

## LICHENS FROM THE LITTORAL ZONE HOST DIVERSE ULVOPHYCEAN PHOTOBIONTS<sup>1</sup>

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**Crustose Verrucariaceae lichens form a distinctive black belt on seashores all over the world. This lifestyle is apparently enabled by a specific set of photobionts. However, their diversity is understudied. We sampled these lichens from the northern Patagonian Pacific coast of Chile. Using molecular markers, we identified both mycobionts and photobionts. The lichens, belonging to the genus *Hydropunctaria* and to the *Wahlenbergiella* group, hosted solely Ulvophycean photobionts. *Pseudendoclonium submarinum* (Kornmanniaceae, Ulvales) was the most common, but representatives of other closely related, yet undescribed, lineages were also found. *Undulifilum symbioticum* gen. et sp. nov. is described within Kornmanniaceae based on culture morphology and DNA sequence data. Furthermore, the free-living macroscopic genus *Urospora* (Acrosiphoniaceae, Ulotrichales) is reported as a lichen photobiont for the first time and is the first of its kind in the order. These results indicate that undescribed algal diversity is waiting to be uncovered in seashore lichens.**

**Key index words:** Chile; *Hydropunctaria*; intertidal rocks; *Pseudendoclonium*; symbiosis; *Undulifilum symbioticum* gen. et sp. nov.; *Urospora*; *Verrucaria*

**Abbreviations:** ASW, artificial sea water medium; BI, Bayesian inference; BBM, Bold's Basal Medium; CAUP, Culture Collection of Algae of Charles University in Prague; CTAB, cetyltrimethylammonium bromide; MCMC, Markov Chain Monte Carlo; ML, maximum likelihood; Pi, parsimony informative; PP, posterior probability; PRC, Herbarium collection of the Charles University in Prague; SAG, Culture Collection of Algae at Goettingen University; SDSF, standard deviation of split frequencies; SPRI, solid phase reversible immobilization; V, variable positions

The remarkable ability of lichens to tolerate abiotic stresses allows them to dominate various hostile habitats, most notably not only bare rocks and soil in arctic and alpine regions (Beckett et al. 2008) but also rocky seashores all over the world (Fletcher 1973, Brodo and Slone 2004). Some seashore lichen species can be found as low as the littoral zone, where they undergo periodic submersion or continual splashing by waves, acclimatizing to both inundation and exposure, associated with high solar radiation, desiccation, osmotic stress, and also wave action. They often form distinctly colored vertical zones. The so-called black belt on the littoral fringe, formed almost exclusively by black crustose Verrucariaceae lichen species, is so striking that it is often confused with oil contamination (Dobson 2014).

The ecology of lichens is significantly influenced by their photobionts (Helms 2003, Peksa and Škaloud 2011). Expansion of the ecological niche of a lichen is often facilitated by its ability to switch to a photobiont adapted to specific environmental conditions (Ertz et al. 2018, Rolshausen et al. 2018, Vančurová et al. 2018). It has also been shown that the pool of adapted photobionts is shared by lichens with a similar ecology, regardless of their taxonomy (Rikkinen et al. 2002). It might be expected that the thriving of lichens on seashore rocks is enabled by a specific community of photobionts.

General knowledge of lichen photobionts from coastal and seashore habitats is scarce and can be briefly summarized as follows. Watanabe et al. (1997) isolated and morphologically identified 13 Trebouxiophyceae photobionts along with one Ulvophycean, *Pseudendoclonium arthrophyreniae*, from lichens from the supralittoral zone. Studies of the littoral zone revealed photobionts from three eukaryotic classes: various “*Dilabifilum*” strains, *Halo-filum ramosum*, *Paulbroadya petersii*, and species of *Pseudendoclonium* (Tschermak-Woess 1976, Thüs et al. 2011, Darienko and Pröschild 2017, Gasulla et al. 2019) from Ulvophyceae; *Heterococcus caespitosus* (Parra and Redon 1977) from Xanthophyceae, and *Petroderma maculiforme* (Gueidain et al. 2011)

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from Phaeophyceae, but also cyanobacterial *Rivularia* spp. (Ortiz-Álvarez et al. 2015).

In the present study, we focused on the diversity of photobionts of crustose Verrucariaceae lichens from the littoral zone at various sites in Chile. Using DNA sequencing of markers, we identified both the mycobionts and photobionts. Exclusively Ulvophycan photobionts were found, and *Pseudendoclonium submarinum* was the most common. We describe a new genus within the Kornamniaceae, Ulvales, and a filamentous alga from the Ulotrichales not previously known as a lichen photobiont.

#### MATERIALS AND METHODS

**Sampling.** Lichens were collected in February 2019 on the Northern Patagonian Pacific Coast in Chile. Nineteen specimens from nine sites are included in this study (Fig 1, Table S1 in the Supporting Information). Collection data are shown in Table S1. Air-dried lichens were transported to the laboratory in paper bags. Afterward, they were stored in a refrigerator at 4°C until processed.

**Photobiont culturing.** About 2 months after the collection, thalli were cut with a sterile razor blade under a stereomicroscope, and about 60-µm pieces that visibly contained photobiont cells were extracted with a sterile needle and placed onto petri dishes with solid artificial sea water (ASW, Starr and Zeikus 1993). Thalli were kept at 16.5°C with 12:12 h light:dark regime. Isolated colonies were then transferred to liquid ASW. The identity of the cultures was confirmed by sequencing nuclear ITS rDNA (see below).

**Sequencing and phylogenetic analyses.** DNA was isolated using the CTAB protocol (Cubero et al. 1999), with an additional washing step with 96% ethanol, directly from pieces of the lichen thalli or from cultures. Specific primers were used to amplify the nuclear small subunit (nuSSU) and nuclear large subunit (nuLSU), and mitochondrial small subunit (mtSSU) rDNA genes of the mycobiont and nuclear SSU and ITS rDNA regions, and plastid *rbL* gene of the photobiont. The primer pairs used were NS1 and 18L (Hamby et al. 1988) for fungal nuSSU; LR0R and LR6 (Vilgalys and Hester 1990) for fungal nuLSU; mrSSU1 and mrSSU3R (Zoller et al. 1999) for fungal mtSSU; newly designed 18S-Ulvo-F (5'-CCATGCATGTCTAAGTA-3', P. Škaloud, this study) and 18S-Ulvo-R (5'-ACCTTGTTACGACTTCWCCT-3', P. Škaloud, this study) for algal nuSSU; KlebsF (Škaloud and Rindi 2013) and ITS4 (White et al. 1990) for algal nuITS; and *rbL*-203F and *rbL*-991R (Nelsen et al. 2011) for chloroplast *rbL*. The PCR conditions are given in Table S2 in the Supporting Information. A negative control was used in each PCR run. PCR products were purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter) and sequenced by MacroGen Europe, Amsterdam, the Netherlands, using the same primers. GenBank accession numbers of the newly obtained sequences are given in Table 1.

Datasets (see below) were aligned separately for each locus using MAFFT v.7 (Kato et al. 2017), using the Q-INS-I method and manually checked. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and eliminated. Substitution models were estimated with JModelTest v. 2.1.4 (Darriba et al. 2012) using Bayesian Information Criterion and are given below.

The phylogenetic position of the mycobionts within the Verrucariaceae was verified based on nuSSU rRNA gene, nuLSU, and mtSSU rRNA gene. A dataset was created (Table S3 in the Supporting Information) to include

representative taxa of all the main groups and lineages of the family Verrucariaceae following Gueidain et al. (2007), Savić et al. (2008) and Pérez-Ortega et al. (2018). Because, according to BLAST searches, our samples matched either the genus *Hydropunctaria* or the *Wahlenbergiella* group sensu Pérez-Ortega et al. (2010), the following taxa were also added: all nine currently recognized species of *Hydropunctaria* (Orange 2012, Spribille et al. 2020) and all taxa reported to belong to the *Wahlenbergiella* group (Gueidain et al. 2009), including *Mastodia tessellata* and five *Verrucaria* spp. from the Chilean coast (Pérez-Ortega et al. 2010). *Capronia* (Chaetothyriales) was used as the outgroup. The final concatenated alignment contained 1,008 nuSSU rRNA gene positions, of which 200 were variable (V) and 118 parsimony informative (Pi), 930 nuLSU (308 V, 231 Pi) and 641 mtSSU rRNA gene (238 V, 180 Pi) positions, and consisted of 74 taxa, including our specimens. The selected substitution models were TIM1ef+I+G (gamma shape 0.489) for nuSSU rRNA gene, TIM3+I+G (0.641) for nuLSU, and TPM3uf+I+G (0.64) for mtSSU rRNA gene.

