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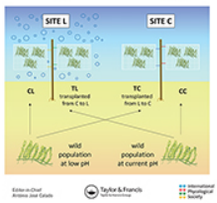

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Morphological variation in Northwest Pacific *Devaleraea mollis* and description of *D. inkyuleei sp. nov.* (Palmariaceae, Rhodophyta)

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ABSTRACT

We assessed the range of morphological variation in *Devaleraea mollis* from the Northwest Pacific. Genetic analyses based on the 5′ end of the genes coding for cytochrome *c* oxidase I (COI-5′), cytochrome oxidase *b* (cob) and photosystem I reaction centre apoprotein A1 (*psa*A), as well as the ITS1–5.8S–ITS2 (ITS) fragment of rDNA, revealed that the morphospecies *D. mollis* includes two species. The alga from Japan previously identified as '*Palmaria palmata*' resolved as sister to *D. stenogona* and is here described as *D. inkyuleei sp. nov*. The species is morphologically similar to *D. mollis*, but it has a more southern range. *Devaleraea inkyuleei* is only known from Japan (Honshu and Hokkaido) and from Aniva Bay (Sakhalin Island, Russia).

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cob; COI-5′; DNA barcoding; ITS rDNA; Russian Far East; Systematics of algae

INTRODUCTION

An important goal of phycological research is to establish an accurate taxonomy of the various algal groups. Unfortunately, high morphological plasticity and the existence of few diagnostic traits can make it difficult to identify species merely on the basis of morphological and anatomical characteristics, particularly bladed red algae. The use of molecular tools is a more objective method for resolving taxonomic issues in many seaweed species with simple or convergent morphologies. Such an approach has been successfully applied to investigate species diversity of many genera and families of Rhodophyta (e.g. Lindstrom 2008; Clarkston & Saunders 2013; Filloramo & Saunders 2016; Koh & Kim 2018). Molecular studies have revealed a previously underestimated species diversity and resulted in identification of cryptic species in nearly all red algal groups (Lindstrom *et al.* 2011, 2015; Clarkston & Saunders 2013; Filloramo & Saunders 2016, 2018; Griffith *et al.* 2017; Schneider *et al.* 2017; Yang & Kim 2017; Zuccarello *et al.* 2018; Vis *et al.* 2020; Díaz-Tapia *et al.* 2021).

Devaleraea mollis (Setchell & N.L. Gardner) G.W. Saunders, C.J. Jackson & Salomaki is one of nine species of the genus *Devaleraea* Guiry (Palmariaceae, Palmariales) listed in Guiry & Guiry (2022). It was initially described as *R. palmata* f. *mollis* Setchell & N.L. Gardner, an infraspecific taxon of *Rhodymenia palmata* (Linnaeus) Greville. In 1975, Guiry proposed a new combination for this taxon, *Palmaria palmata* f. *mollis* (Setchell & N.L. Gardner) Guiry, and noted some differences in sizes of reproductive structures (tetrasporangia and spermatangia) and medullary cells between *P. palmata* (Linnaeus) F. Weber & D. Mohr from the Atlantic and *P. palmata* f. *mollis* from the North Pacific (Guiry 1975). Later, based on obvious differences in the development of a female gametophyte, particularly in the position and number of carpogonia per gametophyte, as well as unsuccessful attempts to hybridize *P. palmata* from the North Atlantic with *P. palmata* f. *mollis* from the eastern Northeast Pacific Ocean, van der Meer & Bird (1985) suggested that there was genetic isolation between the two forms and raised *P. palmata* f. *mollis* to specific status as *P. mollis* (Setchell & N.L. Gardner) van der Meer & C.J. Bird. A molecular-phylogenetic study revealed that *P. mollis* was genetically more closely related to *D. ramentacea* (Linnaeus) Guiry, the generitype of *Devaleraea*, than to *P. palmata*, the generitype of *Palmaria* Stackhouse (Lindstrom *et al.* 1996; Saunders *et al.* 2018), which led to its transfer to the genus *Devaleraea* (Saunders *et al.* 2018).

Devaleraea mollis has been reported in the North Pacific from Pacific Grove, California, along the North American coast to the Aleutian Islands (Guiry 1975; Mondragon & Mondragon 2003), and along the Asian coast from the Commander Islands (Selivanova & Zhigadlova 2013; Klochkova *et al.* 2021), Olutorskiy Gulf (Selivanova 2011), and the coast of Kamchatka (Selivanova & Zhigadlova 2009, 2010) to Urup Island in the Kurile Islands (Gusarova 1975). A comprehensive examination of *D. mollis* (as *P. palmata* f. *mollis*) from the eastern Pacific indicated that this species has a high morphological diversity (Guiry 1975). Such information was not available for western populations. The high-

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resolution images of herbarium specimens labelled as *D. mollis* or *P. mollis* available on the Macroalgal Herbarium Portal (2022; https://macroalgae.org/portal) support the idea that the descriptions by Guiry (1975) and Hawkes & Scagel (1986) did not adequately cover the variations in morphology throughout the species purported range, and that more research was needed to assess this variability.

Examination of specimens housed in the National Museum of Nature and Science, Tsukuba, Japan (TNS), and the review of the checklist of the Japanese seaweeds (Yoshida *et al.* 2015) showed that only '*P. palmata*' has been recorded for Japan. Although these specimens resembled *D. mollis* morphologically and anatomically, sequences of multiple DNA loci (mitochondrial genes, ITS, plastid genes) from Japanese '*P. palmata*' (Lindstrom *et al.* 1996; Kumagai *et al.* 2019a) did not allow the alga to be assigned to *D. mollis*. On the other hand, the ITS sequence of Japanese '*P. palmata*' was close to that of *D. stenogona* (Perestenko) Skriptsova & T.L. Kalita from the Russian coast of the Sea of Japan (Skriptsova & Kalita 2020; Skriptsova *et al.* 2021).

