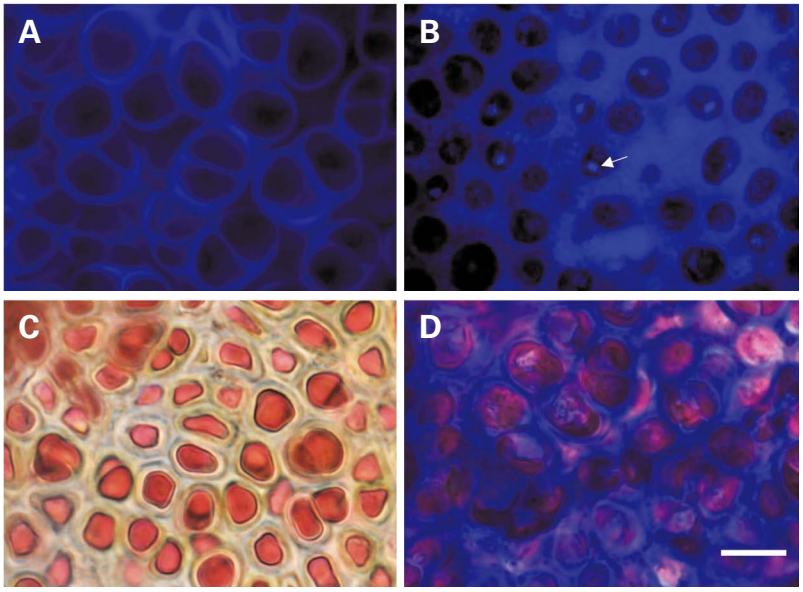

Fig. 2. Fluorescent staining of *Madagascaria erythrocladioides*. A. Calcofluor white stains the matrix of the thallus. B. DAPI staining C. Eosin Y staining. D. Methylene blue staining. Scale bar: 10 μm.

Table 2. List of epiphyte host

	Species	Monospore attachment	No.
Chlorophyta	*Bryopsis plumosa*	+	Collected, Korea, 2003
	Codium fragile	−	MGARB200241
Rhodophyta	*Aglaothamnion callophyllidicola*	+	KNU000165
	Ahnfeltiopsis flabelliformis	+	KNU000109
	Bostrychia moritziana	+	KNU000027
	Colaconema daviesii	−	KNU000474
	Erythronychia sp.	−	Collected, Korea, 2018
	Heterosiphonia pulchra	+	KNU000185
	Pachymeniopsis lanceolata	+	KNU000129
	Pterocladiella capillacea	+	KNU000107
	Pyropia yezoensis	+	KNU000212
	Stenogramma guleopoense	−	KNU000458
Ochrophyta	*Ectocarpus siliculosus*	−	KNU000012

M. erythrocladioides cells formed a thin membrane on the surface of culture dish (Fig. 1F–H), mature crustose thalli with free ends also developed from the membrane (Fig. 1H).

Fluorescent staining of the thallus with Calcofluor-white and DAPI showed that the matrix forming membranes are composed of cellulosic materials (Fig. 2A, B). Eosin Y staining showed that the matrix contained little amount of proteins (Fig. 2C). Methylene blue staining showed that polysaccharides covered the crusts (Fig. 2D). Host preference was assayed by co-culturing *M. erythrocladioides* with 13 different species of algae (Table 2). Monospores did not attach to some algal hosts during co-culture for one month even when whole surface of culture dish was covered with *M. erythrocladioides* cells (Fig. 3). Red algal hosts, *Colaconema daviesii*, *Stenogramma guleopoense* and *Erythrotrichia*