BLAST searches of our photobiont sequences matched taxa of either Ulotrichales or Ulvales, so we performed phylogenetic analyses of both orders. Datasets of Škaloud et al. (2018) were simplified so that all families, genera, and main lineages were represented (Tables S4, S5 in the Supporting Information). The phylogeny of Ulotrichales was based on nuclear SSU rRNA gene and ITS and chloroplast *tufA*, downloaded from GenBank. Additional *Urospora* species were included (Table S4) because BLAST searches of the samples US5215, 5232L, and 5244 matched various species of the genus. Because the occurrence of *Urospora* within lichens was unexpected, DNA was isolated again from ethanol-surface sterilized pieces of thalli of these samples, and amplification and sequencing were repeated. Each time we obtained chromatograms with single distinct peaks. *Desmochloris molenhaueri* (Chlorocystidales), *Halochlorococcum moorei* (Oltmannsiellopsidales) and *Pseudoneochloris marina* (Ulvales) were used as the outgroup. The final Ulotrichales alignment contained 1,729 nuSSU rRNA gene (198 V, 110 Pi), 519 nuITS (277 V, 220 Pi), and 790 *tufA* (388 V, 238 Pi) positions. Substitution models selected for Ulotrichales were K80+I+G (gamma shape 0.727) for nuSSU rRNA gene, TIM2ef+G (0.760) for ITS1, K80+I for 5.8S, TIM2ef+G (0.639) for ITS2 and F81+G (0.1530), TrN+I+G (0.509), and TIM2+I for the first, second, and third codon position of *tufA*, respectively.

The phylogeny of Ulvales was based on nuclear SSU rDNA, and chloroplast *tufA* and *rbL* genes. *Neoclonium akinetum*, *Ulothrix zonata*, and *Sarcinofilum mucosum* (all Ulotrichales) were used as the outgroup. The final Ulvales alignment consisted of 1,729 nuSSU rRNA gene (390 V, 308 Pi), 763 *tufA* (385 V, 284 Pi), and 1,126 *rbL* (329 V, 224 Pi) positions, and the substitution models selected were TrNef+I+G (0.501) for nuSSU rRNA gene, TPM3+G (0.250), HKY+G (0.340) and TIM2+G (0.480) for the first, second, and third codon position of the *tufA* gene, respectively, and JC+I, TPM3uf+I, and TVM+I for the first, second, and third codon position of the *rbL* gene, respectively. All of our samples within Ulvales were placed in the family Kornmanniaceae, so an additional analysis of the family, based on nuSSU rRNA gene and nuITS, was performed. All taxa of the family (Darienko and Pröschold 2017, Škaloud et al. 2018) with available DNA sequence data were included. For taxa that are known to be both free-living and lichenized, a sequence from both strains was included, if available (Table S5). *Ctenocladus circinatus* (Ulvales) was used as the outgroup. The concatenated alignment consisted of 1,021 nucleotides for the nuSSU rDNA gene (142 V, 86 Pi) and 468 nucleotides for the nuITS region (231 V, 187 Pi), and the substitution models were TrNef+I+G (0.63) for nuSSU rRNA gene, HKY+G (0.928) for nuITS1, K80+I for nu5.8S, and TPM2uf+G (0.627) for nuITS2.

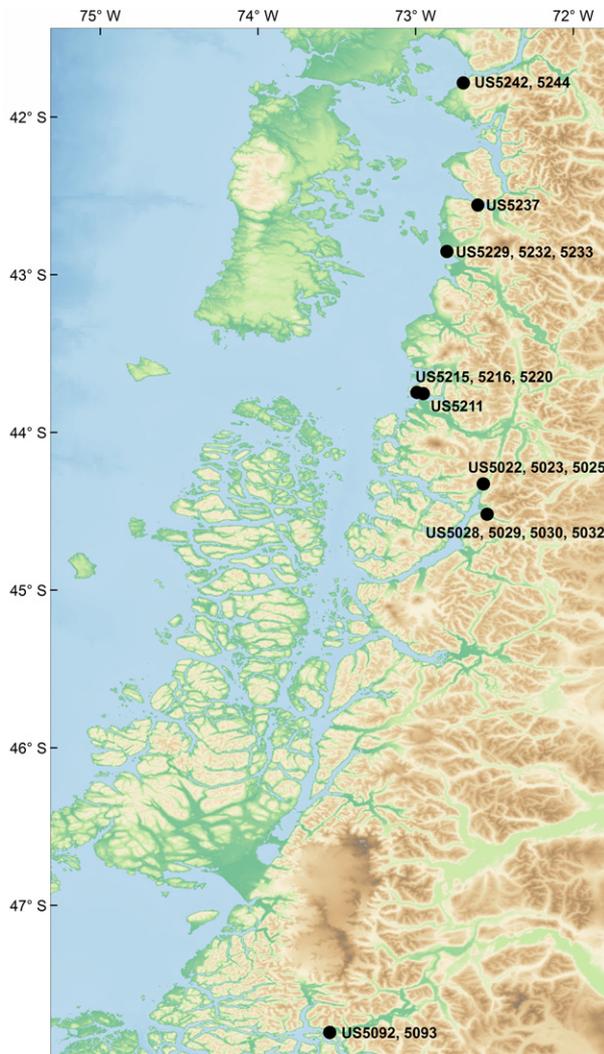


FIG. 1. Map of sampling sites on the Northern Patagonian Pacific Coast in Chile. For details, see Table S1. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Separate analyses of each marker gave congruent results for all the datasets, so they were concatenated. The phylogenetic trees were inferred by Bayesian Inference (BI) in MrBayes v. 3.2.6 (Ronquist et al. 2012) using partitioned datasets. Two parallel Monte Carlo Markov Chain (MCMC) runs, with one cold and three heated chains, were carried out. Trees and parameters were sampled every 100 generations. Convergence of the chains was verified by the convergent diagnostic of the potential scale reduction factor using the sump option, and it approached 1 in all cases. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). For Verrucariaceae, it was run for 26 million generations (SDSF 0.00211), for Ulotrichales for 5 million generations (SDSF 0.001949), for Ulvales for 6 million generations (SDSF 0.001406), and for Kornmanniaceae for 8 million generations (SDSF 0.00145). The first 25% of the trees were discarded as burn-in in each run. 50% majority rule consensus trees were obtained using the sumt option. Bootstrap analyses were performed by maximum likelihood (ML) using RAxML v. 8.2.12 (Stamatakis 2014).

It consisted of 1,000 rapid bootstrap inferences with automatic termination. Analyses were run on the CIPRES Science Gateway v. 3.3 web portal (Miller et al. 2010). The resulting trees were visualized using FigTree v. 1.4.3 (Rambaut 2016).

The associations between the mycobiont and photobiont were visualized with `phytools::cophylo` function in the free software R v. 4.1.0 (R Core Team 2021) using the option to rotate the nodes of both trees to optimize vertical matching of the tips (Revell 2012). For this purpose, simplified ML trees that included only our samples and an outgroup (*Dermatocarpon miniatum* in the case of the mycobiont and *Desmochloris molenhaueri* in the case of the photobiont) were calculated in GARLI (Zwickl 2006).