The purpose of this study was to determine the extent of morphological variation in *D. mollis* across the Northwest Pacific, as well as to determine the identity of the Japanese '*P. palmata*'. *Devaleraea* specimens that were anatomically similar to *D. mollis* but morphologically different were discovered in a recent extensive collection of *Devaleraea* from the Northwestern (NW) Pacific. These specimens could not be easily attributed to any recognized species of *Devaleraea*. We hypothesized that these specimens were divergent *D. mollis* morphotypes. The goals of the this study were to: 1) identify the species affinity of these algal specimens; 2) describe the morphological variability of *D. mollis* in the NW Pacific; and 3) expand the species description in accordance with the extent of this morphological variability. To do this, we sequenced the COI-5′ and ITS regions of our specimens to assign them to known species, followed by a morphological analysis to understand the variability of *D. mollis* anatomical and morphological traits.

MATERIAL AND METHODS

Samples of *Devaleraea* were collected at nineteen locations along the Russian Pacific coast in 2019 and 2021 (Table S1; Fig. 1). All samples were collected by hand from the intertidal zone or by SCUBA-diving from the subtidal zone. Specimens exhibiting noticeable differences in thallus shape, colour, thickness, or consistency were classified as separate morphotypes. For molecular analyses, at least one specimen of each morphological type from each place was used. The vouchers are kept in the Herbarium of the Museum of A.V. Zhirmunsky National Scientific Center of Marine Biology, FEB RAS (MIMB, Vladivostok, Russia) or in A.V. Skriptsova's personal collection (ASKR). We also examined the specimens labelled '*P. palmata*' from the National Museum of Nature and Science, Tsukuba, Japan (TNS), and obtained molecular data from eight of these specimens (Table S2). A total of 120 specimens were morphologically examined in the study.

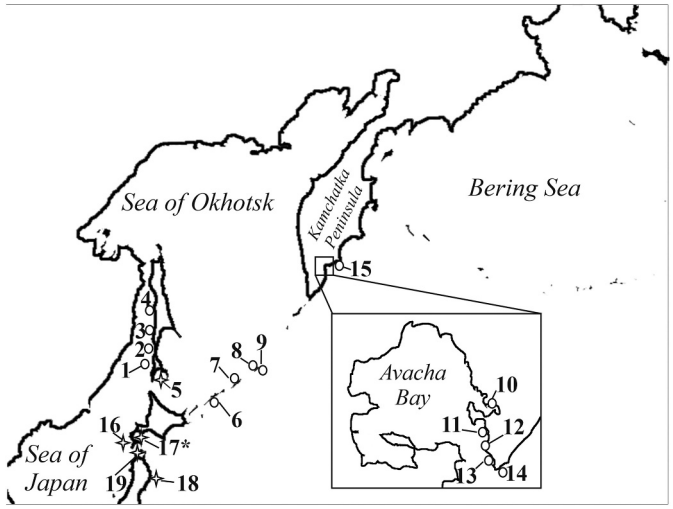


Fig. 1. Sampling locations of *Devaleraea mollis* and *D. inkyuleei* in the North-West Pacific. Sea of Japan, Tatar Strait, Sakhalin Island: 1, near Gornozavodsk village; 2, near Nevel'sk town; 3, near Yablochnoe village; 4, Cape Orlova. Sea of Okhotsk, Sakhalin Island: 5, Aniva Bay. Sea of Okhotsk, Kuril Islands: 6, Iturup Island, Kasatka Bay; 7, Urup Island, Smugliy Bay; 8, Simushir Island, Milna Bay; 9, Simushir Island, Broutona Bay. South-eastern Kamchatka: 10, Avacha Bay, Signal'nyi Peninsula; 11, Avacha Bay, Malaya Lagernaya Bay; 12, Avacha Bay, Bol'shaya Lagernaya Bay; 13, Shlyupochnaya Bay; 14, Avacha Gulf, Cape Mayachnyi; 15, Avacha Gulf, Krasheninnikova Island. Japan: 16, Hokkaido, Hakodate City; 17, Hokkaido, Muroran City (* type locality of *D. inkyuleei*); 18, Honshu, Iwate Prefecture, Yamada town; 19, Honshu, Aomori Prefecture, Hashinohe City. Circles, *D. mollis*; Stars, *D. inkyuleei*.

Morphological analysis

For each specimen, data on blade length and width, blade shape, and the presence and position of proliferations were collected. Cross sections were made by hand in the middle part of the thalli, using a razor blade. An AxioVert 200 M microscope (Zeiss, Oberkochen, Germany) was used to examine the sections. The size of medullary cells, tetrasporangia, and numbers of cell layers in the cortex and medulla were determined from at least five replicates of each thallus.

DNA extraction, PCR amplification and sequencing

For DNA analyses, one to three specimens of each morphotype from different locations were chosen. Total DNA was extracted from silica gel-dried specimens by the CTAB-extraction method (Wang *et al.* 2006). The cytochrome *c* oxidase subunit I (COI-5′) fragment was amplified using the forward primer DevF1 (Bringloe & Saunders 2019) and the reverse primer M13Rx (Saunders & Moore 2013), as described by Saunders & Moore (2013). ITS1 (forward) and JO6 (reverse) primers were used for PCR amplification and sequencing of ITS (ITS1–5.8S rDNA and partial ITS2; Lindstrom *et al.* 1996). We designed external forward psaA130DF (5′-AACCACGACGTGGATTTGGA-3′) and reverse psaADR (5′-CACGACCTGGACCATCACAAG-3′) primers to amplify and sequence photosystem I reaction centre apoprotein A1 (*psa*A); additionally, internal primers DpsaAF (5′-TTGGGGYATAGGACATAGYA-3′) and DpsaAR (5′-ARTTGAGCRTGCCAAGAAGT-3′) were used to sequence *psa*A. Cytochrome *b* (cob) was amplified and sequenced with the use of the pair of forward DcobF (5′-ARTAGTAAACGAYCA

TYTRGTRGA-3′) and reverse DcobR (5′-ACRGGCATRCCY CCTATYCA-3′) primers, designed by us. For *psa*A, the PCR profile was 95°C for 3 min, followed by 5 cycles of 94°C for 30s, 60.5°C annealing for 30s, 72°C extension for 1 min; then 35 cycles of 94°C for 30s, 62°C annealing for 30s, 72°C extension for 1 min; and 72°C final extension for 7 min. The PCR profile for cob was 95°C for 2 min; followed by 35 cycles of 94°C for 30s, 52°C annealing for 30s, 72°C extension for 1 min, and 72°C final extension for 7 min.