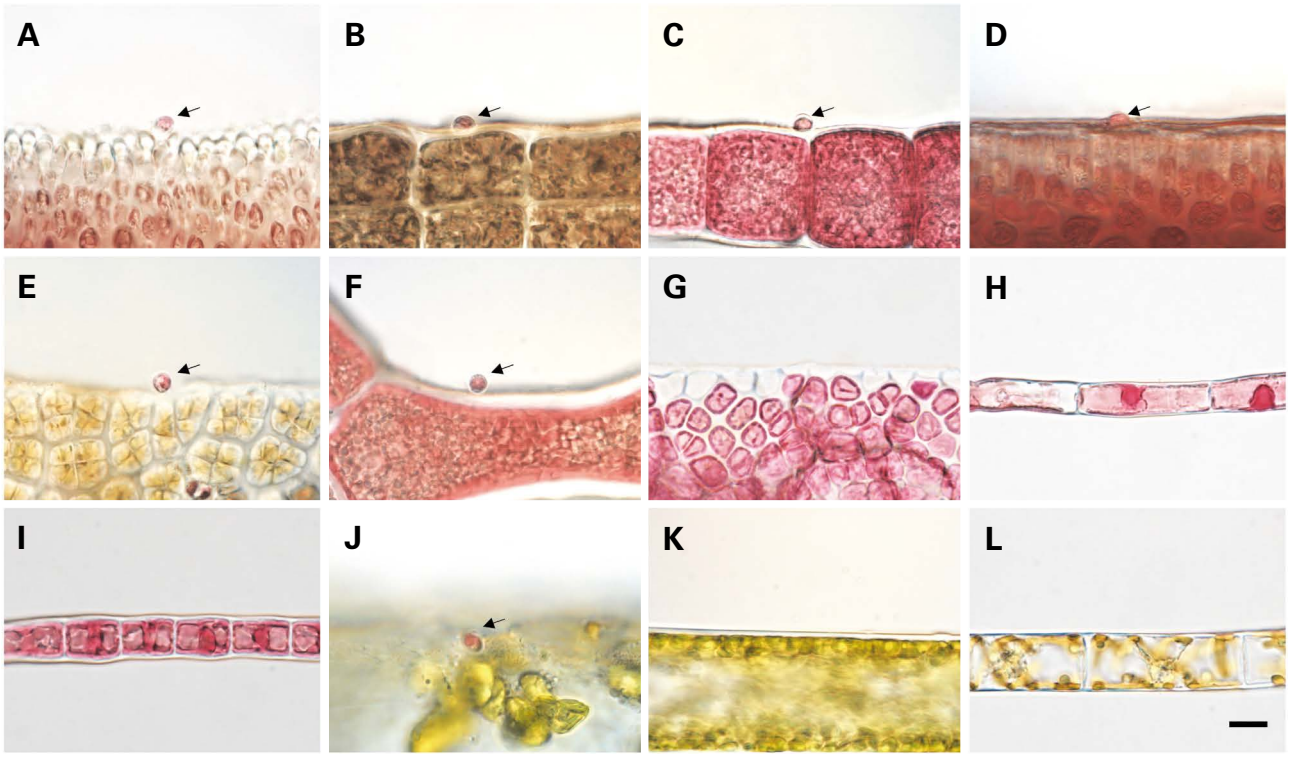

Fig. 3. Epiphyte host of *Madagascaria erythrocladioides*. A–F, J. Monospore of *M. erythrocladioides* attached to the cell walls of *Pachymeniopsis lanceolata*, *Bostrychiamoritziana*, *Heterosiphonia pulchra*, *Ahnfeltiopsis flabelliformis*, *Pyropia yezoensis*, *Aglaothamnion callophyllidicola*, *Bryopsis plumosa*. G–I, K, L. No monospore attached to *Stenogramma guleopoense*, *Colaconemadaviesii*, *Erythronychia* sp., *Codium fragile*, and *Ectocarpus siliculosus*. The arrow refers to the attached monospores. Scale bar: 10 μm.

