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ORIGINAL PAPERS
Phycology

Molecular Phylogeny of the Marine *Prasiola* and *Rosenvingiella* Species (Chlorophyta: Prasiolales) from Southeastern Kamchatka

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Abstract—Molecular phylogenetic tools are often useful in distinguishing cryptic species with similar morphologies, but they can also be helpful in identifying morphotypes of a species, which displays completely different shapes. We performed molecular-phylogenetic analysis of supra-tidal green algae in the Kamchatka peninsula that belong to the order Prasiolales (Trebouxiophyceae, Chlorophyta). Based on *rbc*L sequences results, two new species were recorded for the first time in Kamchatka. Approximately 1.4% of the field-collected *Rosenvingiella constricta* had a unique uniseriate hood-like blade shape, although their *rbc*L sequences were 100% identical with typical multiseriate filamentous plants. Kamchatka's population of *R. constricta* showed 99.4–99.6% identity in *rbc*L sequences with the populations from Canada and New Zealand. Another similar-looking *Rosenvingiella* species collected from the same locality had 93.5% identity of the *rbc*L gene sequence with *R. constricta*. Morphological and geographical analyses also suggested that this species might be a new species of the genus *Rosenvingiella*. *Prasiola delicata* was recorded for the first time in Kamchatka. The Kamchatka population of *P. delicata* showed 100% identity in *rbc*L gene sequence with the population from Vancouver, but differed from the Canadian population morphologically.

Keywords: *rbc*L, Kamchatka, molecular phylogeny, marine algae, distribution, *Prasiola*, *Rosenvingiella*
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INTRODUCTION

Representatives of the order Prasiolales (Trebouxiophyceae, Chlorophyta) belong to one of the most common groups of green algae including marine, freshwater, and soil species from the Polar, Antarctic, cold- and warm-temperate, and tropical regions [4–6, 25, 26, 35, 40]. In this order, the genus *Prasiola* (C. Agardh) Meneghini is the most abundant; *Prasiola* contains 34 species of marine, freshwater, and soil algae [15]. It is most closely related to another genus from this order, *Rosenvingiella* Silva, which includes six species [15]. The morphology of these algae is characterized by a certain variety and high phenotypic plasticity, which largely depends upon the environment [39]. In this group, cryptic species were recorded, which do not differ from each other morphologically and anatomically and usually grow in the same locality, but display significant genetic differences that allow their description as new species [31]. In nature, plants of *Prasiola* and *Rosenvingiella* have often been found together, while in a laboratory culture of their representatives, a replacement of "rosenvingiella"-like morphology with "prasiola"-like morphology was recorded. This allowed some researchers to suggest that *Rosenvingiella* is a developmental stage or a form of *Prasiola* [1, 8–11, 13, 14]. However, this idea was rejected based on molecular-phylogenetic differences between different species of Prasiolales [31, 40].

The systematics of Prasiolales is intensively studied. Attention to this algal group is due to the fact that they are used to study the impacts of extreme environmental factors [20, 22, 23, 27, 29] and are of interest in industrial biotechnology [16, 21, 24]. It would be fair to say that the use of molecular–phylogenetic methods gave rise to rather more questions than answers, because the morphological characteristics of species in this taxonomic group often contradicted the results of genetic analysis. In this group, the most frequently used DNA marker is the *rbc*L gene [40]; however, in the case of controversial taxonomic situations the *psa*B and *tuf*A genes were also analyzed [30–32].

During the field studies on the southeastern coast of Kamchatka, we found species that were preliminarily identified as *Prasiola delicata* Setchell et Gardner and *Rosenvingiella constricta* (Setchell et Gardner) Silva. These species possessed a unique ability to recover viability and reproduce by autospores after a prolonged extreme desiccation [5]. During the laboratory culture of *R. constricta,* we found two different

morphotypes. The first morphotype showed typical multiseriate filaments with constrictions, while the second morphotype had the form of uniseriate hood-like blades, but they occurred in the same population in nature. Under laboratory culture conditions, the plants from both morphotypes formed autospores, which developed into the same filamentous thalli [5]. Thus, we proposed that in Kamchatka two different morphotypes of *R. constricta* develop in nature; however, to prove this precisely, DNA analysis of plants from each morphotype was required. In this paper, we present the molecular-phylogenetic analysis of *Prasiola* and *Rosenvingiella* species from Kamchatka.

