Research Article

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Brown algae (Phaeophyceae) from Russian Far Eastern seas: re-evaluation of *Laminaria multiplicata* Petrov et Suchovejeva

Tatyana A. Klochkova[1,*], Gwang Hoon Kim[1], Kyung Min Lee[2], Han-Gu Choi[3], Mihail N. Belij[4] and Nina G. Klochkova[5,*]

[1] *Department of Biology, Kongju National University, Kongju 314-701, Korea*
[2] *Department of Biology, Chungnam National University, Daejeon 305-764, Korea*
[3] *Korea Polar Research Institute, Songdo Technopark Get-Pearl Tower 7-50, Incheon 406-840, Korea*
[4] *Magadan Scientific-Research Institute of Fishery and Oceanography (MagadanNIRO), Magadan, Portovaya 36/10, 685014, Russian Federation*
[5] *Kamchatka State Technical University (KamchatGTU), Petropavlovsk-Kamchatsky, Klyuchevskaya 35, 683003, Russian Federation*

Eight unusual individuals of a laminariaceaen species were collected from the Sea of Okhotsk in 1974 and described as a new species, *Laminaria multiplicata* Petrov et Suchovejeva in 1976. Since that time no new information, including pictures and numerical data, has been provided, although the species was cited in floristic lists of the Sea of Okhotsk based on the first record in 1976. We investigated a type and 3 paratypes of *L. multiplicata* and strongly believe that they were wrongfully identified abnormal plants of *L. gurjanovae* A. Zinova. Therefore, the species *L. multiplicata* needs to be closed.

Key Words: endemic species; flora; *Laminaria gurjanovae*; *L. multiplicata*; Sea of Okhotsk

INTRODUCTION

The genera *Laminaria* and *Saccharina* are the most diverse among kelps that have attracted research interest from the phycological and industrial points of view. They produce high biomasses and extensive covers on rocky shores of the Russian Far Eastern area (Klochkova and Berezovskaya 1997, Klochkova et al. 2009).

Studies on the kelp flora from the Sea of Okhotsk, especially its northern areas, have proceeded slowly due to difficulties in transportations and harsh weather conditions. Moreover, the continental coast line of the northern areas of the Sea of Okhotsk is sparsely populated. Among early phycological research, remarkable contributions were made by Ruprecht (1850), Schapova (1948), Zinova (1953), Zinova (1954), Blinova (1968), Zinova et al. (1980), Petrov and Vozzhinskaja (1966, 1970), and Petrov and Suchovejeva (1976), who were among the pioneers of Russian phycology and provided the first floristic lists of seaweeds or described new and endemic species from the Sea of Okhotsk. More recent works have described new and endemic kelp genera and species from the Sea of Okhotsk (Klochkova and Krupnova 2004, Cho et al. 2006).

The Sea of Okhotsk was reported to be inhabited by several endemic species, including *Laminaria appressizhiza* Petrov et Vozzhinskaja, *L. inclinatorhiza* Petrov et Vozzhinskaja, *L. multiplicata*, *Phyllariella ochotensis* Petrov et Vozzhinskaja, *Pseudolessonia laminarioides* (Postels et Ruprecht) Cho, Klochkova, Krupnova et Boo, and *Tauya*

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[*]**Corresponding Author**
E-mail: tatyana1905@rambler.ru, ninakl@mail.ru
Tel: +82-41-850-8504, Fax: +82-41-850-8504

basicrassa Kloczcova et Krupnova. However, the status of a number of rare or endemic species of laminariaceaen algae from the Russian Pacific was questioned, including the generic affiliation of *L. appressizhiza, L. inclinatorhiza*, and *L. multiplicata* among those currently attributed to the genus *Laminaria* (Selivanova et al. 2007). In this paper, we attempted to clarify the question raised by the latter study regarding one rare endemic species from the Sea of Okhotsk, *L. multiplicata*, which was described as a new species (Petrov and Suchovejeva 1976) based on eight plants collected from the Sea of Okhotsk in 1974.

MATERIALS AND METHODS

Sample collection and specimen observation

One of the authors (M.N. Belij) has scuba-dived in various northern localities of the Sea of Okhotsk for many years, including the type locality of *L. multiplicata*, Ejrinejskaya Bay, Bolshaya Molta, and has conducted underwater algal collections in different seasons of the year, gathered abundant kelp seaweed samples, and taken underwater photographs. Another author (N.G. Klochkova) participated in several hydrobiological expeditions in the Sea of Okhotsk and worked in its northern continental areas in 2006 and 2008.

Of the eight plants collected in June 1974 (Petrov and Suchovejeva 1976), we studied dry specimens of three plants (type and two paratypes) that are currently held in the Komarov Botanical Institute of Russian Academy of Sciences (KBI RAS, Saint-Petersburg, Russia) and one piece of plant (paratype 3) kept by Dr. M. Suchovejeva in the Pacific Institute of Fishery and Oceanography (TINRO-center, Vladivostok, Russia). It is noteworthy to mention that the type and paratype 1 were not kept in the form they were displayed in Petrov and Suchovejeva's paper (1976), but were re-hydrated again after initial photography, then folded and dried. The blade of paratype 1 was detached from its holdfast.