## RESULTS

**Phylogeny of mycobionts.** The three loci phylogeny of the family Verrucariaceae (Fig. 2) placed our samples within the genus *Hydropunctaria* or within the *Wahlenbergiella* group. Samples US5022, 5023, 5028, 5029, 5030, 5092, 5093, and 5237 belonged to the genus *Hydropunctaria* with full Bayesian posterior probability (PP) support. The rest of the samples were placed in the *Wahlenbergiella* group with full support, but none matched the genus *Wahlenbergiella*, which has a basal position within the group (in accordance with Pérez-Ortega et al. 2010). Samples US5220, 5232H, 5232V, and 5229 formed a lineage with *Verrucaria cf. serpuloides* MAF-Lich 16296 (Pérez-Ortega et al. 2010), but with low support (PP = 0.64); samples US5211 and 5216 formed a sister lineage to the former, the relationship being fully supported; US5215, 5232L, and 5244 were related to *M. tessellata* with full support; US5242 formed a fully supported lineage with *Verrucaria sp.* MAF-Lich 16297 and *Verrucaria cf. degelii* MAF-Lich 16298 (Pérez-Ortega et al. 2010); and finally, the position of US5025 within the group is not clear (Fig. 2). The taxonomic identity of the mycobionts will be treated in more detail elsewhere.

**Phylogeny of photobionts.** The phylogeny of Ulotrichales based on nuSSU rRNA gene, ITS, and *tufA* (Fig. 3) placed our samples US5215, 5232L, and 5244 within the Acrosiphoniaceae, specifically within *Urospora* with full support. They form a lineage with *Urospora wormskioldii*, but with low support (PP = 0.65). *Urospora wormskioldii* and *Urospora penicilliformis* are virtually indistinguishable based on both ITS and the part of the nuSSU rRNA gene we used for the analysis (Lindstrom and Hanic 2005). The difference between our sequences and *U. wormskioldii* was 0–1 bp in nuSSU rRNA gene and 2–3 bp in ITS; and 1–2 bp in nuSSU rRNA gene and 2–3 bp in ITS between our sequences and *U. penicilliformis*. We refrain from giving a species name to the *Urospora* photobionts for now.

The phylogeny of Ulvales based on nuSSU rRNA gene, *tufA*, and *rbcl* placed our samples within the Kornmanniaceae (Fig. S1 in the Supporting Information) with full support. Further phylogeny of the

TABLE 1. List of newly obtained sequences and their GenBank accession numbers.

Sample code	Mycobiont	Genbank accessions			Photobiont	Genbank accessions		
		nuLSU	nuSSU	mtSSU		nuITS	nuSSU	<i>rbcL</i>
US5022	<i>Hydropunctaria</i> group	OL3429959	OL342977	OL342987	<i>Pseudodoclonium submarinum</i>	OL619283	OL342950	OL684554
US5023	<i>Hydropunctaria</i> group	OL342960	OL342978	OL342988	<i>Pseudodoclonium submarinum</i>	OL619284	OL342951	OL684555
US5025	<i>Wahlenbergiella</i> group	OL342961	—	OL342989	<i>Pseudodoclonium submarinum</i>	OL619285	—	—
US5028	<i>Hydropunctaria</i> group	OL342962	—	OL342990	<i>Pseudodoclonium aff. arthropyreniae</i>	OL619286	—	OL684556
US5029	<i>Hydropunctaria</i> group	OL342963	—	—	<i>Pseudodoclonium submarinum</i>	OL619287	—	—
US5030	<i>Hydropunctaria</i> group	OL342964	—	—	<i>Pseudodoclonium</i> sp.	—	OL342952	—
US5092	<i>Hydropunctaria</i> group	OL342965	—	—	<i>Undulifilum symbioticum</i>	OL619288	—	—
US5093	<i>Hydropunctaria</i> group	OL342966	—	OL342991	<i>Pseudodoclonium submarinum</i>	OL619289	—	—
US5211	<i>Wahlenbergiella</i> group	OL342967	OL342979	OL342992	<i>Undulifilum symbioticum</i>	OL619290	—	—
US5215	<i>Wahlenbergiella</i> group	OL342968	OL342980	OL342993	<i>Urospora</i> sp.	OL619291	OL342953	OL684557
US5216	<i>Wahlenbergiella</i> group	OL342969	—	OL342994	<i>Pseudodoclonium submarinum</i>	OL619292	OL342954	OL684558
US5220	<i>Wahlenbergiella</i> group	OL342970	OL342981	OL342995	<i>Undulifilum symbioticum</i>	OL619293	OL342955	—
US5229	<i>Wahlenbergiella</i> group	OL342971	OL342982	OL342996	<i>Pseudodoclonium submarinum</i>	OL619294	—	—
US5232H	<i>Wahlenbergiella</i> group	—	OL342983	OL342997	<i>Undulifilum symbioticum</i>	OL619295	—	—
US5232L	<i>Verrucaria cf. tessellatula</i>	OL342972	OL342984	OL342998	<i>Urospora</i> sp.	OL619296	OL342956	OL684559
US5232V	<i>Wahlenbergiella</i> group	OL342973	OL342985	OL342999	<i>Undulifilum symbioticum</i>	OL619297	OL342957	—
US5237	<i>Hydropunctaria</i> group	OL342974	—	OL343000	<i>Pseudodoclonium submarinum</i>	OL619298	—	—
US5242	<i>Wahlenbergiella</i> group	OL342975	—	OL343001	<i>Pseudodoclonium submarinum</i>	OL619299	—	—
US5244	<i>Wahlenbergiella</i> group	OL342976	OL342986	OL343002	<i>Urospora</i> sp.	OL619300	OL342958	OL684560

family based on nuSSU rRNA gene and ITS (Fig. 4) showed that most of our samples belonged in *Pseudodoclonium*. Specifically, samples US5022, 5023, 5025, 5029, 5093, 5216, 5229, 5237, and 5242 belonged to *P. submarinum*; US5030 also belonged to this genus, this position is not clear due to inability to amplify the ITS sequence; sample US5028 is related to *P. arthropyreniae*, with a difference of 17 bp in nuSSU rRNA gene in comparison to *P. arthropyreniae* SAG 467-2. Samples US5092, 5211, 5220, 5232H, and 5232V (Fig. 4) form a completely new lineage within the family, here referred to as *Undulifilum symbioticum*, gen. et sp. nov.

**Photobiont morphology.** The photobionts did not present any diagnostic characteristics that would allow identification within the thallus (Fig. 5). Cells of lichenized *Urospora* were irregularly spherical of variable size ( $6.2 \times 5.5$ – $11.5 \times 10.1 \mu\text{m}$ ), unevenly scattered within the thallus, forming vertical columns in some parts (Fig. 5, a and b).

*Pseudodoclonium* photobionts were organized in vertical columns in all specimens examined, a characteristic feature of the lichen genera *Hydropunctaria* and *Wahlenbergiella* (Gueidain et al. 2009). Lichenized *P. submarinum* (Fig. 5c) formed individual cells of 4.5–7  $\mu\text{m}$ . Rarely, two-celled filaments were observed. The lichenized *U. symbioticum* (Fig. 5d) photobionts were found in the form of filaments of up to six elongated cells ( $6.5$ – $9 \times 2.5$ – $5.2 \mu\text{m}$ ), also organized vertically.

Only three photobiont cultures were obtained, probably due to the delay between collection and isolation, specifically *Pseudodoclonium submarinum* from samples US5216 and 5242 and *Undulifilum symbioticum* from US5220. In the culture of *P. submarinum* in liquid ASW (Fig. 6), cells in the prostrate filaments are close to spherical, 6.4–12  $\mu\text{m}$  in diameter; cells in the erect filaments are cylindrical 8.8–19  $\times$  2.5–4.5  $\mu\text{m}$ . The chloroplast is parietal with a pyrenoid. For culture detail of *U. symbioticum*, see the description below.