MATERIALS AND METHODS

Algal samples were collected on August 7, 2011 from Starichkov Island (Avachinsky Inlet) and on July 13, 2013 from a small rock located at the entrance to the Avacha Bay (also called as Avacha Bay's throat). In Starichkov Island, the following algae were collected from the supra-tidal zone: *Prasiola delicata* (narrow ribbon-like blades) and *Rosenvingiella constricta* (multiseriate filaments with constrictions and uniseriate hood-like blades). Species *Rosenvingiella* sp. was collected at the entrance to the Avacha Bay. Before the laboratory culture, all plants were carefully separated into groups to avoid mixing individuals of different morphotypes. Petri dishes containing samples were transferred to 2 m^3 incubating chamber and maintained there at a constant temperature of 15°C with 12 : 12 h L:D cycle and 30 μmol m^{-2} s^{-1} light intensity. The detailed method of cultivation was provided in Klochkova et al. [5].

In nature, over 99% of the plants in the population of *P. delicata* belonged to this species (without any *R. constricta* plants being present); these grew in an isolated place. In another population that consisted only of *R. constricta* plants 98.6% and 1.4% of the plants belonged to the "constricta" and "prasiola" morphotypes, respectively. Thus, occasional single plants of the "prasiola" morphotype could be easily distinguished and separated. All plants were thoroughly washed with a fine brush in sterile marine water. Along with plants from the natural populations, we also used juvenile plants that developed in the laboratory culture of both morphotypes of *R. constricta*.

The DNA was extracted using an Intron DNA extraction kit (Intron Biotech, Korea) according to the manufacturer's protocol. The *rbc*L gene was amplified using the primers PF2 (TTCGTATGACTCCTCAATCAG) and PR2 (TTACATGCTGCACGAATA) [40]. The following PCR program was used: initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were extracted from the polyacrylamide gels using the Gel extraction kit (Qiagen, United States). Sequencing was performed using the ALFexpress AutoRead Sequencing Kit (Amersham Pharmacia Biotech) and CycleReader™ Auto DNA Sequencing Kit (MBI Fermentas).

The BLASTn option (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for related sequences in the NCBI website [34]. Our new nucleotide sequences and sequences from the GenBank were aligned using MUSCLE Alignment in the Geneious program (ver. 7.1.8, Biomatters, Auckland). A molecular–phylogenetic tree was generated using Bayesian phylogenetic analysis (MrBayes 3.2.2; [42]) with the GTR substitution model, 3000000 generations and a burn-in length of 300000 generations. The maximum likelihood analysis used RAxML 7.2.8 [44] using the GTR + gamma model. Bootstrap support values (%) were calculated based on 500 bootstrap replicates.

Our new sequences of Kamchatka samples were deposited in GenBank under the accession numbers KX108855 (*R. constricta* morphotype "constricta"), KX108856 (*R. constricta* morphotype "prasiola"), KX108857 (*P. delicata*), and KX108858 (*Rosenvingiella* sp.).

RESULTS

The Morphology of the Investigated Species

In the natural population of *Rosenvingiella constricta* from Starichkov Island, 98.6% of the plants belonged to the "constricta" morphotype. The remaining 1.4% belonged to the "prasiola" morphotype (Figs. 1a–1c). Thalli of the "constricta" morphotype were filamentous, 1–2 (3) mm in length, with multiple (4–7) narrow constrictions on each filament (Figs. 1h–1i). The filaments were uniseriate in the basal portion and becoming multiseriate and broader upwards. The plants grew in bundles and attached to the substrate by an elongated initial cell. The hood-like blades of the "prasiola" morphotype were up to 1.2 mm in length and 400–800 μm wide in the widest part, with or without a small stipe, and always grew as separate single plants (Figs. 1a–1c). All of the collected plants of both morphotypes were sterile, although some had ragged upper portions, implying the formation and liberation of autospores.