For microscopic observations, we used type and paratype of *L. multiplicata* from KBI RAS. A dry piece of plant was re-hydrated in sterilized seawater, cut with a fine razor blade, and observed under a microscope. Micrographs were taken with Olympus DP50 digital camera affixed to an Olympus BX50 microscope (Olympus, Tokyo, Japan) using Viewfinder Lite and Studio Lite computer programs.

Collections of dry specimens of Russian laminariaceaen algae kept in KBI RAS, TINRO-center, and Kamchatka State Technical University (KamchatGTU, Russia) were also studied. The list of all studied specimens is given in Table 1.

Table 1. List of specimens in this study

Species	Collection site	Collection date	Collector
Laminaria digitata (Hudson) Lamouroux	Ny-Ålesund, Svalbard. Plant cast ashore	06.15.2009	Klochkova T. A. & Kim G. H.
L. gurjanovae **(type specimen)**[a]	Sakhalin Island, Aniva Inlet, Ozeretzkoe village, Russia (formerly USSR). Sublittoral zone	09.07.1955	Vozzhinskaja V. B.
L. gurjanovae f. *lanciformis*	Korf Inlet, Kamchatka, Russia. Plant found on littoral zone during syzygial tide	05.22.1998	Klochkova T. A.
L. gurjanovae f. *lanciformis*	Attargan Bay, Sea of Okhotsk, Russia. Scuba diving	07.15.2008	Klochkova N. G. & Belij M. N.
L. gurjanovae f. *lanciformis*	Taujskaya Bay, Sea of Okhotsk, Russia. Plants cast ashore	07.14.2008	Klochkova N. G.
L. multiplicata **(type specimen)**	Ejrinejskaya Bay, Bolshaya Molta, Sea of Okhotsk, Russia (formerly USSR). Scuba diving	06.27.1974	Suchovejeva M.V.
L. multiplicata **(paratypes 1-3)**	Ejrinejskaya Bay, Bolshaya Molta, Sea of Okhotsk, Russia (formerly USSR). Scuba diving	06.27.1974	Suchovejeva M.V.
Saccorhiza sp.	Ny-Ålesund, Svalbard. Plant cast ashore	06.15.2009	Klochkova T. A. & Kim G. H.

[a]While describing *L. gurjanovae*, Zinova (1964) did not provide picture of the type specimen, but published it in a subsequent paper (Zinova 1969).

DNA extraction

For DNA extraction, we used type of *L. multiplicata* from KBI RAS. DNA extraction was performed in January 2009, thus the material was approximately 35 years old. The DNA was extracted using 1) i-genomic DNA Extraction Mini Kit (iNtRON Biotechnology, Seongnam, Korea) following the manufacturer's instructions and 2) hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). During the stages of incubation in 100% isopropanol (CTAB method), the samples were incubated at -20°C for 1-4 weeks. Polymerase chain reaction (PCR) was performed using primers displayed in Table 2, with an initial denaturation at 94ºC for 4 min, followed by 35 cycles of amplification (denaturation at 94ºC for 30 s, annealing at 45ºC for 30 s and extension at 72ºC for 1 min) with a final extension at 72ºC for 10 min.

Table 2. Oligonucleotide primers used to amplify internal transcribed spacer region in *Laminaria multiplicata*

Primer	Direction	Sequences	Reference
LB1	Forward	5' CGC GAG TCA TCA GCT CGC ATT 3'	Yoon et al. 2001
YB1	Forward	5' TTG CAG AAT CCA GTG AAT CAT C 3'	Yoon et al. 2001
BC2	Reverse	5' CGA GTG GTG TCA ACA GAC ACT CC 3'	Saunders and Druehl 1993
LB2	Reverse	5' AGC TTC ACT CGC CGT ACT GG 3'	Yoon et al. 2001

RESULTS AND DISCUSSION

Laminaria multiplicata Petrov et Suchovejeva

Basionym: *Laminaria multiplicata* Petrov et Suchovejeva 1976.
Russian name: Ламинария многоскладчатая. ***Herein:*** *многоскладчатая* – having many folds.
Synonym: None.
Type: Sea of Okhotsk, Ejrinejskaya Bay, Bolshaya Molta (59º22' northern latitude 145º48' eastern longitude, Figs 1-3), depth 3 m, 27.VI.1974, col. by Suchovejeva M.V., kept in Botanical Institute of Russian Academy of Sciences, Leningrad (currently KBI RAS, Saint-Petersburg).