The PCR products were sequenced bidirectionally on an ABI 3130x sequencer (Applied Biosystems). FinchTV software v1.4.0 (Geospiza Inc., Seattle, Washington, USA; http://www.geospiza.com) was used to view the sequences. All sequences were aligned in MEGA X (Kumar *et al.* 2018), and any necessary changes were made manually. The sequences obtained in this study have been submitted to GenBank with the accession numbers provided in Table S2.

Genetic identification

The COI-5′ and ITS were used to identify species based on molecular criteria. Sequences of *Devaleraea* spp and *Palmaria* spp from GenBank and BOLD (Ratnasingham & Hebert 2007) systems, Lindstrom *et al.* (1996), and own sequences were used (Table S2). All newly obtained sequences were identified using distance and topological criteria on the basis of the reference database. The inter- and intraspecific COI-5′ distances (%) were calculated in MEGA X using the uncorrected *p*-distance. This software was also used to reconstruct a Neighbour-joining (NJ) tree from the COI-5′ dataset using an uncorrected *p*-distance. To estimate node support, 1,000 bootstrap (bs) replicates were done. *Palmaria palmata* was used as outgroup. To simplify the NJ tree, identical sequences were removed from the alignment before analysis, and only unique haplotypes were used. The Maximum Likelihood (ML) and phylogenetic tree was built based on the alignment of 75 ITS1-5.8S-ITS2 in MEGA X using a Tamura-Nei+G substitution model, complete deletion for gaps/missing data treatment, and 500 replications for bootstrap estimation. In addition, an NJ tree was built based on ITS and bs was added to the ML tree.

Phylogenetic analyses

To clarify phylogeny and species diversity of *Devaleraea*, Bayesian inference (BI) and maximum likelihood (ML) methods were used on concatenated COI-5′+cob+*psa*A+ITS sequences. MrBayes v3.2.6 (Huelsenbeck & Ronquist 2001) was used for Bayesian inference tree reconstructions and ML analysis was performed using RAxML v8.2.12 (Stamatakis 2006). PartitionFinder v2.1.1 (Lanfear *et al.* 2012) was used to identify the best-fit partitioning scheme and models separately for ITS and each codon position for *psa*A, COI-5′ and cob using the greedy algorithm with linked branch lengths for the corrected Akaike information criterion. The best-fit model for second codon positions of *psa*A was F81 (Felsenstein 1981), whereas the best-fit model for second codon of COI-5' and cob genes was F81+I. HKY+G (Hasegawa *et al.* 1985) was the best-fit model for ITS and the third codon position of cob. GTR+I (Tavaré 1986) was the best model for the first codon positions of the *psa*A, COI-5' and cob loci, while GTR+G was the best-fit for the third codon positions of *psa*A and COI-5' genes.

Species delineation

For species delineation, the Automated Barcode Gap Discovery (ABGD; Puillandre *et al.* 2012) and Multi-rate Poisson Tree Processes (mPTP; Kapli *et al.* 2017) methods were used. The ABGD was implemented using the Web interface available at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html, and the mPTP was implemented on a Web server (http://mptp.h-its.org/#/tree).

Molecular operational taxonomical units (MOTUs) were classified as putative species if they satisfied the following criteria: 1) the MOTUs were compatible in the ABGD and mPTP solution; and 2) the clades were well supported or were not contradicted in the concatenated gene tree.

RESULTS

We analysed 170 COI-5′ sequences (including 25 sequences generated in this study) from *Devaleraea* and *Palmaria* specimens collected on the North Atlantic and North Pacific coasts of Canada, in Japan, and along the Pacific coast of Russia. The majority of the sequences in the NJ tree were assigned to the *D. mollis* cluster (Fig. 2). This cluster included specimens that morphologically corresponded to the description of *D. mollis* (Hawkes & Scagel 1986), as well as specimens with unusual morphologies, such as abundant proliferations from blade margins and the thallus surface, or specimens with nearly formless blades (Fig. 2). The specimens from Japan (including the sequence AP019296 of '*P. palmata*') and the southern coast of Sakhalin Island with typical *D. mollis* morphology formed a well-supported (bs = 95%) cluster that was sister to *D. stenogona* (bs = 91%) and distant from *D. mollis* (Fig. 2). Phylogenetic analysis using ITS sequences yielded similar results (Fig. S1). As a result, the specimens from Japan and southern Sakhalin were considered as an undescribed species of *Devaleraea*.

The maximal COI-5′ intraspecific divergence was 0.4% in group *D. mollis sensu stricto* and 0.2% among Japanese specimens. The interspecific divergence ranged from 2.3% to 6.9%. The putative new species diverged from *D. mollis* by 2.8%, and it diverged from *D. stenogona* by 2.3%, whereas *D. mollis* and *D. stenogona* differed by 3.3%.

The ABGD and mPTP analyses revealed seven species: *D. stenogona, D. ramentacea, D. callophylloides* (M.W. Hawkes & Scagel) G.W. Saunders, C.J. Jackson & Salomaki, *D. titlyanoviorum* Skriptsova & T.L. Kalita, *Palmaria decipiens* (Reinsch) R.W. Ricker, *D. mollis* and a group of specimens from Japan and the southern Sakhalin (Fig. 2).