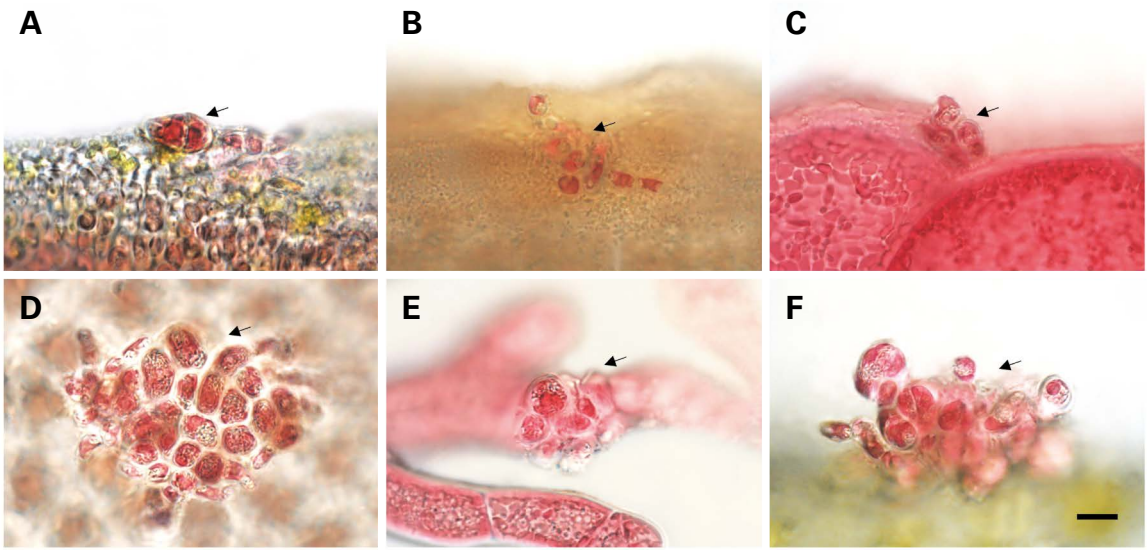

Fig. 4. Development of monospores of *Madagascaria erythrocladioides* on different algal hosts. A–F. Monospore of *M. erythrocladioides* grew on the cell walls of algal hosts *Pachymeniopsis lanceolata*, *Bostrychiamoritziana*, *Heterosiphonia pulchra*, *Pyropiayezoensis*, *Aglaothamnioncallophyllidicola*, and *Bryopsis plumosa*. The arrow refers to the attached *M. erythrocladioides*. Scale bar: 10 μm.

