

Ralfsia longicellularis (Ralfsiales, Phaeophyceae): a Far East Asian endemic brown alga from Korea

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Abstract: *Ralfsia longicellularis* is known as an endemic species in Far East Asia. In this study, we report *R. longicellularis* as a new record from Korea based on morphological and molecular analyses. Molecular analyses based on plastid-encoded *rbcL* gene sequences and morpho-anatomical studies were undertaken on *Ralfsia* species, a poorly studied genus from Korea. *Ralfsia longicellularis* is mainly characterized by a dark brown thallus; 770–1200 μm thick, curved cells in the creeping and ascending parts of the filaments; basal layer and erect filament cells with a width-to-length ratio of 1 : 1.5 to 10; narrow-shaped sporangia on-stalk cells at the base of the paraphyses; and mostly uniseriate plurilocular reproductive organs capped with 1–2 sterile cells. The *R. longicellularis* samples from Korea in this study were similar to ones collected from the type locality (Peter the Great Bay, Russia) in morphology. The *rbcL* analyses also revealed that our Korean *R. longicellularis* samples were placed in the same clade with Russian materials, within a *Ralfsia* clade but distinct from the congeners.

Keywords: *rbcL*, *Ralfsia longicellularis*, Ralfsiales, Phaeophyceae

INTRODUCTION

The crustose brown algal genus, *Ralfsia*, was described by Berkely (in Smith and Sowerby 1843). It is characterized by having thalli crustose, flat and expanded, more or less circular in outline, attached to the substratum with or without rhizoids; erect vegetative filaments tightly adherent, straight or curved, branched or simple, arising from a basal layer; unilocular sporangia clavate, ovoid to pyriform, sessile or pedicellate arising from the supporting cells of paraphysis; plurilocular reproductive organs intercalary, arising from a cell located in the submerged part of the erect filament, composed of one to four rows of plurilocular reproductive filaments with one to several terminal sterile cells; chloroplasts mostly plate-shaped, generally one per cell; hairs in tufts, scattering over thallus (Setchell

and Gardner 1924; Hamel 1931–1939; Hollenberg 1969; Abbott and Hollenberg 1976; Tanaka and Chihara 1980; Fletcher 1987; Womersley 1987; Parente and Saunders 2019). Currently, 17 *Ralfsia* species are recognized worldwide (Guiry and Guiry 2020). Of them, four *Ralfsia* species were reported from Korea: *Ralfsia fungiformis* (Gunnerus) Setchell and N.L. Gardner, *R. verrucosa* (Areschoug) Areschoug, *R. confusa* Hollenberg, and *R. integra* Hollenberg (Lee and Kang 1986; Lee and Kang 2002; Keum 2010).

Ralfsia longicellularis was described from Peter the Great Bay, Far East Russia by Perestenko in 1980. It is mainly characterized by a thick dark brown thallus, curved cells in the creeping and ascending parts of the filaments forming a unilateral symmetry, basal layer and erect filament cells' width to length ratio of 1 : 1.5–10, and narrow-shaped spo-

rangia on stalk cells at the base of paraphyses (Perestenko 1980). Since its description, *R. longicellularis* has only one report worldwide (Guiry and Guiry 2020). In the analysis of the biogeographical structure of flora in Vostok Bay, Sea of Japan, it was reported as part of Asian low-boreal species (A1B) (Kozhenkova 2009). This may allude to the fact that *R. longicellularis* is endemic to the northern region of Far East Asia.

We collected the unidentified samples that fit the genus *Ralfsia*'s description, from coasts of Korea. We observed their detailed morphology and analyzed molecular data based on the plastid-encoded *rbcL* gene for their phylogenetic relationships. In this study, we add *Ralfsia longicellularis* to the Korean marine algal inventory.

MATERIALS AND METHODS

1. Morphology

Samples of *Ralfsia* were collected from shallow intertidal zones of Korea (Taeon, Buan, and Wando) and Russia (Vladivostok). Vouchers were air-dried and preserved in silica gel for morphological and molecular studies. For morphology, samples were detached from substrate by use of a single-edged blade, then embedded in a matrix (O.C.T., CellPath, Ltd., Newtown, Wales, UK) and sectioned (8–10 μm thickness) using freezing microtome (Shandon Cryotome FSE, Thermo Shandon, Ltd., Loughborough, UK), stained in a 1 : 1 mixture of aqueous aniline blue and acetic acid. Photomicrographs were taken using an Olympus DP71 camera mounted on an Olympus microscope (BX51TRF; Olympus, Tokyo, Japan) and a digital camera (Nikon D40; Nikon, Japan). Representative voucher specimens examined in this study are deposited in the herbarium of Chosun University (CUK) and the National Institute of Biological Resources (NIBR), Korea.