*Patterns in photobiont occurrence.* *Pseudendoclonium submarinum* was the most common photobiont, regardless of the mycobiont identity (Fig. 7). It was found in nine of 19 samples belonging to various lineages of *Hydropunctaria* (US5022, 5023, 5029, 5093, and 5237) and *Wahlenbergiella* group (US5025, 5216, 5229, and 5242). Its occurrence was not affected by geography, substrate type, orientation, exposure, or vertical position of the lichen (see Table S1). *Undulifilum symbioticum* was found in four *Wahlenbergiella* group samples (US5211, 5220, 5232H, 5232V; Fig. 7), belonging to one lineage within the group (Fig. 2) and also in one *Hydropunctaria* sample (US5092). The four *Wahlenbergiella* samples were collected from horizontal surfaces of schist coastal rocks in distinct zones; US5220 was observed in the upper eulittoral zone (within the barnacle zone), US5232H and US5232V in lower littoral fringe (just above the barnacle zone), and US5211 in the mesic-supralittoral zone (at the level of *Mastodia tessellata*), *Hydropunctaria* US5092 was found in the upper littoral fringe of a sileaceous seashore. *Urospora* was noted to be the photobiont of three samples related to *M. tessellata* (Figs. 2, 7) within the *Wahlenbergiella* group (US5215, 5232L, and 5244). All of them were found in the littoral fringe, two just above the barnacle zone, one in the black belt (Table S1).

*Taxonomic description.* *Undulifilum* Škaloud, Černajová et Schiefelbein **gen. nov.**

*Description:* Thallus brush-like and crust-forming, composed of both prostrate and erect filaments. Young thalli consist of a prostrate system of irregularly branched uniseriate filaments, composed of long cylindrical cells possessing a single, parietal, plate-like chloroplast. Most of the filaments are regularly wavy. Lateral branches are formed near the apical part of the cylindrical cells, just below the transverse cell wall. Mature thalli consist of a central prostrate system of densely packed cells surrounded by numerous branched filaments radiating outwards. The terminal cells of the branches are usually significantly longer than those found close to the thalli center. Usually, a well-developed prostrate system of branched filaments is formed on the central prostrate system. Reproduction occurs by vegetative division. Neither zoospores nor sexual reproduction were observed.

Differs from other genera by 18S rRNA sequences and by a wavy habit of the filaments.

*Etymology:* ‘Unduli’ refers to the wavy nature of cells and filaments; ‘filum’ refers to the filamentous habit.

*Type species* (designated here): *U. symbioticum* sp. nov.

*Undulifilum symbioticum* Škaloud, Černajová et Schiefelbein **sp. nov.** (Figs. 8, 9).

*Description:* Colonies in both liquid and agarized BBM medium large, up to 0.2(–0.3) mm in diameter, consisting of prostrate and erected filaments. Young thalli are formed by branched prostrate filaments with relatively long, cylindrical cells attached to the surface,

3.5–4.5(–6)  $\mu\text{m}$  in width and 9–68(–80)  $\mu\text{m}$  in length. During filament growth, cells begin to bend axially, leading to the formation of wavy filaments. In mature thalli, basal cells of filaments transform into short, subglobose, densely packeted cells, 3.5–9  $\mu\text{m}$  in diameter. Cell packets up to 14  $\mu\text{m}$  in diameter. These cells may divide in different directions, forming a pseudo-parenchymatous mass. The cells possess a single parietal chloroplast with a bulged margin and a single pyrenoid. Asexual reproduction by thallus fragmentation into cell packets, which unipolarly germinate into new filaments. Neither zoospores nor sexual reproduction was observed.

*Etymology:* The name refers to the symbiotic lifestyle of the species.

*Holotype* (here designated): PRC 4719.

*Ex-type culture:* CAUP J 1801 (Culture Collection of Algae of Charles University in Prague).

*Habitat:* Photobiont of Verrucariaceae lichens on seashore rocks.

*Type locality:* Chile, Aysén, Puerto Raúl Marín Balmaceda, Rada Del Palena, S43.74625 W72.99108333.

*GenBank accession numbers:* OL343003 for ITS and OL343004 for nuSSU rDNA.

*Additional material examined:* US5092, US5211, US5232H, 5232V.

## DISCUSSION

Coastal rock habitats host about 700 lichen species (Hawksworth 2000), however, only a small portion of them are able to survive in the littoral zone. They have been primarily studied in temperate regions of the Northern hemisphere, but are still understudied (Hawksworth 2000). Therefore, it is not surprising that only one of our Chilean samples could be identified as a known lichen species. Our results suggest that the real diversity of black Verrucariaceae from the littoral zone substantially exceeds the diversity currently described.

At higher taxonomic ranks, lichen mycobiont-photobiont associations are quite stable, where whole lichen families or even orders have preferences for specific genera of photobionts (Miadlikowska et al. 2006). The most common, by far, is *Trebouxia* (Trebouxiophyceae), followed by *Nostoc* (Cyanobacteria), *Trentepohlia* (Ulvophyceae), and *Asterochloris* (Trebouxiophyceae; dePriest 2004, Miadlikowska et al. 2006). However, these photobionts are rarely found in Verrucariaceae, a family in which most of the amphibious lichens (both freshwater and marine) belong. Instead, Verrucariaceae host a plentitude of other algae, as summarized by Tschermak-Woess (1989), Thüs et al. (2011), and Sanders and Masumoto (2021).

Members of the Ulvophyceae family Kornmaniaceae are the most often reported photobionts of amphibious lichens. Formerly, it was the genus *Dilabifilum* (Binz and Vischer 1956, Tschermak-Woess 1976, 1989, Thüs et al. 2011) whose taxonomy was