Original description by Petrov and Suchovejeva (1976) in Russian language: In June 1974, the scuba divers of the scientific-research ship of the Pacific Scientific-Research Institute of Fishery and Oceanography (TINRO) have collected 8 plants, which were later named as *L. multiplicata* (Petrov and Suchovejeva 1976). The plants had elliptical or wide cuneiform blades of 56-102 cm long and 21-40 cm wide and up to 1.5 mm thick, with numerous narrow longitudinal folds (Rus.: складки). The folds were 0.5-33 mm in width and arose over the blades surface at 0.6 mm, making it thickened or sagged. The blades had 20-30 or more folds. In fresh plants, a wide middle stripe was distinguished. The blades' edges had small waving. The medullar layer occupied 0.4-0.5 of the blade's total width. Mucilage channels were present in the cortex layer and sometimes in the intermediate layer. Holdfast was thin, 17-28 cm long, with a ring of mucilage channels and lacunas; rhizoids had a ring of mucilage lacunas. Sporangia appeared as narrow and short lines on the folds, later forming spots on both sides of the blade coinciding in shape. This species grew at 3 m depth. It was similar to *L. gurjanovae* A. Zin. in 1) sporangia sori shape that coincided on both sides of the blade, 2) blade shape, and 3) length of holdfast. Perhaps, it originated from *L. gurjanovae* A. Zin., however differed from this and all other species of *Laminaria* by having numerous folds. However, it could not be attributed to *Cymathere* due to small thalli sizes and presence of numerous folds, and also because it was similar looking with one of the *Laminaria* species (herein: *L. gurjanovae*).

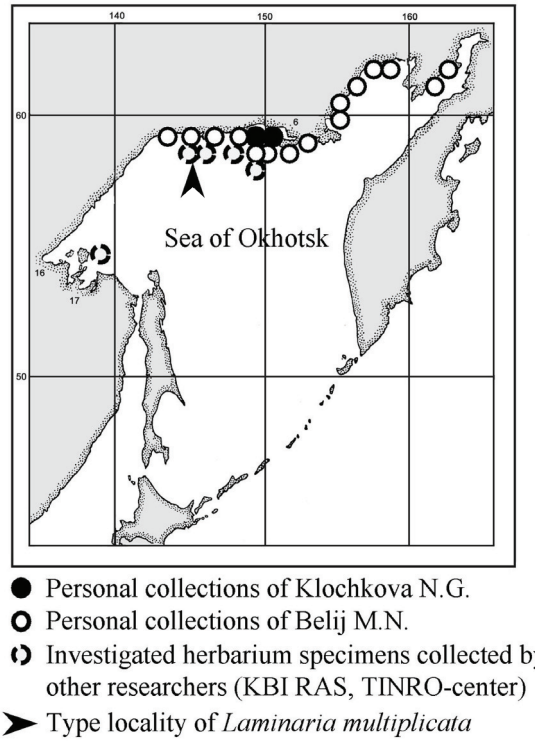

Fig. 1. Map of the research area.

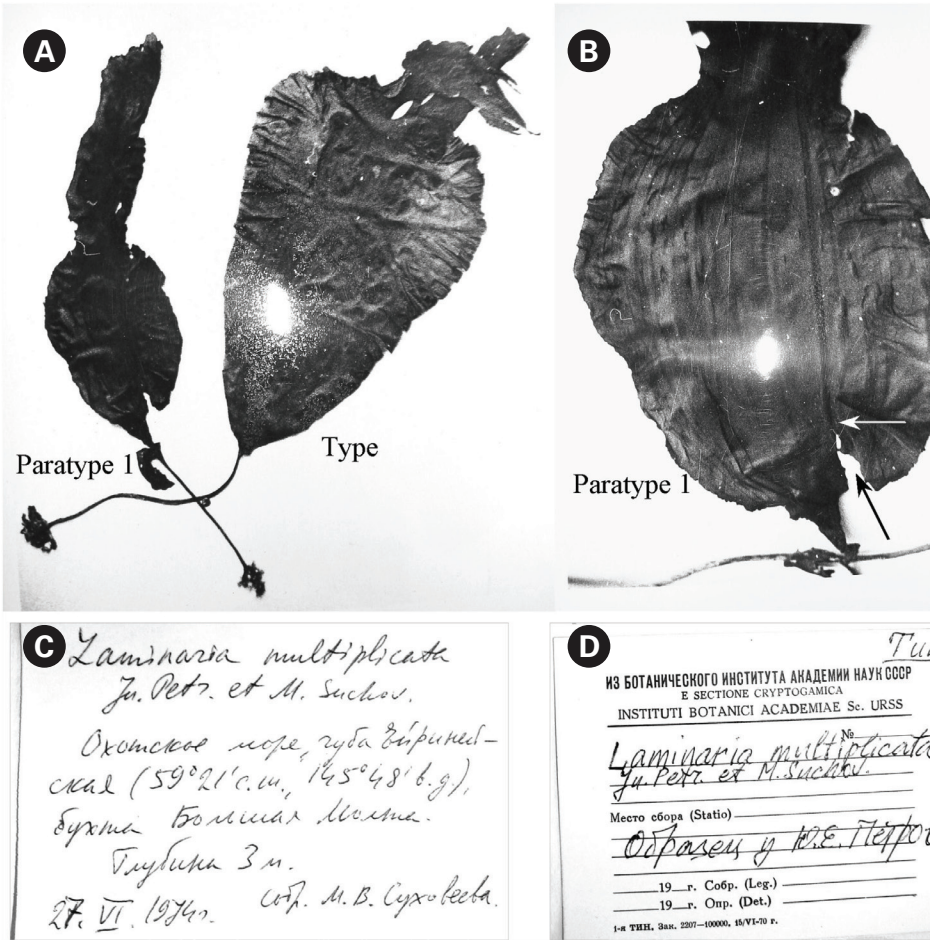

Fig. 2. Type specimens of *Laminaria multiplicata* and original labels. These specimens (type and paratype) were also displayed in Novosti Sistematiki Nizshih Rastenii (News on Systematics of Non-vascular Plants) by Petrov and Suchovejeva (1976). The specimens (A, B) were re-photographed from black-and-white images arranged in a plate currently kept in KBI RAS. (B) An enlarged paratype. Arrows point to an area where the blade started to tear longitudinally. In Petrov and Suchovejeva's paper (1976), this photograph was differently cropped. (C) Original label by Suchovejeva, specifying collection site, depth, date, and collector. (D) New label made in KBI RAS specifying 'Type. Specimen kept by Petrov'.