In the laboratory, plants belonging to different morphotypes were separated from each other and transferred to sterile marine medium to initiate unialgal culture. At 2 weeks later, the hood-like blades that belonged to the "prasiola" morphotype started to segregate into separate autospores (Fig. 1d), whereas filaments of the "constricta" morphotype separated into packages consisting of two to six (8) autospores (Figs. 1j and 1k). In the "prasiola" morphotype, autospores were of different sizes and shapes, but they germinated in the same way. In the germlings, the elongated initial cell was always covered with an undulate

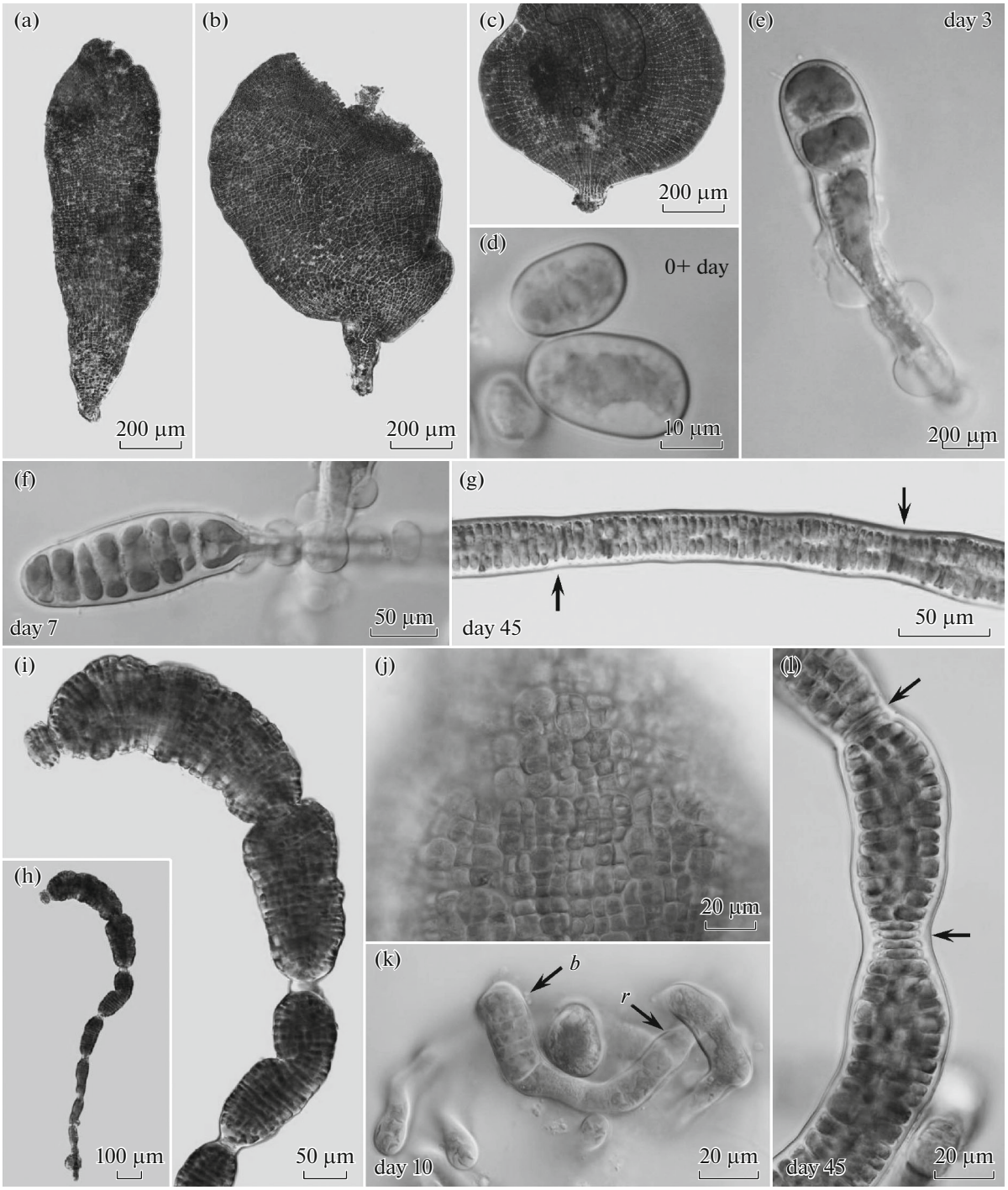

Fig. 1. Morphology of *Rosenvingiella constricta* specimens used in this study and their autospores. (a–g) *R. constricta* morphotype "prasiola": (a–c) three hood-like plants from the same sample differ from each other in shape and size of the blade; (d) autospores were liberated and germinated separately from each other; (e–f) development of juvenile plants from the autospores on the 3rd and 7th days after their attachment to the substrate; (g) fragment of the blade that developed from an autospore on the 45th day; minute constrictions developed in some parts of the blade (shown with arrows); (h–l) *R. constricta* morphotype "constricta": (h–i) an adult plant and enlarged part of its filament showing typical constrictions; (j) the upper part of the plant with packages of autospores localized under the cuticle; (k) development of juvenile plants from the autospores on the 10th day after their attachment to the substrate; arrows and letterings point to rhizoidal outgrowth (*r*) and a developing filament (*b*); (l) fragment of the filament that developed from the autospore after 45 days; constrictions are shown with arrows.