2. Molecular study

Genomic DNA was extracted using a NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). The extracted DNA was stored at -20°C and used to amplify *rbcL*. The *rbcL* gene was amplified using the primer combinations ND*rbcL*2-DRL1R and DRL2F-R3A (Kogame *et al.* 1999; Hwang *et al.* 2005) with HelixAmp Ready-2x-Go Series (NanoHelix Co., Ltd., Daejeon, Korea). All PCR amplifications were carried out with a Veriti 96 well Thermal cycler

(Applied Biosystem). PCR products were purified using a PCRquick-spinTM PCR product purification kit (iNtRON Biotechnology, Inc, Seongnam, Korea). New *rbcL* sequences obtained from *Ralfsia longicellularis* have been deposited in EMBL/GenBank under the accession numbers MT000915 (CUK18752), MT000917 (CUK18820), MT000918 (CUK19216), MT000916 (CUK19269) and MT000914 (CUK20024).

Nineteen *rbcL* sequences (781 bp) were aligned, including sequences of other species of Ralfsiales previously published in GenBank and two outgroup taxa, *Tilopteris mertensii* (Turner) Kützing and *Sargassum muticum* (Yendo) Fensholt, with ClustalW (Thompson *et al.* 1994). Phylogenetic analyses were conducted using raxmlGUI1.5b2 (Silvestro and Michalak 2012). Maximum likelihood analyses were conducted using the GTR+G+I model, with 1,000 bootstrap replicates. Bayesian inference was performed using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Markov chain Monte Carlo runs were conducted for 2 million generations, each with one cold chain and three heated chains using the GTR+ Γ +I evolutionary model and sampling and printing every 1,000 generations. Summary trees were generated using a burn-in value of 800.

RESULTS AND DISCUSSION

1. Morphological observations

Class Phaeophyceae F.R. Kjellman, 1891 갈조강
Order Ralfsiales Nakamura ex P.-E. Lim & H. Kawai in
Lim *et al.*, 2007 바위딱지목
Family Ralfsiaceae W.G. Farlow, 1881 바위딱지과
Genus *Ralfsia* M.J. Berkeley, 1843 바위딱지속

Ralfsia longicellularis L. P. Perestenko, 1980

긴세포바위딱지 (신칭) (Fig. 1A–E)

Material examined. NIBROR0000001763 (deposited in the National Institute of Biological Resources), CUK19216 (= MBRB0103TC19216D1), MT000918, Pado-ri, Sowuon-myeon, Taeon-gun, Chungcheongnam-do, Korea ($36^{\circ}43'05.65''\text{N}$, $126^{\circ}07'33.79''\text{E}$), November 3, 2018, T.O. Cho and B.Y. Won, at 1 m depth by hand; CUK18752 (= MBRB0103TC18752D1), MT000915, Pado-ri, Sowuon-myeon, Taeon-gun, Chungcheongnam-do, Korea ($36^{\circ}43'05.65''\text{N}$, $126^{\circ}07'33.79''\text{E}$), March 3, 2018, T.O.