The Bayesian phylogenetic tree reconstructed on the basis of concatenated sequences of COI-5′, cob, ITS and *psa*A loci revealed seven distinct genetic species groups (including the outgroup *P. palmata*). Specimens from Japan constituted a well-supported clade (BPP = 1, bs = 86%) sister to *D.*

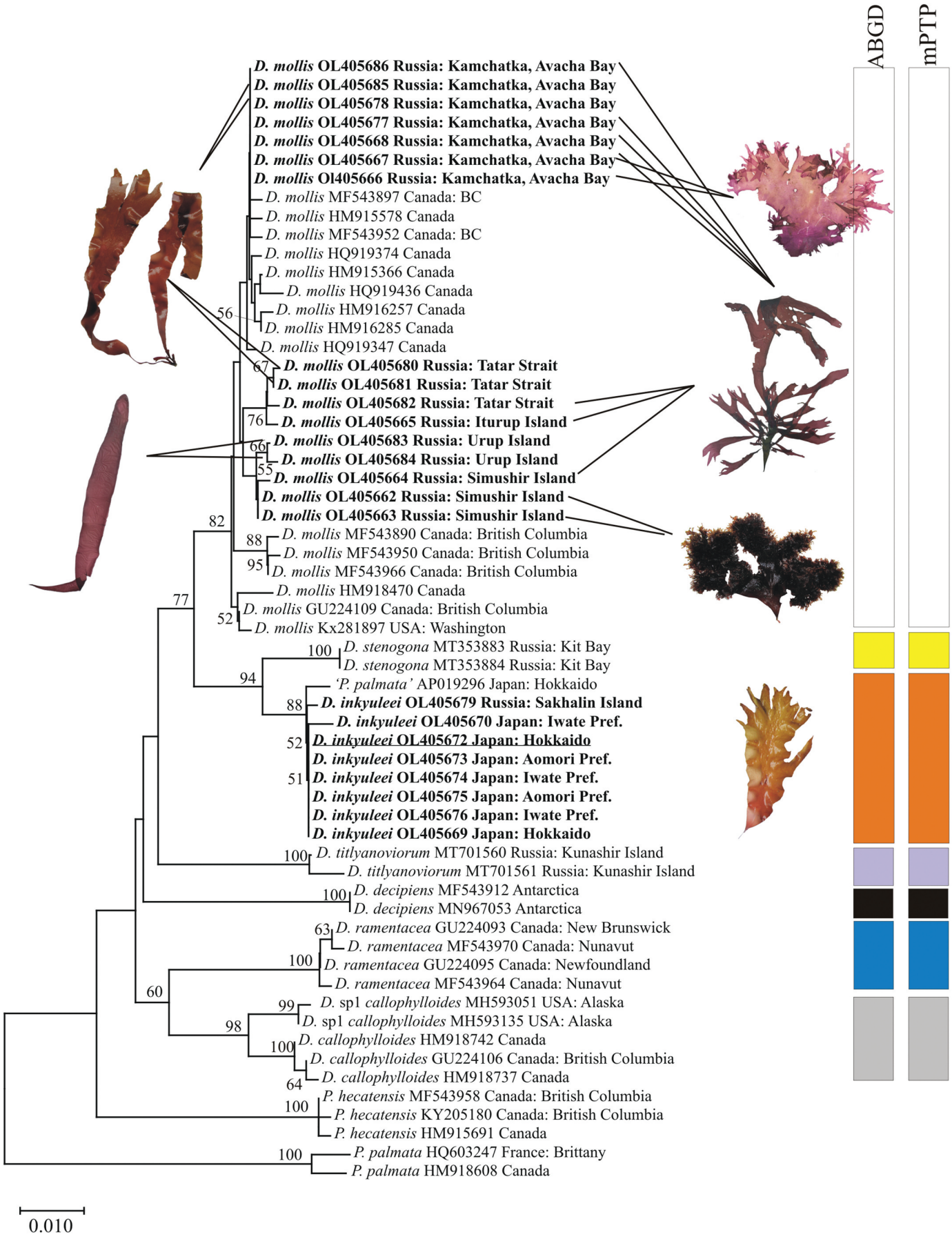


Fig. 2. Neighbour-Joining tree for COI-5' data indicating genetic groups resolved in the genus *Devaleraea*. Numbers on nodes indicate bootstrap values. Sequences generated in this study are indicated in bold. The holotype of *D. inkyuleei sp. nov*. is underlined. The different morphotypes of *D. mollis* connected with their sequences are shown. Columns on the right show results of the Automated Barcode Gap Discovery (ABGD) and Multi-rate Poisson Tree Processes (mPTP) analyses; various colours mean separate genetic species.

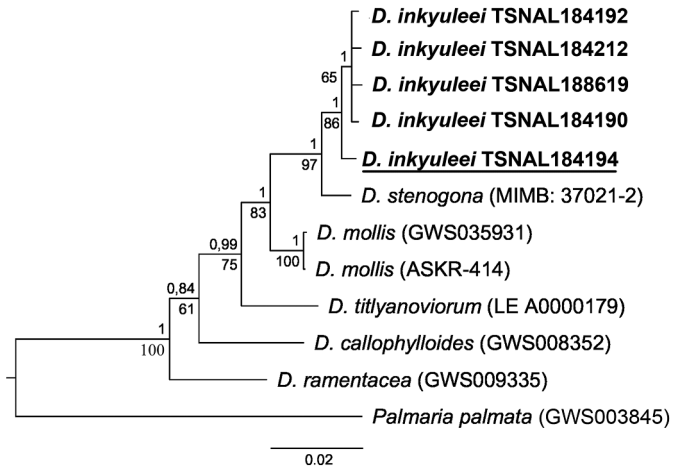


Fig. 3. Bayesian tree of *Devaleraea* inferred from concatenated COI-5′, cob, ITS and *psa*A sequences. The holotype of *D. inkyuleei* is underlined. Numerals above tree nodes are Bayesian posterior probabilities; below nodes, ML bootstrap values.

stenogona, with which they formed a highly supported clade (BPP = 1, bs = 97%; Fig. 3). Based on phylogenetic and DNA-barcoding data, we conclude that *Devaleraea* from Japan is a distinct species, which we name *D. inkyuleei*.