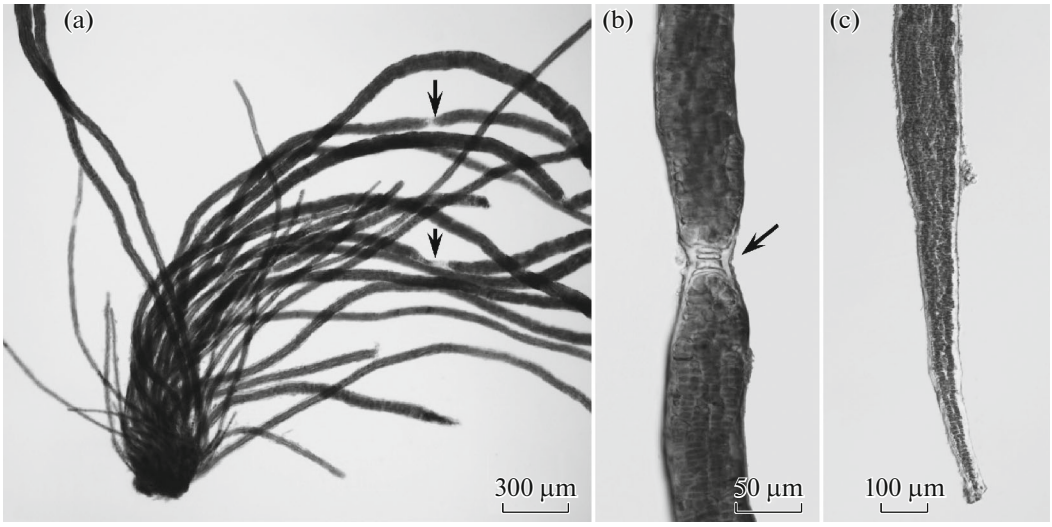

Fig. 2. The morphology of *Rosenvingiella* sp. and *Prasiola delicata* used in this study. (a) A bundle of *Rosenvingiella* sp. plants; arrows point to two plants with constrictions; (b) an enlarged view of *Rosenvingiella* sp. filament with constriction (shown with arrow); (c) the lower part of the narrow ribbon-like blade of *P. delicata*.

cuticle (Figs. 1e and 1f); 1.5–2 months after autospore liberation, young germlings developed into filamentous plants, 1.0–1.5 mm long (Fig. 1g) and then stopped growing. In the progeny plants, narrow characteristic constrictions were absent; however, they displayed insignificant differences in the width of filaments, which were located at nearly the same distances from each other and looked similar to constrictions (Fig. 1g). In the "constricta" morphotype, packages of the autospores germinated into narrow filamentous thalli 1.0–1.5 mm long, with constrictions (Fig. 1l). Another species from Kamchatka that we found, *Rosenvingiella* sp. (Fig. 2a), was morphologically most similar to *R. constricta*, however it differed from it in having a longer filamentous thallus (up to 6–7 mm long) and a lack of constrictions. In this species, constrictions developed in less than 1% of plants and only one constriction occurred per filament (Figs. 2a and 2b). Kamchatka's population of *Prasiola delicata* contained narrow uniseriate ribbon-like blades up to 5–6 mm long, with ragged upper portions and attached to the substrate with a basal cell (Fig. 2c).

Molecular Identification

Analysis of the *rbc*L gene of each morphotype of *R. constricta* and young thalli that developed from their autospores in the laboratory culture showed that hood-like blades had 100% affinity with filamentous plants with constrictions; thus, they belonged to the same species (Fig. 3). The samples from Kamchatka of both morphotypes appeared in the clade formed by *R. constricta* from New Zealand (HQ174315) and Canada (AF189067) and *Prasiola* sp. from England (AY694197). The similarity of nucleotide sequences of Kamchatka's samples of *R. constricta* with other samples of this species was 99.4% (HQ174315) and 99.6% (AF189067, AY694197), respectively.