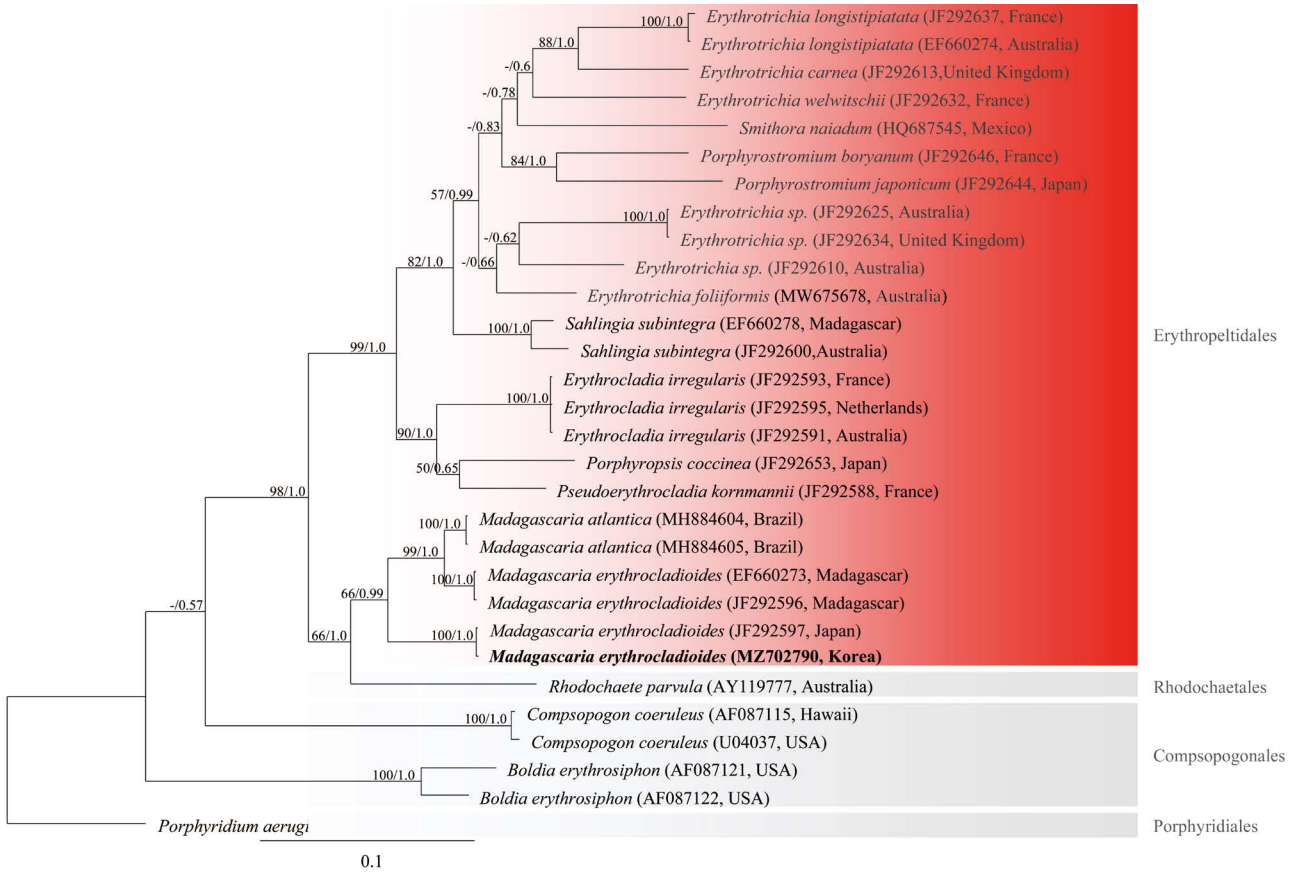

Fig. 5. Phylogenetic tree of *rbcL* from *Madagascaria erythrocladioides* and other algae. A phylogenetic analysis was performed using MrBayes 3.2.6 and RAxML 8.2.11 methods for Bayesian phylogenetic analysis and Maximum likelihood analysis. Only values ≥ 50 are shown. Sequences generated in this study are highlighted in bold.

sp. did not bound with monospores. Some monospores attached to a green algal host *Bryopsis plumosa*, but not to *Codium fragile*, and *Ectocarpus siliculosus*, a brown algal host (Fig. 3J–L). Once tight binding occurred between the epiphyte and host typical crustose thalli developed on the surface of hosts (Fig. 4).

Phylogeny. Phylogenetic analysis based on *rbcL* sequence showed that *M. erythrocladioides* from Korea nests in the same clade with original species described in Japan by with a genetic distance of 0.0–0.1% (Fig. 5).

DISCUSSION

An epiphytic red alga, *Madagascaria erythrocladioides*, was first reported in Korea. Although sexual reproduction was not observed in laboratory culture, vegetative characteristics of Korean strain was in good agreement with the original description of the species. Phylogenetic characteristics of Korean *Madagascaria erythrocladioides* also conformed well with Japanese strain described by Zuccarello *et al.* (2010) and nested in the same clade with other *Madagascaria* species (Soares *et al.* 2019).

Host specificity is important for finding an environment in which the epiphytic species can survive and thrive. As previous studies have shown, their attachment to substrate never happens randomly (Seo *et al.* 2020). Our results showed there is a relatively broad host preference in monospore attachment and epiphyte development of *Madagascaria erythrocladioides*. Epiphytic thalli did not develop on the surface of some algae including three red algae (*Colaconema daviesii, Erythronychia* sp., *Stenogramma guleopoense*), one green algae (*Codium fragile*), one brown algae (*Ectocarpus siliculosus*), after one month of co-culture. The close association between epiphytes and particular host species appears to begin with the tight binding of monospores

to the host surface, but host preference did not appear to be affected by phylogenetic relationships with the hosts. Further studies are needed to elucidate the role of the host role in the epiphytic development of this species.

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