In this paper, we use the names *L. gurjanovae* A. Zinova (1964, 1969) and *L. gurjanovae* f. *lanciformis* Petrov (1972) instead of *Saccharina gurjanovae* (A. Zinova) Selivanova, Zhigadlova et Hansen and *S. gurjanovae* f. *lanciformis* (Petrov) Selivanova, Zhigadlova et Hansen throughout the text. The reason is as follows. Selivanova et al. (2007) stated that they re-named and re-authorized species and consecutively its form on the basis of molecular-phylogenetic data, but did not provide evidence of those data from 2007, such as sequences in GenBank (http://www.ncbi.nlm.nih.gov, searched on April 23, 2010). Also, their paper did not contain detailed methods used for molecular analysis or pictures of the plants. Omissions of these basic criteria make the nomenclatural combinations doubtful and the claims unsupported. Whoever provides trustable evidence such as sequences in GenBank of *L. gurjanovae* and *L. gurjanovae* f. *lanciformis* and proves their positioning in the *Saccharina* clade will be the author of new nomenclatural combination. Until then the case should remain open and the names existing until 2007 should be used, cf. *Laminaria gurjanovae* A. Zinova and *L. gurjanovae* f. *lanciformis* Petrov.

It is noteworthy to mention that description of *L. gurjanovae* f. *lanciformis* was given by Petrov (1972) in violation of the condition of valid publication of names according to International Code of Botanical Nomenclature (Art. 42, McNeill et al. 2006), since a diagnosis in Latin language was given and no illustration provided. Strictly speaking, the name should be regarded as *nomen invalidium*, although we still have used it in this paper. Figs 4D & 5A-C depict plants that have historically been