Two prasiolalean species from Kamchatka were recorded for the first time (Figs. 2 and 3). We found a population of *P. delicata* on Starichkov Island (Fig. 2c) and *Rosenvingiella* sp. (Figs. 2a and 2b) on a small rock located at the entrance to Avacha Bay. Identification of Kamchatka's population of *P. delicata* was based on 100% identity of the *rbc*L gene sequence of our samples with a population of this species from Vancouver. *Rosenvingiella* sp. is genetically different from all representatives of this genus (93.4–96.7% differences in the *rbc*L sequence); it may potentially be a new species. Its affinity with *R. constricta* from the same locality in Kamchatka is 93.5%. The *rbc*L gene sequence of *Rosenvingiella* sp. is most similar (96.7%) to *Rosenvingiella australis* Heesch et Nelson from New Zealand and *R. tasmanica* Moniz, Rindi et Guiry from Tasmania.

DISCUSSION

In the order Prasiolales, one of the most important taxonomic problems has been the elucidation of whether the genus *Rosenvingiella* was a developmental stage or a form of *Prasiola*. Analyses of the *rbc*L gene sequences of different representatives of these genera show separation into two distinct groups of species [30, 40], i.e., they belong to different genera. During the course of our study, we found that at least one species of *Rosenvingiella*, that is, *R. constricta*, can develop a unique hood-like morphology, which is not typical for this genus and is characteristic of the genus *Prasiola*. We found a population of *R. constricta* on the coast of Kamchatka that consisted of plants of two morphotypes; moreover, the "constricta" morphotype was