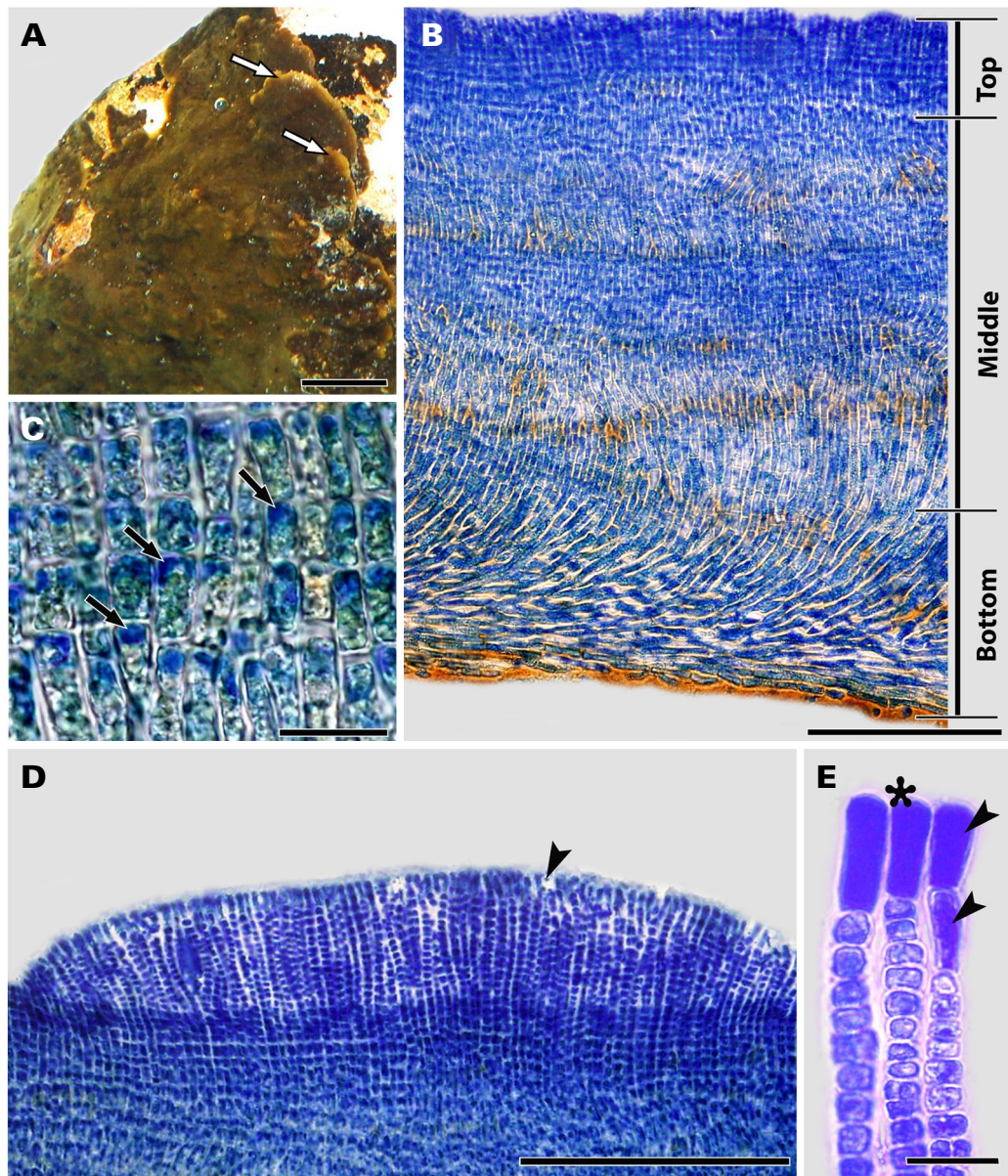


Fig. 1. *Ralfsia longicellularis* CUK18820 (MT000917) from Jeongdo-ri, Wando-eup, Wando-gun, Jeollanam-do, Korea. A. Dark brown crustose thallus with overlapping lobes on the margin. B. Radial longitudinal section showing the apical part of erect vegetative filaments (top) and the mid-portion of erect filaments (middle), both having cells longer than wide, and the basal portion of the thallus (bottom), presenting curved cells. C. Chloroplasts (arrows), parietal and single per cell. D. Sorus (arrowhead) with plurilocular reproductive organs. E. Plurangial reproductive filaments capped with one (asterisk) to two (arrowheads) sterile cells. Scale bars: A = 1.0 cm; D = 200 μ m; B = 100 μ m; C & E = 20 μ m.

Cho and B.Y. Won, at 1 m depth by hand; CUK18820 (= MBRB0103TC18820D1), MT000917, Jeongdo-ri, Wando-eup, Wando-gun, Jeollanam-do, Korea (34°17'50.04"N, 126°42'08.14"E), April 1, 2018, T.O. Cho and B.Y. Won, at 1 m depth by hand; CUK19269 (= MBRB0103TC19269D1), MT000916, Chaeseokang, Byeonsan-

myeon, Buan-gun, Jeollabuk-do, Korea (35°37'27.04"N, 126°27'56.88"E), January 26, 2019, T.O. Cho and B.Y. Won, at 1 m depth by hand; CUK20024 (= MBRB0103TC20024D1), MT000914, Ulitsa Leytenanta Shmidta, Vladivostok, Russia (43°06'21.78"N, 131°52'11.07"E), October 10, 2019, T.O. Cho and B.Y. Won, at 1 m depth by