Devaleraea inkyuleei Skriptsova & Mas. Suzuki *sp. nov.*

Figs 4–13

HOLOTYPE: TNS-AL 184194, 42°18′N, 140°58′E, Denshinhama, Honcho, Muroran, Hokkaido, Japan, collected 20 May 2012 by M. Suzuki, deposited in the National Museum of Nature and Science, Japan (TNS).

HOLOTYPE DNA BARCODE: COI-5′, OL405672; *psa*A, OL405653; cob, OL405659; ITS, OL352699.

ISOTYPE: TNSAL184195, collected 20 May 2012 by M. Suzuki (deposited in TNS).

REPRESENTATIVE DNA BARCODES: See Table S2.

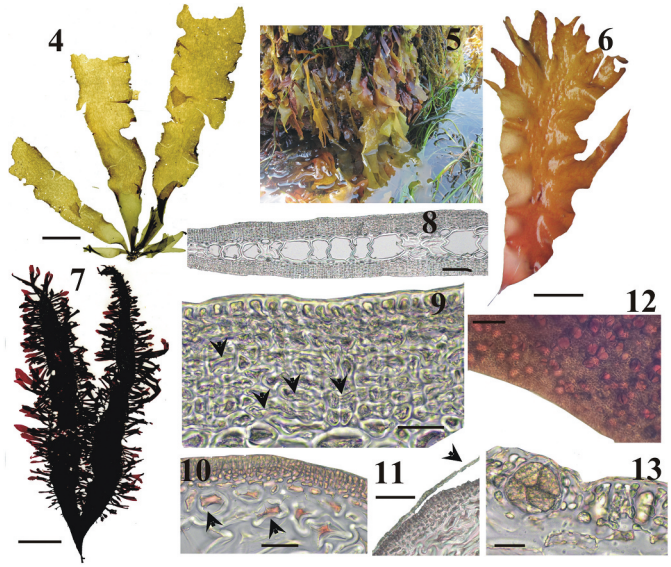
TYPE LOCALITY: 42°18′N, 140°58′E, Denshinhama, Honcho, Muroran, Hokkaido, Japan.

ETYMOLOGY: This species is named in honour of Dr. In Kyu Lee for his contribution to our understanding of the Palmariaceae in Japan.

DISTRIBUTION: Known from Hokkaido and northeastern Honshu, Japan, and the southern coast of Sakhalin Island (Aniva Bay), where it grows on rocks and boulders in the lower intertidal zone.

MATERIAL EXAMINED: Listed in Table S1.

DESCRIPTION: Blades up to (and sometimes exceeding) 30 cm in height, 1.5–6 cm in width and 200–627 µm in thickness. Blades membranous to coriaceous, not hollow, narrowly cuneate, gradually broadening upwards, simple to somewhat palmate, with irregular distal margin, apices rounded; abundantly proliferous along margin when old. Blades reddish-brown in colour, fading to yellowish or yellowish-green (Figs 4–7). Medulla consisting of 1(–2) layers of large colourless cells, 122–167 × 190–440 µm, with thick cell-walls (7–9 µm) and with areas of smaller cells packed among large cells (Fig. 8). Around this central layer, 1–3 layers of smaller (20–52 µm) thick-walled medullary cells present in many specimens. Inner cortex consisting of 1–2 layers of pigmented cortical cells, 8–9 µm in diameter, which are surrounded by 3–4 layers (up to 10 in basal parts of blade and in old plants) of outer cortical

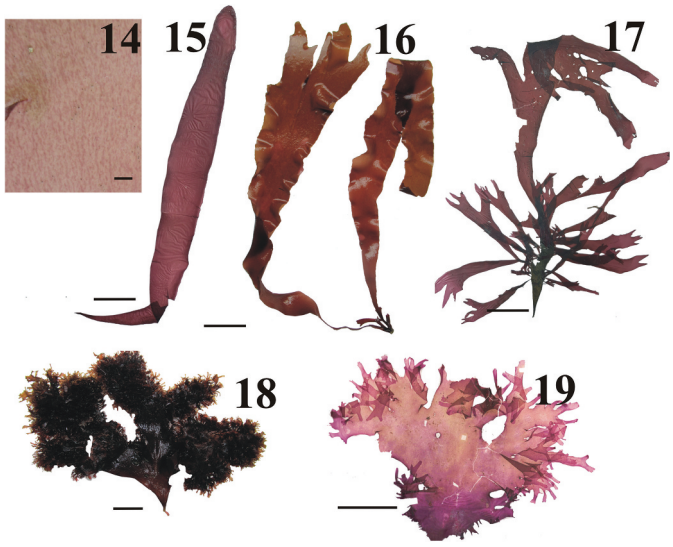


Figs 4–13. *Devaleraea inkyuleei sp. nov.*, morphology and anatomy.
Fig. 4. Holotype of *D. inkyuleei*. Scale bar = 5 cm.
Fig. 5. Algae growing in intertidal zone at Denshinhama, Honcho, Hokkaido, Japan.
Fig. 6. Specimen collected in May 2021 in a low intertidal zone in the Aniva Bay (Sakhalin Island). Scale bar = 5 cm.
Fig. 7. Specimen collected in August 2006 in Kabushima, Hachinohe City, Aomori Prefecture, Japan, with abundant proliferations at the margin. Scale bar = 5 cm.
Fig. 8. Cross-section through vegetative thallus of old plant showing a large-celled medulla and a multi-layered, small-celled cortex. Scale bar = 100 µm.
Fig. 9. Fragment of a multi-layered cortex showing abundant cell fusions (arrows). Scale bar = 20 µm.
Fig. 10. Fragment of the cortex of a young plant. Arrows indicate subcortical cells. Scale bar = 20 µm.
Fig. 11. Cuticle (arrow). Scale bar = 20 µm.
Fig. 12. Surface view on the margin showing a narrow space at the margin not occupied by tetrasporangia. Scale bar = 100 µm.
Fig. 13. Tetrasporangia. Scale bar = 20 µm.