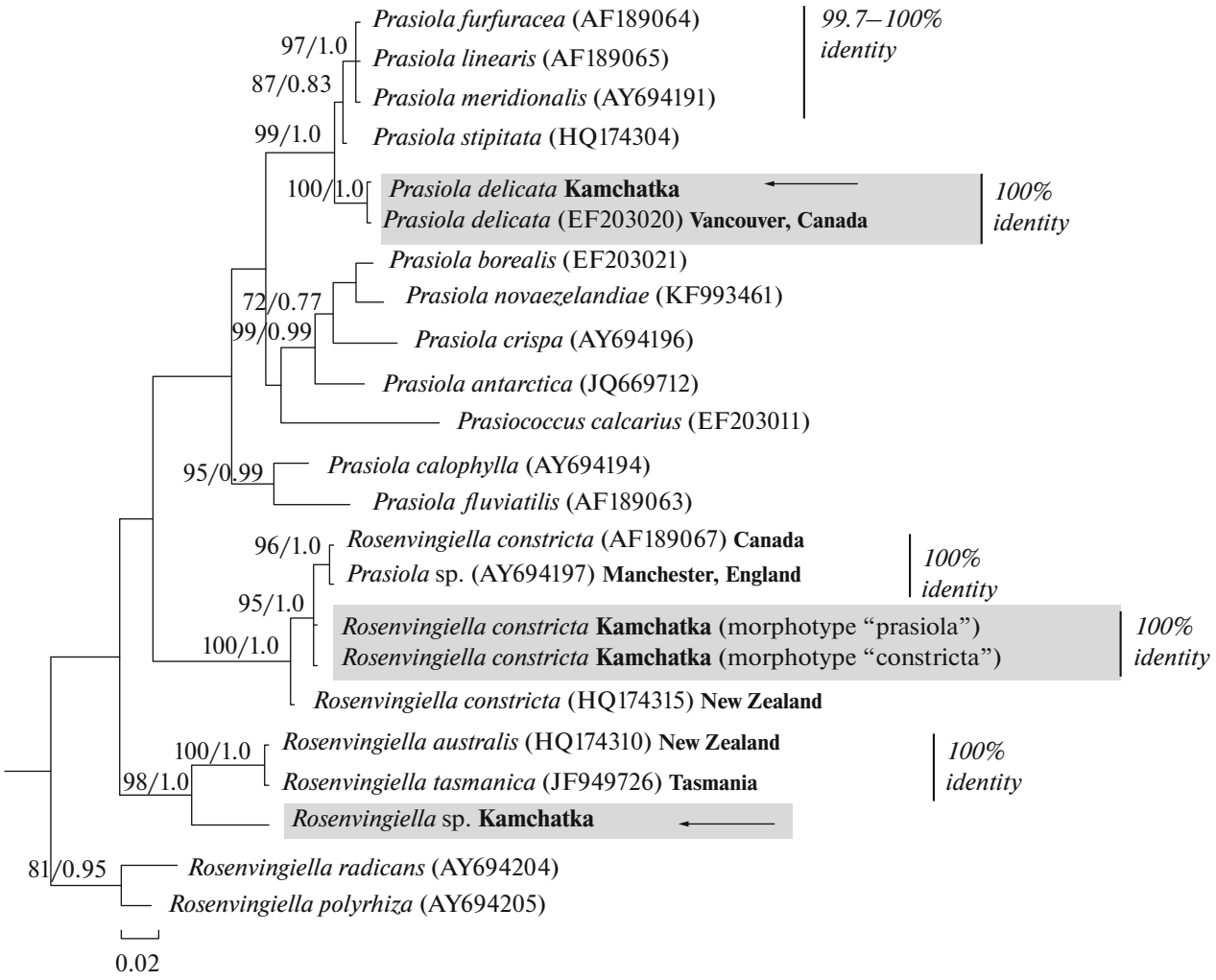

Fig. 3. Bayesian inference of the phylogenies of *Rosenvingiella* and *Prasiola* species based on *rbc*L sequences (the outgroup is *Kornmannia leptoderma*). The labels on the branches are posterior probabilities and consensus support values. Our new sequences are highlighted; other sequences were obtained from the NCBI.

dominant over the "prasiola" morphotype in nature. It is not known what affected their development, because all of the plants grew in the same population under the same environmental conditions. In a laboratory culture, all autospores that formed in the hood-like blades and multiseriate filaments with constrictions developed into the same filamentous thalli. Analyses of the *rbc*L gene sequences showed 100% identity of these plants with each other. Therefore, diagnoses of the genus *Rosenvingiella* and its species *R. constricta* should be extended, because in some rare cases representatives of *Rosenvingiella* can in fact display the *Prasiola*-like morphology.

Kamchatka's population of *R. constricta* has 99.4–99.6% *rbc*L sequence identity with populations of this species from New Zealand and Canada. Considering that all samples were collected from different continents and hemispheres, this difference (0.4–0.6%) is insignificant and implies that these are remote populations of the same species. It is noteworthy that a marine population of *R. constricta* from Canada (AF189067) has 100% *rbc*L sequence identity with *Prasiola* sp. from England (AY694197), although the latter species was collected from the surface of a concrete wall in the center of Manchester at a distance of 60 km from the sea and morphologically was represented by long uniseriate filaments that were 10–18 μm wide, without rhizoids and multiseriate portions of the thallus; moreover, it appeared not to be capable of growing in seawater [40]. In the phylogenetic tree by Rindi et al. [40], this species, which was identified as *Prasiola* sp., grouped with *R. constricta* from Canada (AF189067) forming a separate "border" clade, located as the last clade in the genus *Prasiola*. However, in another phylogenetic tree by Kim et al. [25] and in our tree, this clade appeared in the group of *Rosenvingiella* species. In the phylogenetic tree by Moniz et al. [30], this soil species from England was

included under the name *Rosenvingiella* sp. without any explanation of the change of the generic name, although in the NCBI database it is still registered as *Prasiola* sp. (AY694197) and its morphology corresponds to the diagnosis of *Prasiola*. The absolute identity (100%) of the *rbc*L sequence of this soil species with the marine *R. constricta* is an example of how the morphological and ecological characteristics of a species can contradict the results of genetic analysis. Moreover, this is another indirect confirmation that representatives of *Rosenvingiella* can display *Prasiola*-like morphology.

Many unsolved taxonomic problems remain in the group of prasiolalean algae. As an example, the recent phylogenetic analysis using *rbc*L gene showed that *Prasiococcus calcarius* (Petersen) Vischer, which is a type species of the genus *Prasiococcus* Vischer, appeared in the middle of the group formed by *Prasiola* species [18, 25, 30]. In treatments of similar situations in other taxonomic groups of algae, such genera are divided into several separate genera; however, this has not been done in the case of *Prasiola*. Another problem is the presence of species with 100% identity of sequences that are used as DNA markers, but that differ in morphology and ecology. As an example, *R. australis* from New Zealand (HQ174310; [18]) and *R. tasmanica* from Tasmania (JF949726; [30]) have 100% identity at the *rbc*L gene, although the identity has not been discussed as yet and they remain considered as different species [15]. It should be noted that in the description of these species, special attention was paid to the DNA analysis and in the case of *R. tasmanica* the *rbc*L gene difference was indicated in the Latin diagnosis as a taxonomic character of the species [30]. Perhaps, *R. australis* should be synonymized with *R. tasmanica*; because the latter species was described earlier, it has priority, otherwise there is no sense in indicating the differences of specific genes in the species diagnoses.