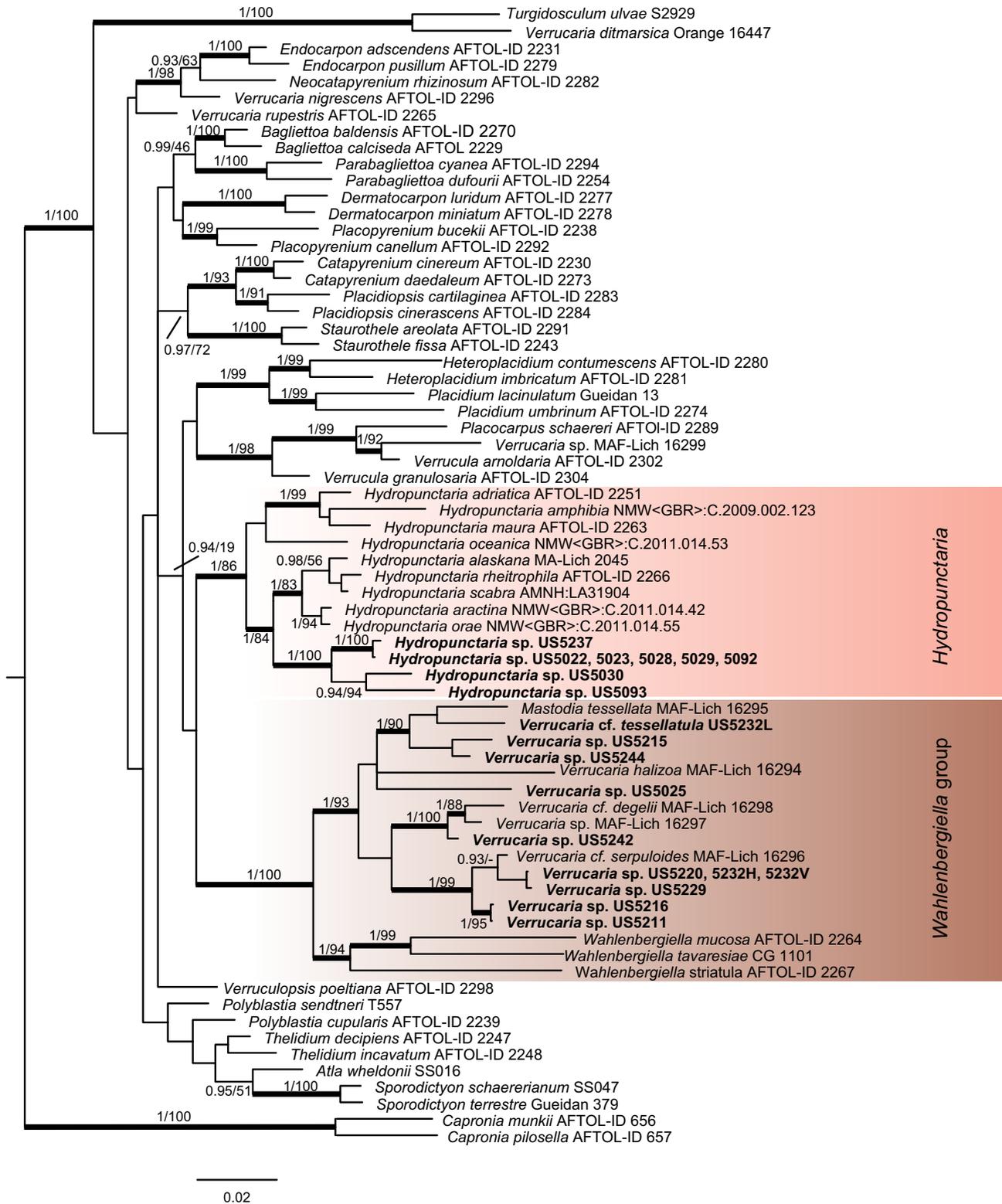


Fig. 2. Phylogeny of the family Verrucariaceae obtained by Bayesian Inference of concatenated nuSSU rRNA gene, nuLSU, and mtSSU rRNA gene. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Specimen/voucher numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Tables 1 and S3. Scale bar represents the expected number of substitutions per site. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

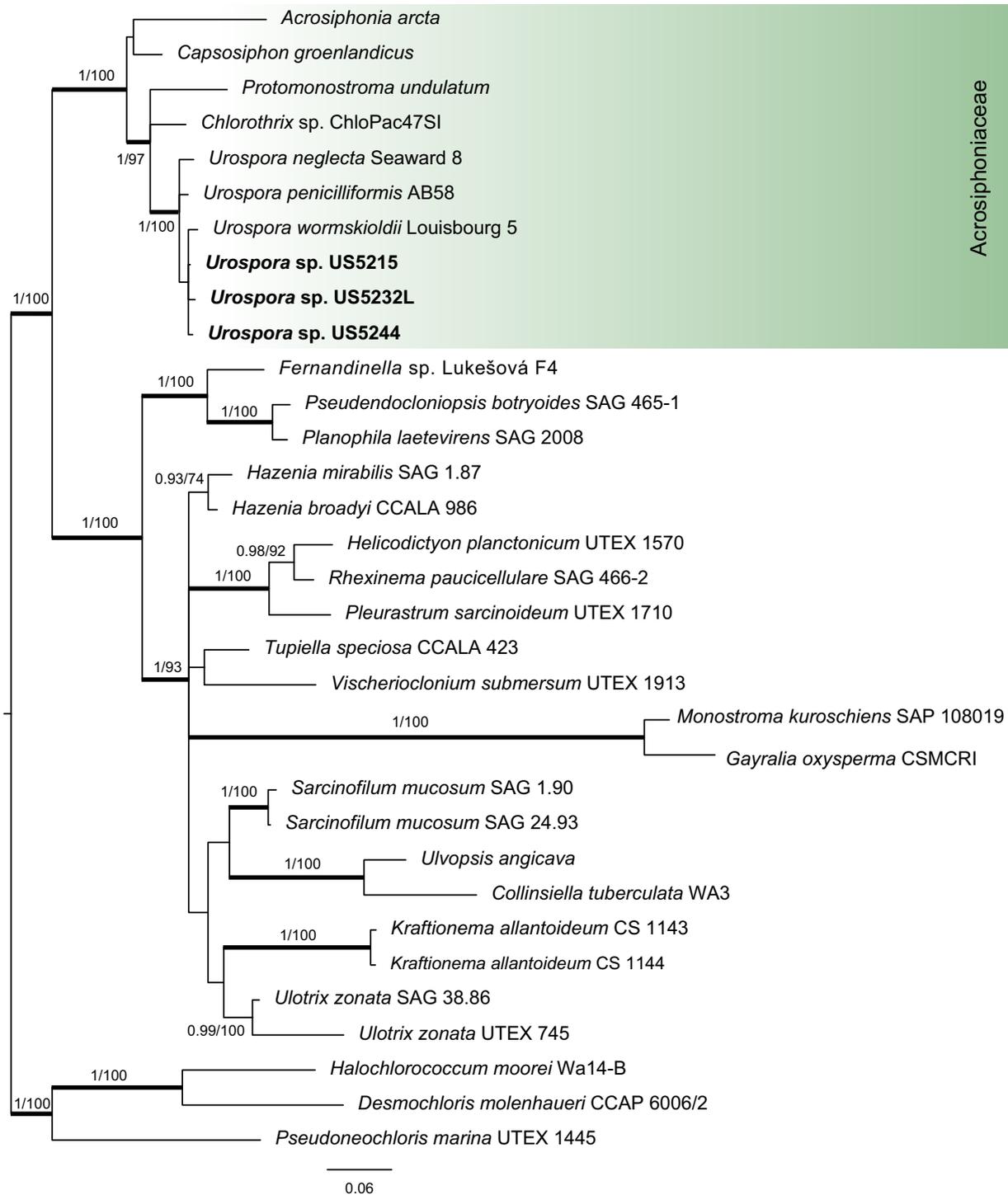


FIG. 3. Phylogeny of the order Ulotrichales obtained by Bayesian Inference of concatenated nuSSU rRNA gene, nuITS, and chloroplast *tu*fA. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Strain numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Tables 1 and S4. Scale bar represents the expected number of substitutions per site. [Color figure can be viewed at wileyonlinelibrary.com]

resolved recently by Darienko and Pröschold (2017). They synonymized *Dilabifilum* with *Pseudendoconium* and placed some of its species into novel, closely related genera *Halofilum*, *Lithotrichon*, and

*Paulbroadya*. A majority of amphibious Verrucariaceae photobionts identified as *Dilabifilum* strains by Thüs et al. (2011) actually also belong to *Pseudendoconium* and *Halofilum*, according to BLAST searches