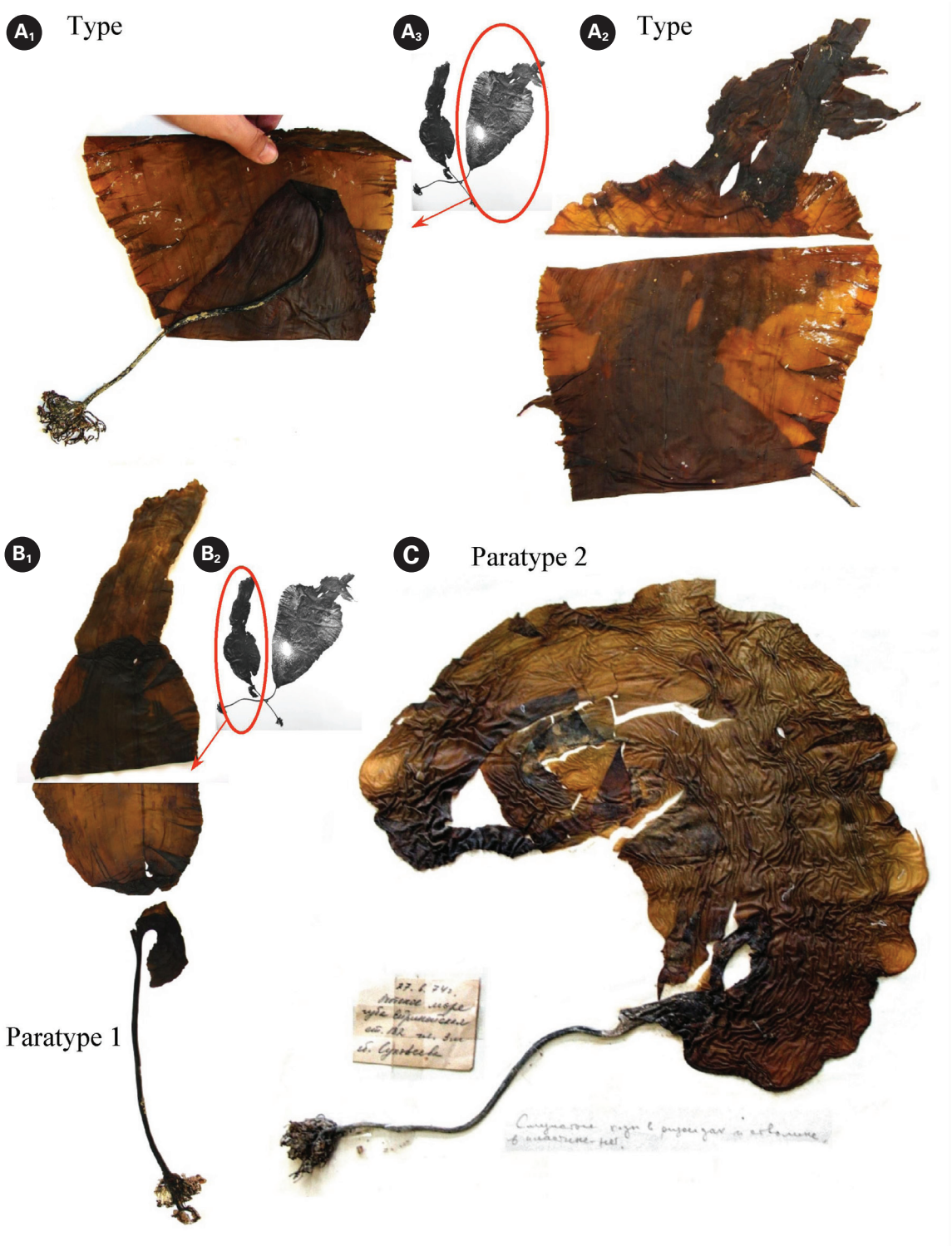

Fig. 3. Photographs of plants kept in KBI RAS. (A_{1-3}) Current appearance of type specimen (A_3, encircled). ($B_{1, 2}$) Current appearance of paratype 1 (B_2, encircled). Apparently, the plants were re-hydrated after initial photography, then folded three times and dried. Moreover, the blade of paratype 1 was detached from its holdfast. (C) Paratype 2 with asymmetric blade bent to one side.

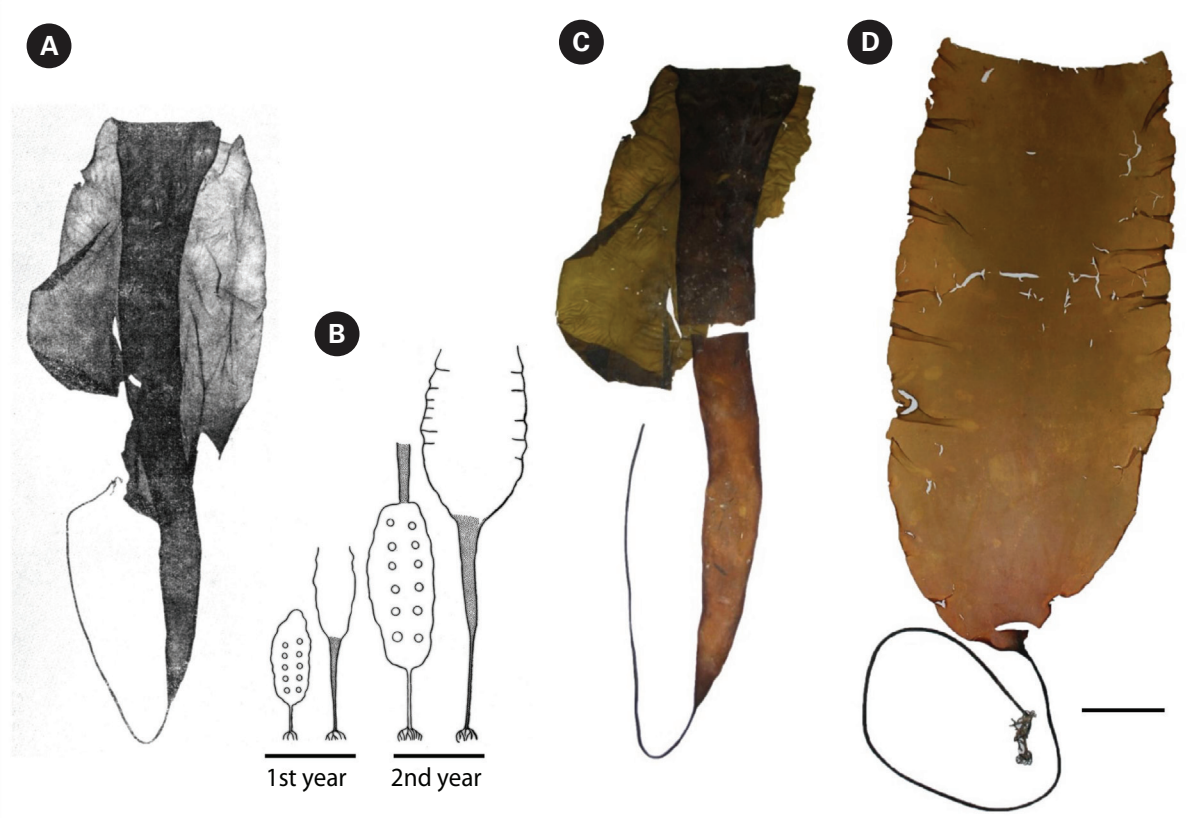

Fig. 4. *Laminaria gurjanovae* (A-C) and *L. gurjanovae* f. *lanciformis* (D). (A) Type specimen. (B) Scheme of thalli changes according to age. (A, B) Reproduced from Zinova (1969) with permission from Novosti Sistematiki Nizshih Rastenii (News on Systematics of Non-vascular Plants). (C) Type specimen re-photographed on 01.04.2008. (D) Plant from Taujskaya Bay, Sea of Okhotsk, with wide cuneiform blade. Scale bar represents 5 cm.