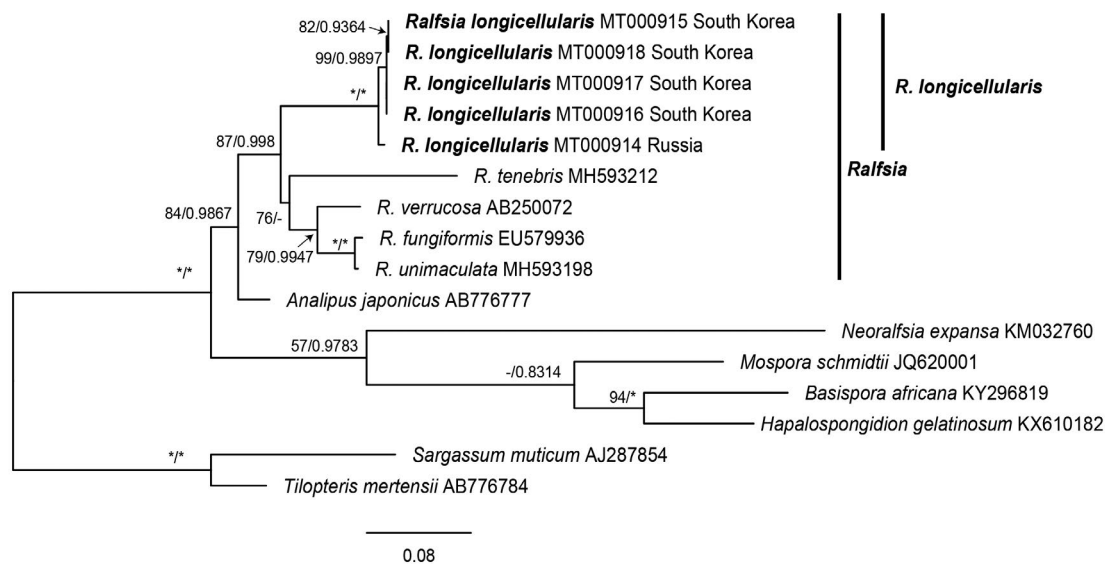


Fig. 2. Phylogenetic tree of *Ralfsia* and other species in Ralfsiales based on Bayesian and RAxML analysis with *rbcL* sequences. Value above the branches = Bayesian posterior probabilities > 0.75, Maximum likelihood bootstrap values in > 50%. Values lower than BPP 0.75 or BS 50 are indicated by hyphens (-). Values of BPP 1.00 or BS 100 are indicated by asterisks (*).

hand.

Description. Thallus thick, crustose, epilithic, dark brown (almost black in the dry state), with uneven surface, may have overlapping lobes (Fig. 1A, arrows) at margins, several centimeters across, 770–1200 μm thick, undersurface irregularly distributed with rhizoids 8.4–14 μm wide. In the creeping and ascending parts of the filaments (Fig. 1B, bottom), the cells are curved, often with oblique partitions, 8.5–11(14) μm width, with a ratio of width to length 1 : 1.5–7, forming horizontal rows. The lower layer of creeping ascending filaments is 0.3 times the thickness of the thallus. The cells of vertical filaments (Fig. 1B, middle) are cylindrical 5.5–8.5 μm width, with a ratio of width to length 1 : 1.5–10. Cells of the upper third (Fig. 1B, top) of vertical filaments taper to the top with 4–8 μm width and a ratio of width to length 1 : 1–5. Chloroplasts (Fig. 1C, arrows) parietal, single per cell, mostly on the apical part of the cell. Reproductive portions of sori (Fig. 1D, arrowhead), scattered throughout the thallus. Narrow-shaped unilocular sporangia, on 1–2(3) stalk cells, 22.5–31 \times 65–92 μm . Paraphyses of 10–13 cells, 190–210 μm long. Apical cells of paraphysis 8.5–10 μm wide. Plurilocular reproductive organs 90–265 μm long, uniseriate, occasionally biseriate, capped with one (Fig. 1E, asterisk) to two (Fig. 1E, arrowheads) sterile cells. Sterile cell 5–8 μm width and 1.8–3 times as long as the width. Hair unknown.

Habitat. Epilithic at intertidal zone.

World Distribution. Asia; Russia (Far East) (Guiry and Guiry 2020) and Korea.

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2. Phylogenetic analyses

The 781 *rbcL* bp portion was sequenced for *Ralfsia longicellularis*. Phylogenetic analyses revealed that both Korean and Russian *R. longicellularis* sequences were placed within the same clade (Fig. 2). The analyses revealed *R. longicellularis* differs from its nearest neighbor *R. tenebris* by 7.7–7.9% and the genotype, *R. fungiformis*, by 8.7–8.8% gene sequence divergence. Intra-specific genetic divergence within *R. longicellularis* was 0.0–1.0%.

Remarks. *Ralfsia longicellularis* was originally described by Perstenko (1980) from Russia. This species has only been described and reported (Kozhenkova 2009) from Russia. Most characteristics from the protologue of *R. longicellularis* from Peter the Great Bay, Russia (Perstenko 1980) are an exact match with our Korean samples. However, the protologue had unilocular sporangia, while Korean samples presented plurangial reproductive structures. This shows that both types of reproductive organs are borne on separate thalli. The original description had no molecular analysis. Our study included molecular analyses. The *rbcL* genetic divergence between Korean samples and those from Vladivostok (within Peter the Great Bay) was 1%.

Therefore, Korean samples are the same species as Russian ones. We add this species as *Ralfsia longicellularis* in list of Korean macroalgal flora.

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