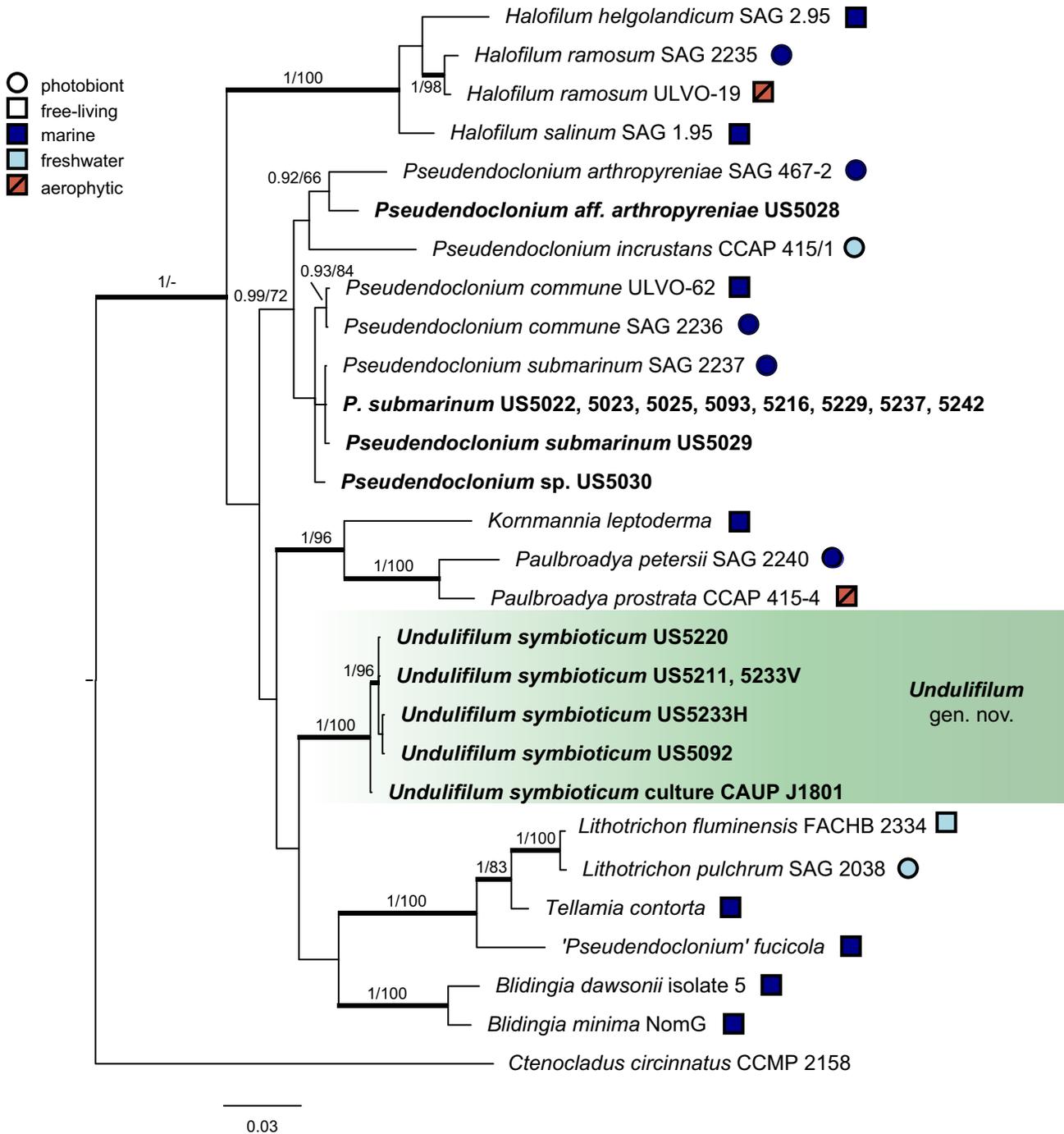


FIG. 4. Phylogeny of the family Kornmanniaceae obtained by Bayesian Inference of concatenated nuSSU rRNA gene and ITS. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Strain numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Table 1 and Table S6 in the Supporting Information. Scale bar represents the expected number of substitutions per site. [Color figure can be viewed at wileyonlinelibrary.com]

of available nuSSU rRNA gene sequences (not shown). Sixteen of 19 photobionts in our samples belonged to the Kornmanniaceae, *P. submarinum* being the most common species (nine samples). Thus, the family Kornmanniaceae itself is rich in lichen photobionts (Fig. 4), especially of

amphibious Verrucariaceae. In addition to the above mentioned genera and *Urospora symbioticum* described herein, *Blidingia minima* occasionally associates with *Turgidosculum ulvae* (Pérez-Ortega et al. 2018) forming one of the few known peculiar borderline lichens with inverted thallus structure,

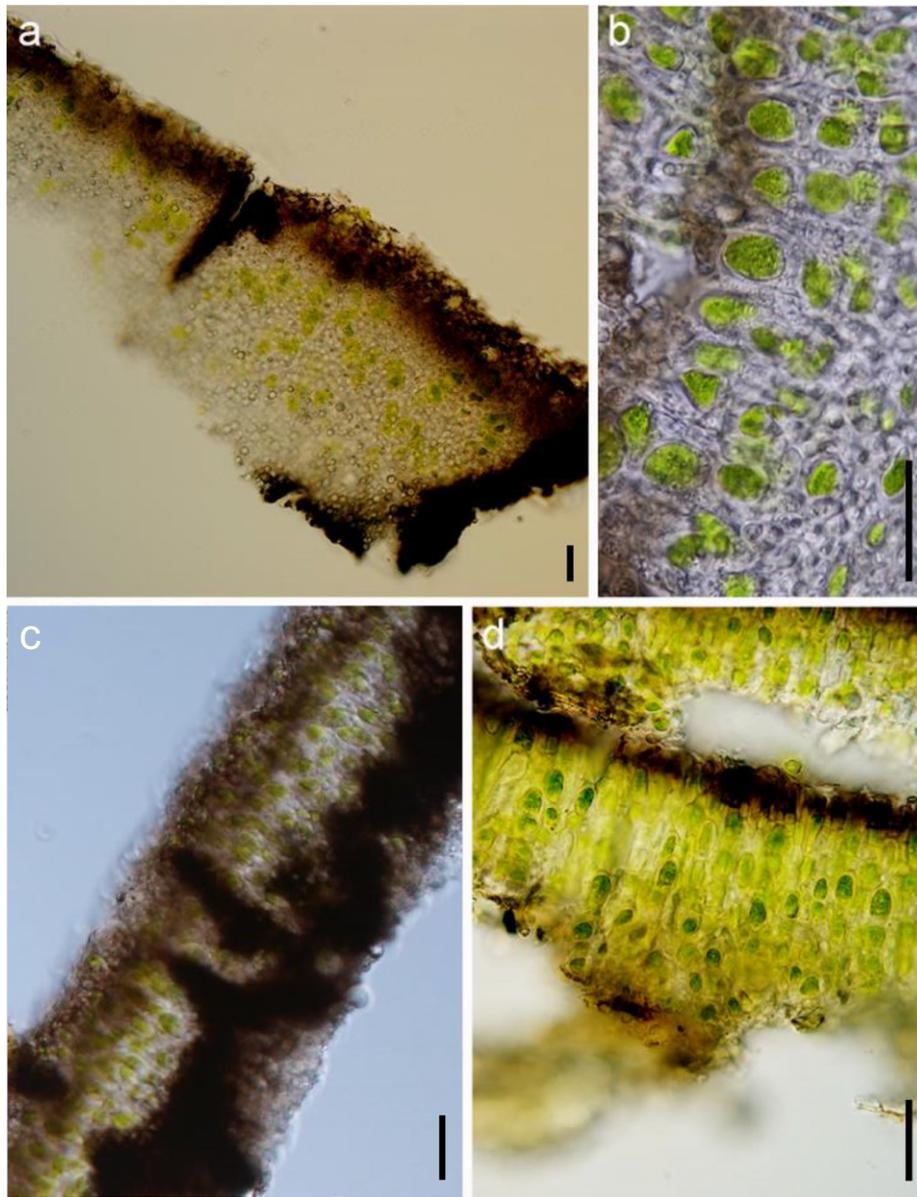


FIG. 5. Cross sections of lichen thalli showing different photobionts. a, b. *Urospora* sp. c. *Pseudendoclonium submarinum*. d. *Undulifilum symbioticum*. Scale bars = 20  $\mu$ m. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

where the photobiont forms the major part of it, and the mycobiont is the inhabitant (Kohlmeyer et al. 2004).

Although the family is predominantly marine, there are also freshwater and aerophytic taxa (Fig. 4). The genera *Kormmannia* and *Tellamia* are exclusively marine, while *Lithotrichon* is exclusively freshwater (Darienکو and Pröschold 2017, Liu et al. 2019) and other genera show wider ecological amplitudes. The two closely related species of *Paulbroadya*, *P. prostrata*, and *P. petersii* are an aerophytic alga and marine lichen photobiont, respectively. *Pseudendoclonium* is mainly marine, but *Ps. incrustans* is a photobiont of freshwater *Verrucaria aquatilis* (Darienکو and Pröschold 2017). At the species level,

for example, the marine *Ps. submarinum*, *Blidingia minima*, and *Banksia marginata* can also be found in brackish estuaries, the *Blidingia* species occasionally even in freshwater habitats (Wille 1901, Škaloud et al. 2018). The coastal photobiont *Ps. arthrophyreniae* is also known as free-living, terrestrial aerophytic on various substrates (Škaloud et al. 2018). However, in these cases, DNA sequence evidence that the different eco-forms actually represent the same species has not been generated. On the other hand, *Halofilum ramosum*, known as the photobiont of marine lichens, has also been isolated from the green biofilm on a wall of ruins, its identity verified by DNA sequence data (Darienکو and Pröschold 2017). Physiological experiments found distinct