identified as *L. gurjanovae* f. *lanciformis* by Russian phycologists. We also present photograph of type specimen of *L. gurjanovae* collected from Sakhalin Island in 1955 (Fig. 4A & C). In our understanding, the plant chosen by Zinova (1969) as type specimen of *L. gurjanovae* was at the end of the second year of growth, based on the author's scheme of thalli changes (Fig. 4B). When stating similarity of *L. multiplicata* to *L. gurjanovae*, Petrov and Suchovejeva (1976) did not mention any forms described by Petrov (1972) and did not specify which one resembled *L. multiplicata*. However, as seen from a schematic drawing by Zinova (1969), the morphology of *L. gurjanovae* (cf. *L. gurjanovae* f. *gurjanovae*, Petrov 1972) varies greatly according to age, and its thalli at the end of the first and beginning of the second years of growth do indeed look similar to *L. multiplicata*. Zinova (1964) also stated that young (1-year-old) plants of *L. gurjanovae* had wide cuneiform blades, whereas in older plants the lower part of blade was extended in length, becoming narrow cuneiform. Our field observations of *L. gurjanovae* and *L. gurjanovae* f. *lanciformis* also showed that morphologies could vary according to age and growth locality (Figs 4D & 5A-C).

Observations of available specimens of *Laminaria multiplicata* by the present authors

Petrov and Suchovejeva (1976) showed two plants in one photograph under the legend 'General view of type specimen (a)' without specifying the scale bar (Fig. 2A & B). One plant had the letter 'a' put close to it and, thus, was presumably a type specimen, whereas the second plant was presumably a paratype. To our knowledge, eight plants of this species (type and seven paratypes) have ever been collected since 1974. It was cited in floristic lists of the Sea of Okhotsk on the basis of Petrov and Suchovejeva's paper (Emelyanova 2006). No additional descriptions, photographs, or micrographs have been published to date except for Petrov and Suchovejeva's paper. Also, the latter study did not cite any reference to support their finding.

It is not known how many dry specimens were prepared from the eight plants collected by Suchovejeva in 1974. We were able to find three specimens of *L. multipli-*

Fig. 5. Examples of development abnormalities in some kelp species. (A-D) *Laminaria gurjanovae* f. *lanciformis* from (A_1) Korf Inlet, Kamchatka with single protuberance that developed longitudinally from the blade's basal part towards its tip (arrows). (A_2) Cross section of blade with protuberance (arrow). The blade thickness was approximately 1 mm. (B) Attargan Bay, Sea of Okhotsk with inhibition of growth, scars, folds, and visibly darker color (white dashed square). (C) Taujskaya Bay, Sea of Okhotsk with narrow cuneiform blade, curved and bended to one side and very well distinguished dark stripes (above dashed line). (D) Attargan Bay, Sea of Okhotsk with very well-distinguished dark stripes (arrows), which were not sporangia sori or contaminating attaching organisms on the blade surface. (E, F) Additional rhizoidal clusters (arrows) developing on the holdfasts of *Saccorhiza* sp. (E) and *Laminaria digitata* (F) from Ny-Ålesund, Svalbard. Scale bars represent: A & B, 3 cm; C, 10 cm; D-F, 5 cm.

cata in KBI RAS (Fig. 3). Also, one piece of *L. multiplicata* was in the collection at the TINRO-center. Perhaps, the remaining four paratypes were held by Dr. Petrov. If so, they may be lost to study, since Dr. Petrov has not been an active researcher in the Russian Academy of Sciences from 1990-es. We found a type specimen (Fig. 3A$_{1-3}$) and a broken specimen of paratype 1 (Fig. 3B$_{1, 2}$) in KBI RAS. We analyzed them microscopically (Figs 6 & 7) and attempted to analyze the DNA.

Observation of morphology of available plants and analysis of the original photographs showed that some had development abnormalities. For instance, paratype 1 (Fig. 3B$_{1-2}$) was torn longitudinally from its basal part (Fig. 2B) and the breaking point was right on the 'fold' line described by Petrov and Suchovejeva (1976). Paratype 2 (Fig. 3C) had a long holdfast and asymmetric blade bent to one side; moreover, it did not have the dark-colored projections/protuberances previously designated as folds (cf. Petrov and Suchovejeva 1976). We do not know what the remaining plants looked like, but, among those currently available, only the type looked more or less normal (e.g., with proportional and complete blade), and not appreciably different from a known species, *L. gurjanovae*, except for the so-called 'folds'.

What are these folds? Petrov and Suchovejeva (1976) provided descriptions but did not show microphotographs of the structures they termed folds and used as key character of *L. multiplicata*. In the classical view, a fold is a line or an arrangement made by the doubling of one part over another. Our microscopic observations of *L. multiplicata* revealed that the darker lines on the blades' surface, which were called 'folds' by Petrov and Suchovejeva, were in fact irregularities of the cortex layer's thickness resulting in slight projections/protuberances on its surface (Fig. 6).