The same situation is observed with the samples identified as *Prasiola stipitata* (HQ174304), *P. linearis* (AF189065), *P. meridionalis* (AY694191), and *P. furfuracea* (AF189064); they have 99.7–100% identity in the *rbc*L sequence. In fact, this has been noted before our present study [18, 41]; however, the authors did not amalgamate these species under the name *P. furfuracea*, which has priority, because, in their opinion, these species displayed different morphologies [41]. At the same time, according to the earlier statement of these authors, the morphology of Prasiolales has a high phenotypic plasticity and largely depends upon environmental factors [39].

In modern phycology, the identification of species and genera is based on DNA analysis rather than on morphology; this is clearly demonstrated in the number of publications on the representatives of different algal orders [7, 12, 17, 19, 28, 33, 36–38, 45]. The prasiolalean algae cannot be an exception in such a case. If cryptic species with identical morphology but clear genetic differences have been recorded in this taxonomic group [31], i.e., with the clear demonstration of the advantage of the genetic method over the morphological analysis, then the samples with 100% identity of the genes used as DNA markers should be attributed to the same species regardless of the morphological differences, which are known to occur in different phenotypes.

Analysis of the *tuf*A gene also showed 100% identity between *P. meridionalis* and *P. stipitata*; however, even in this case the authors did not combine these species, because, in their opinion, the genetic identity of these species is the result of trans-Arctic radiation and is associated with the climatic history of the North Atlantic and North Pacific ocean during recent evolutionary time [32]. In our opinion, the impact of continental drift and marine transgression on the species distribution would be a reasonable suggestion in the case of macroalgae, but in the case of very small and highly resistant algae, such as prasiolaleans, it would be logical to assume their introduction to remote habitats through some carrier. Representatives of the order Prasiolales are extremely tolerant to desiccation. As an example, our studies showed that some prasiolalean species remain alive for 3–4 years after losing almost 100% of their intracellular fluids [5].

Four representatives of prasiolalean algae have been recorded in the flora of Russian far-eastern seas, including *Prasiola borealis* Reed, *P. crispa* (Lightfoot) Kützing, *R. constricta* and *R. polyrhiza* (Rosenvinge) Silva [1]; two of them, *P. borealis* and *R. constricta*, were reported from Kamchatka [4]. We found new species for the flora of Kamchatka: *Rosenvingiella* sp. and *Prasiola delicata*. Presently, six species are recorded in the genus *Rosenvingiella* [15] and the *rbc*L genes of five species have been sequenced, whereas the *rbc*L gene of the last species, *Rosenvingiella simplex* Vinogradova collected from the South Shetland Islands in King George Island [2], remains unstudied. Morphologically, *R. simplex* is most similar to *R. constricta* and also to our samples of *Rosenvingiella* sp. However, the *rbc*L gene similarity between *R. constricta* and *Rosenvingiella* sp. is only 93.5%. This case is an example of how morphologically similar species from the same locality are genetically divergent. The morphology of *Rosenvingiella* sp. does not fit the description of *R. simplex* and their habitats are located at a distance of more than 12000 km; however, we cannot describe it as a new species from Kamchatka until *R. simplex* from the type locality is sequenced.

The species *P. delicata* was reported by V.B. Vozzhinskaya from Sakhalin Island [3]; however, according to Vinogradova [1], the notes made by V.B. Vozzhinskaya cast doubt on the species identification. We report for the first time that, based on 100% *rbc*L sequence identity, this species from Kamchatka is genetically identical with the population from Van-

couver, whereas the Kamchatka's and Canadian plants are morphologically different. The original description of this species contained drawings and described wide elongated or cordate blades with a stipe [43], whereas the Canadian population from Vancouver consisted of roundish or cordate blades [41].

In modern phycology, the molecular-phylogenetic approach is more frequently used to distinguish cryptic species with similar morphologies and genetic differences. Our current study showed that this approach can be successfully applied to identify different morphotypes of one species.

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