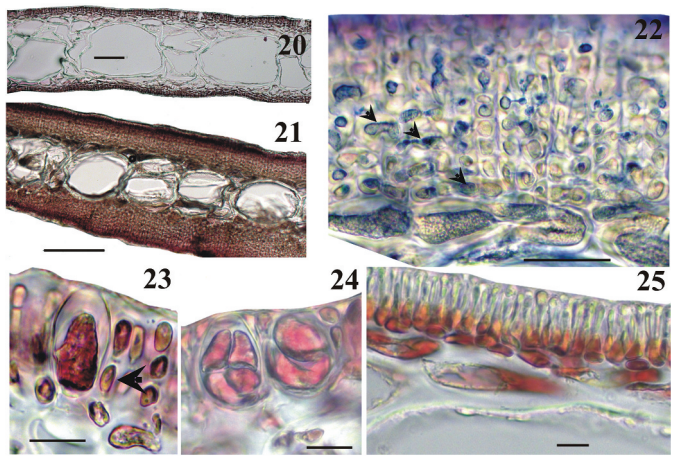
spherical or cuboidal cells (Fig. 10). In young blades the inner cortical layer may be absent. Typically, fusion between adjacent cortical cells occurs in old plants (Fig. 9). Cortex is covered with cuticle (Fig. 11). Rare unicellular epidermal hairs develop from outermost cortical cells. Tetrasporangia sori develop on both blade surfaces except at the extreme (15–90 µm) margin or occur at the margin (Fig. 12). Tetrasporangia subspherical, 34–42 × 36–44 µm, developing among sterile cortical cells in elongated filaments 3–4 cells long (Fig. 13).

Morphological observation of NW Pacific *D. mollis*

An examination of all NW Pacific specimens genetically assigned to *D. mollis* shows that this species is morphologically extremely variable. Although blades were typically reddish-brown in colour, often they were unevenly coloured, with darker red stripes spreading across the entire blade, even in sterile plants (Fig. 14), or with mottled lightly-coloured patches in some tetrasporangial plants. The blade's consistency ranged from soft membranous to firm coriaceous and was determined by cortex development. The thallus shape of specimens from various locations (Figs 15–19) ranged from almost oval, simple, dichotomously or palmately divided into broad lobes (typical for *D. mollis*; Figs 15, 16), to abundantly proliferous (Figs 17, 18) or nearly formless (Fig. 19).



Figs 14–19. Morphological variability of *Devaleraea mollis*.
Fig. 14. Uneven colouration of the blade. Scale bar = 0.5 cm.
Fig. 15. Typical morphology of the species. Plant from Simushir Island collected on 29 June 2019. Scale bar = 5 cm.
Fig. 16. Specimen from the west coast of Sakhalin Island collected on 5 May 2021. Scale bar = 5 cm.
Fig. 17. Plant with proliferations on the margin (collected in May on the west coast of Sakhalin). Scale bar = 5 cm.
Fig. 18. Plants abundantly proliferating from surface, collected in August in Broutona Bay (Simushir Island). Scale bar = 2 cm.
Fig. 19. Plant with a nearly formless blade collected in June 2021 in Avacha Bay (the eastern Kamchatka). Scale bar = 5 cm.



Figs 20–25. Anatomy of *Devaleraea mollis*.
Fig. 20. Cross-section through the vegetative thallus of a young plant shows large-celled medulla surrounded by smaller cells and a one-two-layered, small-celled cortex. Scale bar = 100 µm.
Fig. 21. Cross-section through the vegetative thallus of an old plant showing a multi-layered cortex. Scale bar = 200 µm.
Fig. 22. Fragment of a multi-layered cortex showing abundant cell fusions (arrows). Scale bar = 20 µm.
Fig. 23. Young tetrasporangia among three- or four-celled vegetative cortical filaments (arrow). Scale bar = 20 µm.
Fig. 24. Mature tetrasporangia. Scale bar = 20 µm.
Fig. 25. Spermatangia. Scale bar =10 µm.

Development of proliferations was mostly determined by the age of the plants. Most blades collected in May–June on Kamchatka, Sakhalin Island or the Kurile Islands were large and linear, wide wedge-shaped, simple or palmately divided. The blade length and width varied in a range of 14 to 52 cm and 1.5 to 19 cm, respectively, with length to width ratio of 2–18. However, several plants collected in the same areas in May–June had old blades that had been destroyed to varying degrees, from which new blades regenerated (Fig. 17). Such plants were obviously biennial. New blades developed on the short remnants of the old blade in these plants; they were narrowly wedge-shaped, palmately divided once or twice, or rarely simple; their length was 6–16 (–30) cm and width 0.9–4.5 cm, with length to width ratio of 3–12. In August, after spore discharge, blades were partially destroyed and some proliferated from the blade margin or surface (Fig. 18). These proliferations ranged in size from 0.5 to 2 cm long and 0.2–0.5 cm wide, and in shape from narrowly lanceolate, to linear or obovate, and simple or divided into lobes. The most exotic appearance was observed in the plants collected in the mid-intertidal zone of Broutona Bay, a wave-protected bay covered by ice during winter: short (up to 10 cm) and broad (up to 12 cm) old blades were completely covered with new short (up to 1.2 cm long and 0.5 cm wide) oval blades (Fig. 18). Such plants grew alongside plants with long proliferations from the old blade's margin (Fig. 17).