As appeared from the longitudinal preparations and cross sections of the blade, the thickness of the cortex layer made with brown-colored small cells differed from 112-128 μm to 198-272 μm in the most thickened and projected part, which created longitudinal protuberances on its surface (Fig. 6C-E). Because the brown-colored layer had different thicknesses and was more sloped upward in some places, they appeared darker. Moreover, physodes with concentrated brown pigment were found in places where the blade's cortex layer was thicker (Fig. 7A & B). Physodes are rather common in the kelp species (Klochkova N.G., personal observations), contained as colorless vesicles in young cells and yellow and brown vesicles in old cells, and they contain tannins in the form of chloroglycin and other polyphenols (Petrov 1977).

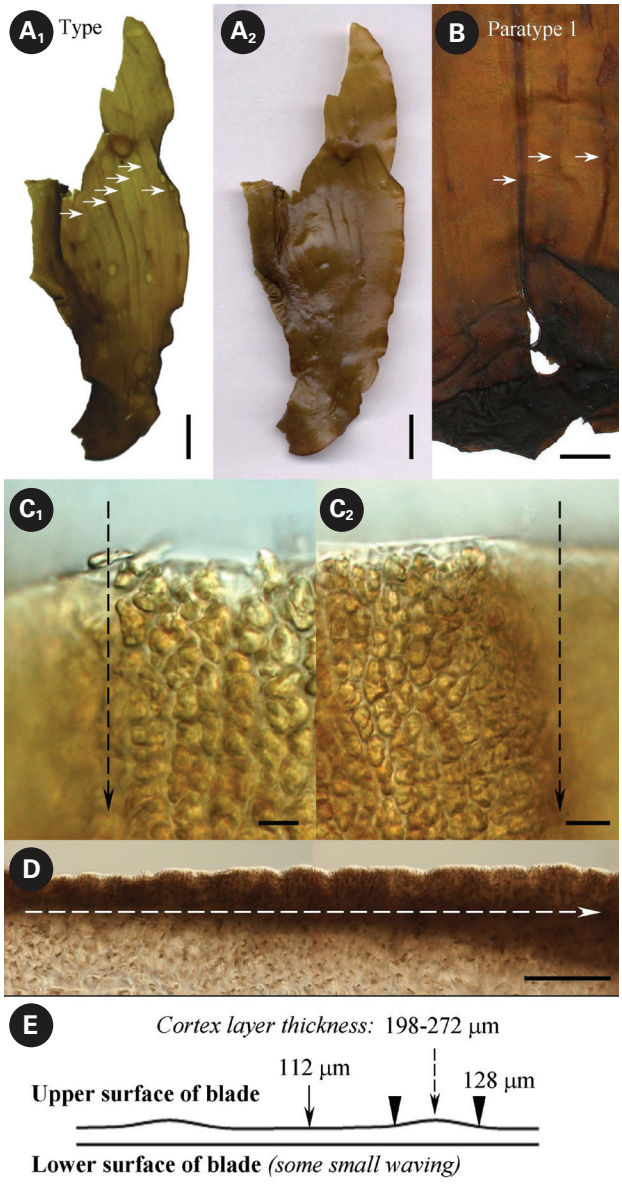

Fig. 6. Morphology of protuberances called folds by Petrov and Suchovejeva (1976). (A$_{1, 2}$) Seawater-saturated fragment of type showing protuberances (arrows), which were said to be the key character of this species. (A$_1$) Specimen was placed on the piece of transparent plastic and photographed so that the light beam came through the blade from beneath. (A$_2$) Same specimen photographed with the light beam directed from above. (B) Enlarged image of the basal part of paratype 1 (dry) showing protuberances (arrows). (C$_{1, 2}$) Protuberance photographed from above, showing its left (C$_1$) and right (C$_2$) borders (dashed arrows). (D) Cross section of the blade, showing different thickness of the cortex layer (dashed arrow). (E) Schematic diagram of the blade's appearance in the investigated plants of *Laminaria multiplicata*. Scale bars represent: Figs A$_{1, 2}$ & B, 1.5 cm; Fig. C$_{1, 2}$, 10 μm; Fig. D, 100 μm.

The protuberances developed longitudinally (Fig. 6A & B) when the unevenness of the cortex thickness started in the blade's basal part during the early stages of thalli growth and proceeded in the same areas when the blades

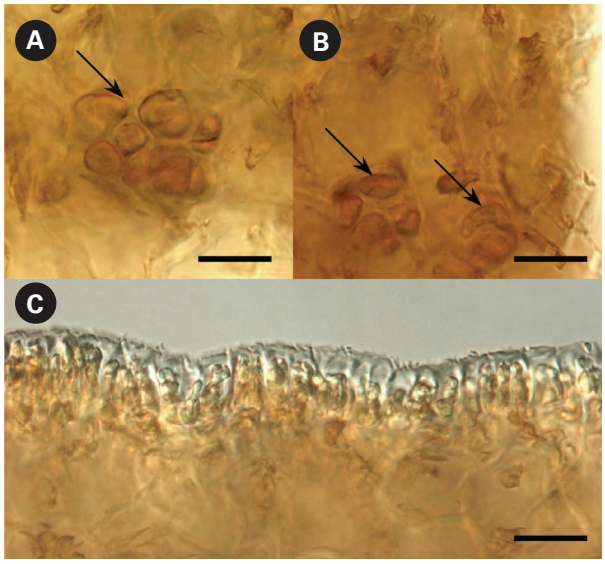

Fig. 7. Cross section of the blade. (A, B) Physodes (arrows). (C) Wavy reverse surface of blade, which did not have dark protuberances. Scale bars represent 10 μm.