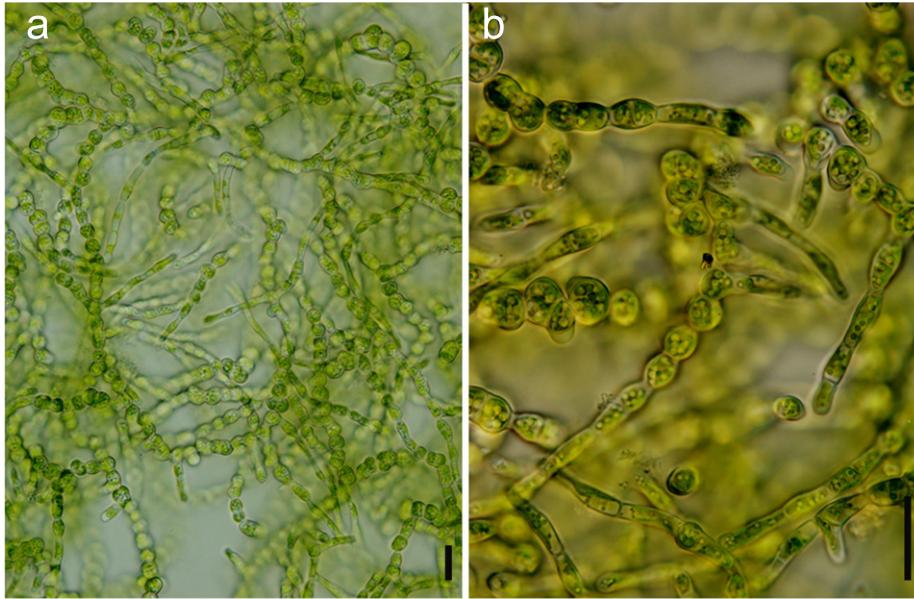


FIG. 6. Morphology of *Pseudendoclonium submarinum* in culture. Scale bars = 20  $\mu$ m. [Color figure can be viewed at wileyonlinelibrary.com]

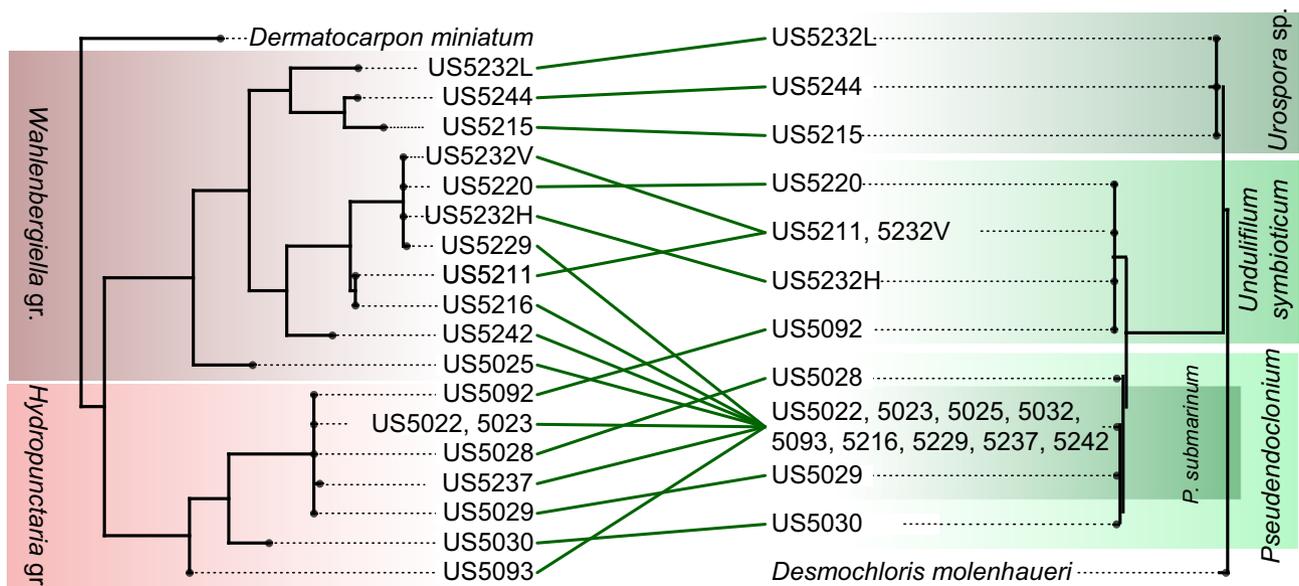


FIG. 7. Interaction network between mycobionts and photobionts considering phylogenetic relationships among them. [Color figure can be viewed at wileyonlinelibrary.com]

osmoregulatory responses between strains isolated from lichens from different vertical zones on the seashore and the hypervariable chloroplast RPL10A region sequence data suggested that the eco-forms might actually represent young sister species (Gasulla et al. 2019).

Taken together, there are multiple transitions from marine to freshwater or aerophytic lifestyles at various levels within the Kornmanniaceae. This capacity may be the reason why Kornmanniaceae are the most common photobionts of lichens in the

littoral zone. Although salinity at a site is more or less constant, lichen thalli deal with huge fluctuations in both salinity and water content, causing considerable changes in osmotic pressure. They are submerged by the sea or washed by waves, and then as the water falls, the lichen dries, and a layer of salt is left on the lichen surface, then if it rains the salt is washed off, and the lichen absorbs fresh water (Dobson 2014). Thus, the flexibility in osmoregulation of the photobiont represents a clear advantage, if not a necessity, for seashore lichens.