Anatomical observations showed that the medulla was typically composed of one layer of large (90–580 µm) transparent cells surrounded by one or two rows of smaller (18–75 × 23–118 µm) cells (Fig. 20). The thickness of the cortex varied within the same blade: in apical and middle parts of the blade, the cortex was typically composed of one to two layers of small rounded cells, and in the basal part and old blades the cortex consisted of three to seven layers of cells in anticlinally oriented rows (Figs 21, 22). We observed cell fusions of two, three and more cells in the multilayered cortex (Fig. 22). Rare unicellular epidermal hairs developed from the outermost cortical cells. Tetrasporangia developed on the entire blade surface except the extreme margin and the basal part. Tetrasporangia were broad-oval or subspherical, 18–49 × 28–72 µm (Fig. 24). They developed among three- or four-celled cortical filaments (Fig. 23). Spermatangia were narrow, 7–10 µm long, in continuous sori on both blade surfaces (Fig. 25).

We did not see any differences between Japanese *D. inkyuleei* and *D. mollis* in morphology or anatomy of macrothalli (Table 1).

DISCUSSION

Determining species boundaries is one of the main tasks of taxonomic studies. This is especially critical in species with simple morphologies, like the Palmariaceae, which lack female reproductive structures on the macrothalli or post-fertilization development, which are hallmarks of red algal classification. Due to the high morphological plasticity of these algae, identifying specimens only on the basis of descriptions available in the scientific literature is sometimes impossible. The study of morphological plasticity of species across their geographic range, in various ecological conditions, and among specimens of different ages, can reveal new data on species morphology

Table 1. Comparison of morphological and anatomical characters of *Devaleraea inkyuleei sp. nov*. with morphologically (*D. mollis)* and genetically (*D. stenogona*) closely related species from the North-West Pacific.

Characters	*D. mollis*	*D. inkyuleei*	*D. stenogona*
Anatomical			
Number of large medullary cell layers	1, occasionally 2–3	1–2	1–2
Large medullary cells: maximal diameter, μm	210 ± 90 (90–580; *N* = 107)	217 ± 86 (80–440, *N* = 72)	50–400
Number of layers of small cells surrounding large medullary cells	typically absent or present, sporadically form a discontinuous layer	typically absent, if present they form 1–2 layers	1–2
Number of cortex cell layers	1–2, in old blades and basal part up to 6–7	2–4(–6), in old blades and in basal part up to 6–12	1–2, to up to 10 in the basal portions or old thalli
Unicellular epidermal hairs	present	present	unknown
Tetrasporangia (width × length, μm)	31.9 ± 6.5 × 42.0 ± 9.6 (18–49 × 28–72, *N* = 66)	33.7 ± 7.1 × 45.2 ± 7.6 (25–55 × 34–60, *N* = 19)	35–60
Morphological			
Shape of blades	linear, wide-cuneiformis, simple, dichotomously and palmately lobed or formless	linear, wide-cuneiformis, simple, dichotomously and palmately lobed in upper part	narrow wedge-shaped, dichotomous, palmately or irregularly branched, terminal branches are linear with acute tips
Blade sizes (length × width, cm; length to width ratio)	14–55 × 1.5–19; 1:2–18	9–25 × 0.8–6; 1:3–14	up to 25(–45) × 1–3
Shape of proliferation	lanceolate, linear, obovate, simple or divided on lobes, developing on the margin or entire blade surface	linear or lanceolate, simple or dichotomously divided, develop on margin	short, linear or hair-like proliferations
Proliferation sizes (length × width, cm)	0.5–16 × 0.2–4.5	0.2–1.7 × 0.1–0.3	nd

nd – no data.

that can be use to expand species descriptions and draw species boundaries. Despite the number of vegetative criteria that have been used to differentiate species of *Devaleraea*, members of this genus can exhibit extensive variability in habit, making accurate species-level identification difficult. This is particularly true of the solid *Devaleraea*. Because anatomical features can be similar in several species, for example in *D. mollis, D. stenogona* and *D. titlyanoviorum*, they cannot be used alone to differentiate species. In such circumstances, the use of molecular tools is essential for accurately assigning specimens to a species.

Based on a study of over 100 morphologically different but genetically determined *D. mollis* specimens, we observed great morphological variation in the Western Pacific. This variability may be related to large-scale regional or local spatial variability of environmental factors, as well as seasonal variation. It has been shown that key abiotic factors that determine morphology of seaweeds are wave exposure, ice abrasion and light availability (Shibneva & Skriptsova 2015). To reduce hydrodynamic drag and protect against breakage and dislodgement, algae in wave-exposed locations typically have long, narrow blades (Gutierrez & Fernández 1992; Shaughnessy *et al.* 1996). High wave velocity and scouring by ice may also alter morphology of seaweeds by damaging and tattering blades, which then regenerate as formless blades. In our investigation, algae with various morphologies (simple, lobed, proliferous or formless blades) were often collected from the same location, whereas morphologically similar *D. mollis* plants were found in separate places. The development of morphologically different blades of *D. mollis* in the same place may be associated with micro-scale environmental variation, when very different current velocity, light intensity and wave activity can be observed across small spatial scales owing to microtopography. Differences in morphology can also originate from factors relating to the biology of the seaweeds themselves, such as algal age and life cycle stage (for review see Shibneva & Skriptsova 2015). We presume that the development of proliferations depends on the age of the plants to a greater extent, and that it is a characteristic of old (after spore release) or perennial blades. Great variation in proliferations was also reported in *D. ramentacea* (Kjellman 1883; Zinova 1955), which was related to wave exposure and depth, as well as to the age of the alga (Kjellman 1883).

Our molecular study revealed that specimens from Japan and the southern Sakhalin, morphologically resembling *D. mollis* are a distinct species genetically closer to *D. stenogona*. Nevertheless, *D. inkyuleei* clearly differs morphologically from *D. stenogona* (Table 1). This discrepancy between morphology and genetics is not a novel phenomenon in the genus. Previously, we reported that *D. stenogona* is a complex of at least two species, with the new species, *D. titlyanoviorum*, being the sister taxon of *D. mollis* rather than *D. stenogona* (Skriptsova *et al.* 2021).

In an attempt to find morphological features that distinguish *D. inkyuleei* from *D. mollis*, we analysed the nearly complete set of qualitative traits (except the position of spermatangia) available for species identification and delimitation in the solid *Devaleraea* and *Palmaria*, based of macrothallus morphology. However, a detailed investigation revealed no obvious qualitative or quantitative trait that distinguishes the two species. The sole difference between these species was the range of variability of some measured characteristics (Table 1). Thus, we regard *D. inkyuleei* as a cryptic species, which can be distinguished from *D. mollis* only genetically or biogeographically.

The distribution patterns of *D. inkyuleei* and *D. mollis* are different (Fig. 1). *Devaleraea mollis* occurs from the Bering

Sea to Sakhalin Island, where it was found on the west coasts. *Devaleraea inkyuleei* is limited in range to Hokkaido and the northeastern Honshu, and southern Sakhalin. Distributions of both species appear to overlap in southern Sakhalin; however, *D. inkyuleei* is typically more southerly in its range. As was shown, species biogeography is an important feature that might aid in species separation in the field (Lindstrom *et al.* 2011). An integrative approach incorporating molecular, morphological, habitat and biogeographic data was used to differentiate species in the genera *Mastocarpus, Pyropia, Rhodymenia* and *Sheathia* (Lindstrom *et al.* 2011, 2015; Filloramo & Saunders 2016; Vis *et al.* 2020).

Red algae species descriptions have traditionally been almost exclusively based on morphological characters, and the species are thus morphospecies; this approach can lead to incorrect estimation of true species diversity in different algal groups due to high intraspecific morphological polymorphism or the failure of the species to diverge morphologically even after they have become separate biological species. The biological species concept is currently being used to determine species boundaries in various algal groups, including Rhodophyta. This concept is closely allied to the evolutionary species concept and focuses on reproductive isolation to distinguish species (for review see Leliaert *et al.* 2014). According to Saunders (2008), genetic species groups are expected to be consistent with biological species because genomes acquire independent mutations when populations become reproductively isolated. When numerous species-level markers (e.g. nuclear and organellar) resolve the same groupings but rigorous morphological study fails, Clarkston & Saunders (2010) suggest that the molecular evidence should take precedence, and separate genetic groups should be recognized as different species. In modern taxonomic phycological studies, the establishment of new species solely on the basis of genetic differences without revealing morphological diagnostic characters is not rare. True cryptic species were described as new species in the red algal genera *Gloipeltis* (Yang & Kim 2017), *Champia* (Griffith *et al.* 2017), *Dasya* (Schneider *et al.* 2017), *Botryocladia* (Filloramo & Saunders 2018), *Sheathia* (Vis *et al.* 2020) and *Lithophyllum* (Caragnano *et al.* 2020). In certain cases, cryptic species may be distinguished by their distribution (Schneider *et al.* 2017; Yang & Kim 2017; Filloramo & Saunders 2018; Vis *et al.* 2020), whereas in other the distribution ranges overlap, or the species coexist in sympatry (Lyra *et al.* 2016; Zuccarello *et al.* 2018; Caragnano *et al.* 2020). Further comprehensive morphological investigations, including culture studies and detailed analyses of reproductive anatomy, can sometimes reveal a crucial morphological difference between cryptic species (Schneider *et al.* 2017; Yang & Kim 2017). It is possible that differences between *D. mollis* and *D. inkyuleei* may be discovered in sizes and position of spermatangia or in the features of female gametophyte and carpogonia development.

It should be noted that *D. inkyuleei* described here is a well-known species in northern Japan, having previously been recognized as *Palmaria palmata* or *Palmaria* sp. and, before that, *Rhodymenia palmata* (Japanese name: Darusu; Tazawa 1975; Lee 1978; Yabu & Yasui 1984; Deshmukhe & Tatewaki 1990; Mine & Tatewaki 1994; Yoshida *et al.* 2015). Some studies on this alga revealed that it differs from true *P. palmata* in terms of chromosome number (Yabu 1971, 1976) and female gametophyte development (Yabu & Yasui 1984; Deshmukhe & Tatewaki 1990). Although Mine & Tatewaki (1994) recognized that the species required a new name, they called it *Palmaria* sp. Later, complete chloroplast genome (AB807662; Kumagai *et al.* 2019b) and mitochondrion (AP019296; Kumagai *et al.* 2019a) sequences were obtained, and phylogenetic analyses performed on the concatenated three-gene alignment (COI-5′+*psb*A+*rbc*L) revealed that Japanese '*P. palmata*' was distinct from Atlantic *P. palmata* and was included in the clade containing *D. ramentacea, D. callophylloides, P. decipiens* and *D. mollis* as a sister branch to *D. mollis* (Kumagai *et al.* 2019a); nevertheless, this species has been until now called '*P. palmata*'. Because Japanese '*P. palmata*' was resolved within the *D. inkyuleei* genetic lineage in our analysis, we concluded that they are the same species.

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AUTHOR CONTRIBUTIONS

A.V. Skriptsova: seaweed collection, original concept, light microscopy, manuscript preparation. M. Suzuki: seaweed collection, DNA sequencing, manuscript preparation. A.A. Semenchenko: DNA sequencing, analysis of molecular data, manuscript preparation.

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