grew in height. They appeared only on one side of the blade and the reverse side did not have such distinct drops of cortex thickness, although its surface was slightly wavy (Fig. 7C). Of course, one might question that the plants were collected approximately 36 years ago and were hardly pressed during specimen preparation. However, if those structures on the surface were indeed numerous narrow longitudinal folds of 0.5-33 mm at width, they would have been dried folded, whereas they were all dried unfolded and slightly projected. Moreover, they were very distinguishable in the dry and re-hydrated thalli, even after almost four decades of preservation. Thus, our microscopic observations show that the structures termed folds, which were the key character of this species, are in fact protuberances resulting in irregularities in the blades' texture. No other key characters were listed by Petrov and Suchovejeva (1976) to distinguish this species from other *Laminaria* spp. Also, they mentioned that *L. multiplicata* might have originated from *L. gurjanovae* because of similarities in sporangia sori shape that coincided on both sides of the blade, the blade shape, and holdfast length. *L. gurjanovae* is an independent species described by Zinova (1964, 1969) who mentioned it from the Sea of Okhotsk, Sea of Japan, and Sakhalin.

Fig. 5A shows *L. gurjanovae* f. *lanciformis* collected from Korf Inlet, Kamchatka. This plant had similar holdfast, rhizoids, and blade shape to *L. multiplicata*. It also had a single longitudinal protuberance starting from the blade's base and proceeding along the entire length; it was present on one side of the blade, appearing in the same manner as in *L. multiplicata* except that in the latter species they were numerous. We previously observed similar irregularities in the blade texture in laminariaceaen algae, especially in plants developing under the influence of anthropogenic pollution (Klochkova N.G., personal observations). We also noted similar stripes in *L. gurjanovae* f. *lanciformis* from the Attargan Bay and Taujskaya Bay in the Sea of Okhotsk (Fig. 5B-D). So, we believe those were developmental abnormalities. As can occur with any plant, kelps may develop various abnormalities (Fig. 5) (Klochkova and Berezovskaya 2001). However, it should not have been mistaken for a key character to describe a new species, *L. multiplicata*.

The on-site collection activities of two of this paper's authors have been described earlier. They collected numerous *Laminaria, Saccharina,* and other kelp species from the Sea of Okhotsk, including rare or endemic species, but none resembling Petrov and Suchovejeva's description of *L. multiplicata*, even in the type locality. There were abundant kelp beds on the bottom of the Ejrinejskaya, Penzhinskaya, Gizhinskaya, and Taujskaya bays. Moreover, they found abundant plants of the endemics of the Sea of Okhotsk such as *L. appressizhiza, L. inclinatorhiza, T. basicrassa,* and etc., but not a single plant resembling description of *L. multiplicata.*

Kelp species such as *Laminaria* and *Saccharina* do not tend to grow as a single isolated plant on a large space of sea bottom, but are rather found in clusters or form beds (e.g., Klochkova and Berezovskaya 1997, Klochkova et al. 2009). They are also perennial plants. Medium-sized kelp such as *L. multiplicata* (cf. 56-102 cm long and 21-40 cm wide) is impossible to miss when one targets it for several consecutive years like we did, and yet it has not been found by anyone since 1974.

We tried to analyze the DNA of type specimen of *L. multiplicata* using conventional extraction with Kit and the CTAB method. Conventional method has failed to yield any DNA, but the CTAB method was partially successful. The amplification of only one internal transcribed spacer region was possible but the result was unclear and did not allow for credible comparison. Thus, the DNA of currently existing specimens of *L. multiplicata* is already damaged and cannot be studied. There could be many reasons for the damage, including 1) material age (ca. 35 years-old at the time we analyzed it), 2) initial preparation of collected samples (rinsing in freshwater for mannitol removal, fast air drying, presence/absence of formalin treatment after collection), and 3) preservation conditions during the past 36 years. It is noteworthy

that we have previously obtained clear DNA results from 20-year-old brown algae using the CTAB method. Thus, the method is applicable to old algal samples. Whatever the reason, the DNA of the plants described as *L. multiplicata* could not be studied. The data files of fragmentary sequences and remaining dry sample are available upon request from the first author; however incomplete sequences have not been registered in any database.

CONCLUSION

We strongly doubt the existence of *L. multiplicata* due to the following reasons: 1) the only key character are abnormalities of development; 2) the blades of paratypes were somewhat abnormal in texture and shape, thus implying that the eight collected plants demonstrated abnormalities of development; 3) no one has succeeded in finding any new plants for approximately 36 years, and we did not see any plants in the type locality falling under its description for several consecutive years, in different seasons; 4) it was originally said to be close to *L. gurjanovae* in morphology and reproduction and speculated to originate from it. We strongly believe that those plants were *L. gurjanovae* bearing some abnormalities of development.

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