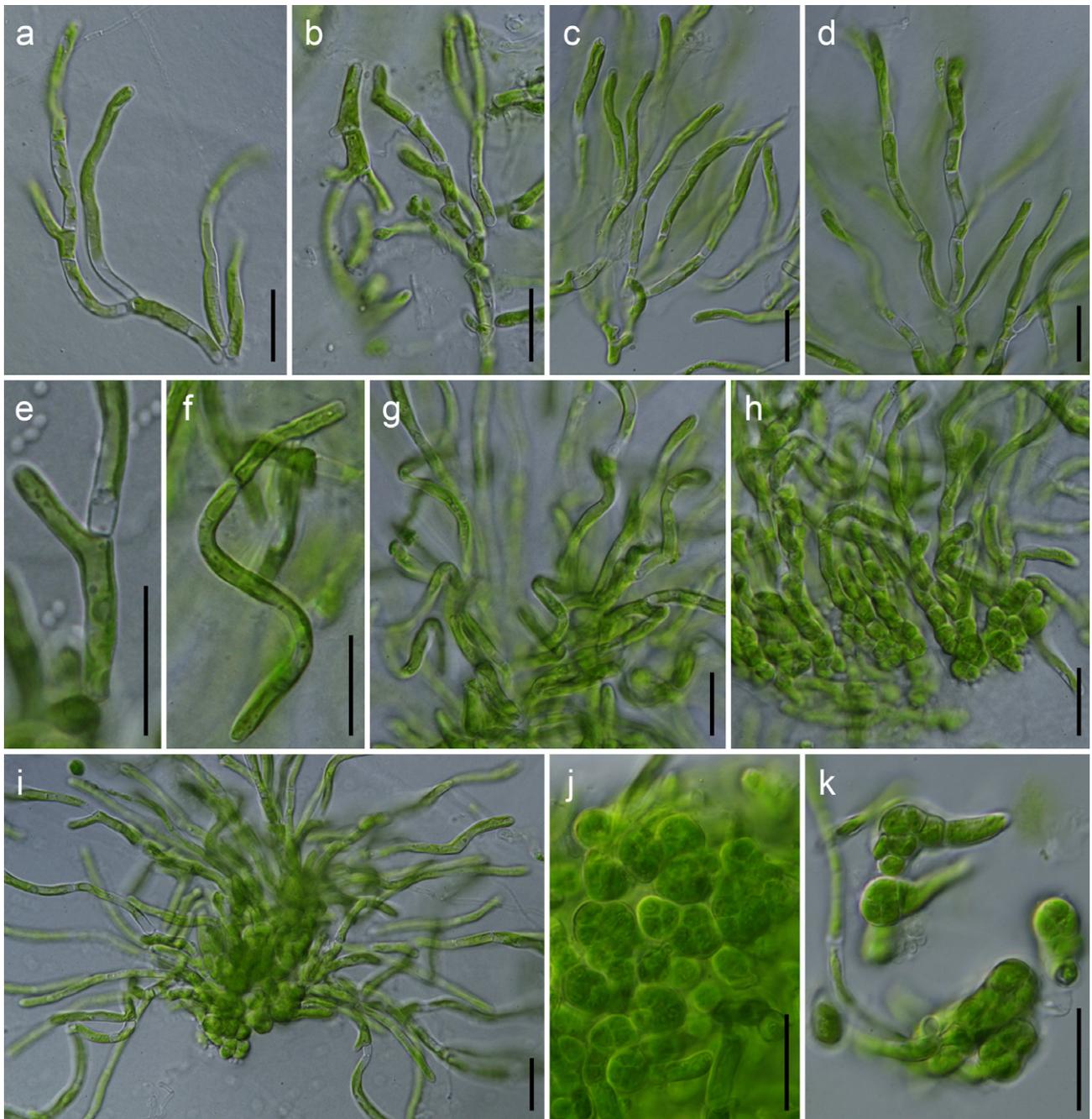


FIG. 8. Morphology of *Undulifilum symbioticum*, gen. et sp. nov. a. Young filaments consisting of elongated, cylindrical cells. b. Production of side branches. c. Growth of branches into elongated cells. d. Formation of wavy filaments. e. Branching cell with a well-visible pyrenoid. f. Axially curved cell. g. Mature filaments formed by significantly curved cells. h. Transformation of basal cells into short, subglobose, densely packed cells. i. Overall view of a mature thallus consisting of a central prostrate system of densely packed cells surrounded by numerous branched filaments radiating outward. j. Close-up view of the central pseudoparenchymatous mass of cells. k. Germination of cell packets into new filaments. Scale bars = 20  $\mu\text{m}$ . [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

No lichen photobiont has previously been reported among the Ulotrichales. In the present study, we observed *Urospora* in association with three related mycobionts (*Mastodia* lineage of the *Wahlenbergiella* group, Table 1, Figs. 2, 7) from three different localities (Fig. 1, Table S1). It was also confirmed by repeated DNA isolation, amplification,

and sequencing. Thus, although unexpected, we consider this finding reliable. The photobionts cannot be assigned any species name because the DNA sequence markers used do not allow for a clear distinction between *Urospora wormskioldii* and *U. penicilliformis* (Lindstrom and Hanic 2005). It might be another, yet unknown, closely related species.

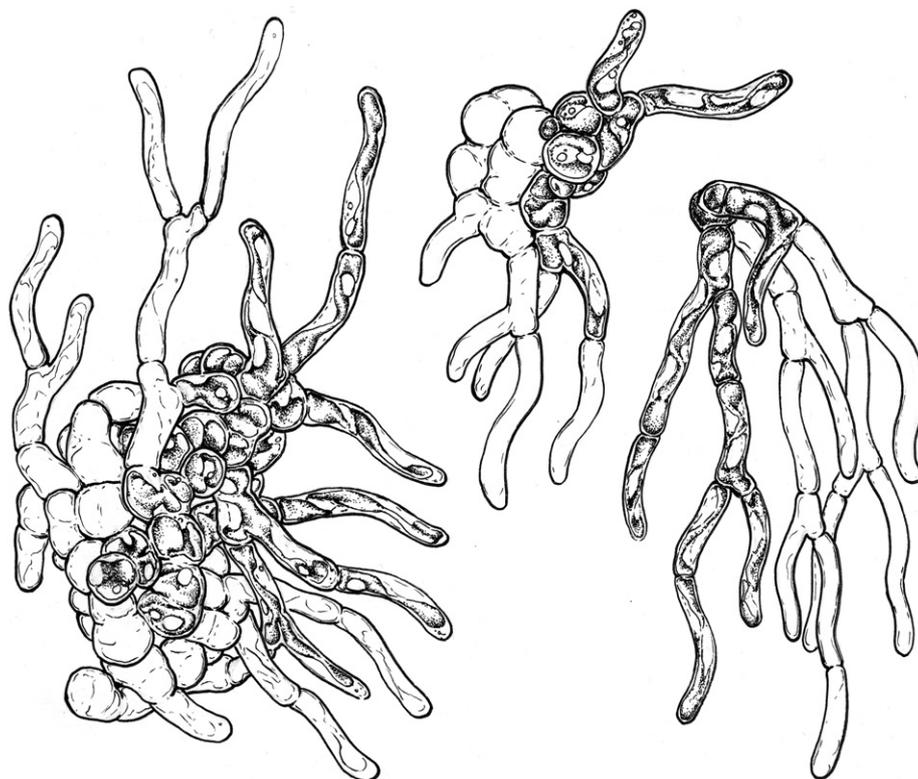


FIG. 9. Illustration of *Undulifitum symbioticum*, gen. et sp. nov.

*Urospora* belongs to Acrosiphoniaceae, the most ancestral lineage within Ulotrachales (Škaloud et al. 2018). *Urospora* species are macroscopic filamentous algae growing in intertidal zones of cold seas (Lindstrom and Hanic 2005). Acquisition of a locally adapted photobiont is reasonable from the mycobiont's point of view, but it is not clear as to what would be the advantage of the lichenized state for an alga, which is successful in the same habitat on its own. *Petroderma maculiforme* (Phaeophyceae), which usually forms free-living crustose thalli, is also known as the photobiont of *Wahlenbergiella tavaresiae*. Where the two forms coexist, the latter also inhabits upper parts of the intertidal zone, while the former is limited to the lower and mid-intertidal zones (Sanders et al. 2004). *Urospora wormskioldii* and *U. penicilliformis* occur in the upper and lower littoral zones, respectively (Hanic 2005). Thus, for now, it cannot be concluded whether the switch to the lichenized state enables ecological niche widening in the case of *Urospora*.

The fact that most of the seashore lichen species seem to be specific toward their photobiont may be the result of lack of data. There is also evidence that the photobiont choice depends on the ecology of a lichen (Ortiz-Álvarez et al. 2015). Gasulla et al. (2019) suggested that the zonation of lichens on the seashore is, at least partly, driven by photobiont physiology. That would concur with the hypothesis of photobiont-mediated guilds (Rikkinen et al. 2002), which expects lichens with the same environmental preferences to

share a set of well-adapted photobionts. However, conclusions cannot be drawn before the diversity of seashore photobionts is generally recognized. Even the very limited sampling of this study revealed novel seashore lichen photobionts, indicating we might be only at the beginning of uncovering their diversity.

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#### AUTHOR CONTRIBUTIONS

**I. Černajová:** Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (equal). **U. Schiefelbein:** Investigation (equal). **P. Škaloud:** Conceptualization (equal); Formal analysis (supporting); Funding acquisition (lead); Methodology (supporting).

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**Figure S1.** Phylogeny of the order Ulvales obtained by Bayesian Inference concatenated nuSSU rRNA gene and chloroplast *tufA* and *rbcL*. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Strain numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Tables 1 and S5. Scale bar represents the expected number of substitutions per site

**Table S1.** Herbarium codes and collection data.

**Table S2.** PCR conditions.

**Table S3.** GenBank accession numbers of the sequences used in the phylogenetic analyses of the Verrucariaceae.

**Table S4.** GenBank accession numbers of the sequences used in the phylogenetic analyses of the Ulotrichales.

**Table S5.** GenBank accession numbers of the sequences used in the phylogenetic analyses of the Ulvales.

**Table S6.** GenBank accession numbers of the sequences used in the phylogenetic analysis of the Kornmanniaceae.

### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site: