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Nuclear rDNA sequences of *Wittrockiella amphibia* (Collins) comb. nov. (Cladophorales, Chlorophyta) and morphological characterization of the mat-like growth form

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Abstract

Cladophora amphibia was found in Yaquina Bay (Oregon, USA) for the first time since the type collection from California in 1903. Vegetative plants were buried in the top centimeter of intertidal mud, partially covered by mats of *Rhizoclonium* and *Chaetomorpha*. *C. amphibia* plants had densely pigmented upright branches penetrating the mud surface and overlying algal mats. Morphological and habitat characteristics of *C. amphibia* strongly overlap with those of *Wittrockiella paradoxa* Wille, and molecular sequences of ribosomal genes, including the variable ITS2 region, indicate very high genetic similarity between these two taxa. The seven point mutations in the ITS2 DNA sequences are regarded as low intraspecific variation. Since *C. amphibia* had been described earlier, *W. paradoxa* Wille becomes a taxonomic synonym of *C. amphibia*, and the new binomial *Wittrockiella amphibia* (Collins) comb. nov. is required. As had been previously reported for *W. paradoxa*, the studied Pacific plants were 'cladophoroid' in form (upright, branched thalli with cylindrical cells), in contrast to the stunted and poorly branched 'rhizoidal' plants mainly found in Europe. The heterotrichous mode of growth is further characterized.

Introduction

Cladophora amphibia Collins 1907 is known only from its type collection from a saltmarsh in central California (Collins 1907, Setchell & Gardner 1920), and it has not been collected or recorded again in more than 100 years. It was described as forming a thin layer on the sediment in the highest intertidal in a manner similar to the heterotrichous system of matted filaments reported for *Wittrockiella paradoxa* Wille 1909 by South (1981). The thallus of *C. amphibia* consists of matted lower parts with irregular to subcylindrical cells (40-70 µm in diameter) and more regular erect branches (30-50 µm in diameter), resembling a *Vaucheria* thallus in habit (Collins 1907). Some cells were reported to be swollen in the middle to 100 µm in diameter. The cross-walls that cut off branches from the mother cell occur at some distance from the base as in *Cladophoropsis*. Overall, this species appears to be morphologically very similar to members of the genus *Wittrockiella* and to *W. paradoxa* in particular.

The brackish-water genus *Wittrockiella* is a small group of ulvophycean green algae. It is a member of the *Aegagropila*-lineage, an assemblage of species-poor, mainly freshwater genera such as *Aegagropila*, *Arnoldiella*, *Basicladia*, and *Pithophora* (Hanyuda *et al*. 2002, Yoshii *et al*. 2004). This lineage is one of the four lineages recognized within the Cladophorales (Leliaert *et al*. 2009a). *W. paradoxa* is the type species of the genus (Wille 1909), the other two species in the genus are *W. lyallii* (Harvey) C. Hoek, Ducker *et* Womersley and *W. salina* V.J. Chapman. The placement of *W. paradoxa* and *W. lyallii* within the same genus has been verified by molecular data (Yoshii *et al*. 2004). Moreover, there is speculation that *W. paradoxa* might be related to *Cladophorella calcicola* Fritsch (van den Hoek *et al*. 1984), a morphologically similar warm-temperate to tropical species that typically occurs semi-terrestrially on moist limestone and has been found in Australia, Bangladesh, and tropical hothouses in Europe (Fritsch 1944, Islam 1964, Cribb 1965).

Wittrockiella paradoxa was originally described from a brackish saltmarsh in southwestern Norway as forming a cartilaginous mucilage-capsule in a cyanobacterial crust. The filaments were short, poorly branched and had very irregular to roundish cells. Cells of both *W. paradoxa* and *W. salina* are known to produce long septate hairs (Wille 1909, van den Hoek *et al*. 1984), but these are often absent. Akinetes or aplanospores can form singly or in short chains within the filaments (Wille 1909).

Additional morphological observations have broadened interpretation of the species, and more densely branched growth forms have been described (Polderman 1976, South 1981). The short, poorly branched filaments consisting of round or irregular cells have been termed the 'rhizoidal growth form'; the more densely branched and upright plants consisting of irregular to cylindrical cells have been termed the 'cladophoroid growth form' (Polderman 1976, South 1981, South 1989). Cell sizes range from 8-20 µm in diameter and 10-100 µm in length in the rhizoidal form, and 20-80 µm in diameter and 80-250 µm in length in the cladophoroid form (Polderman 1976, South 1981).

In addition to the crust-like habit described for the type material, *Wittrockiella paradoxa* can form mats on soft estuarine sediments or occur as as epi- and/or endophytes in saltmarsh plants (Polderman 1976, South 1981, 1989). *W. paradoxa* has a wide distribution in the Northern Hemisphere: records are known from the Wadden Sea (Germany and the Netherlands; Polderman 1976), southern and southwestern England (Polderman 1976), the Atlantic coast of France (Dizerbo & Herpe 2007), southwestern Norway (Wille 1909), northwestern Spain (Calvo *et al*. 1999), northeastern America (Collins *et al*. 1910, South

1989), and the Pacific coast of northwestern America (South 1981). It most likely is more widespread, but it could easily have been overlooked due to its unobtrusive habit. In the Atlantic locations, the rhizoidal form seems to dominate. In the Pacific, the cladophoroid form was mostly reported, occurring as a 'greenish fur' on the surface of mud, but the rhizoidal form was also found (South 1981). Through culture work, it has been shown that the rhizoidal form can develop into the cladophoroid form (Polderman 1976, South 1981).

During surveys of the algal flora of Oregon, patches of *Cladophora amphibia* mats were discovered in the Yaquina Bay estuary. The mats are morphologically and ecologically characterized here. In addition, sequences of nuclear ribosomal genes, including the variable internally transcribed spacer 2 region, have been generated for the Oregon material of *C. amphibia* and for a cultured Washington isolate of *Wittrockiella paradoxa*. Due to our findings, the synonymy of *W. paradoxa* with *C. amphibia* is proposed.

Materials and Methods

The following specimens were investigated morphologically:

(1) Field-collected *Cladophora amphibia* (Leiden L0793284, L0793286). Loc. USA, Oregon, Yaquina Bay, Sally's Bend (44.6288N 124.024W); Leg. G.I. Hansen, 07 May 2006 (L0793286) and 20 November 2009 (L0793284). Both were collected from the surface layer of mud under *Chaetomorpha aerea* (Dillwyn) Kützing/*C. linum* (O.F. Müller) Kützing and *Rhizoclonium* sp. mats in an intertidal estuarine habitat (Figs. 1-2) further characterised by Cyanobacteria and *Vaucheria* spp.

(2) Two pressed isotypes of *Cladophora amphibia* (Leiden L1284, L2989). Loc. USA, San Francisco Bay, Alameda; Leg. W.J.V. Osterhout and N.L. Gardner, 26 September 1903. Both were collected from damp soil among *Salicornia* sp. in a saltmarsh. Vouchers in the Leiden herbarium were distributed as Exsiccatae Phycotheca Boreali-Americana No. 1284 (Collins *et al.* 1905).

(3) Cultures of *Wittrockiella paradoxa* (CCMP1674 culture). Loc. USA, Washington, San Juan Islands, Lopez Island (48.420N 122.900W), 1977. Leg. C. O'Kelly. Original material was endophytic in *Zostera marina* Linnaeus leaves.

For morphological comparison and imaging, parts of dried specimens were re-soaked in water and brought to a boil with a drop of synthetic detergent to allow for full reconstitution of the collapsed cells (van den Hoek 1963). Images were taken with a digital camera (ColorView Illu, Olympus Soft Imaging Systems, Münster, Germany) connected to a Olympus SZX10 stereo-microscope and a Olympus BH2 light microscope (Olympus Optical Co. GmbH, Hamburg, Germany). Fresh collections of *C. amphibia* from Oregon were photographed with a Leica DFC 290 digital camera (Leica Camera AG, Solms, Germany) attached to Zeiss Axioskope and Stemi SR microscopes (Carl Zeiss Jena GmbH, Jena, Germany).

We sequenced the small subunit (SSU) rRNA gene (GenBank GU384872), the partial large subunit (LSU) rRNA gene (GenBank GU384873), and the ITS2 region (GenBank GU384875) of *Cladophora amphibia* (specimen L0793284). For *Wittrockiella paradoxa* (CCMP1674), the SSU sequence was downloaded from GenBank (AB078732), and the partial LSU (GenBank GU384874) and the ITS2 (GenBank GU384876) sequences were newly generated. DNA extraction, PCR amplification and sequencing were performed following Boedeker and Immers (2009), but modified for the SSU and the ITS2 region as follows: the complete SSU rDNA gene was amplified using the primer pairs SR1-SS11H and SSU897-18SC2 (Leliaert *et al.* 2007a), the ca. 530 bp containing most of the 5.8S rRNA gene and the complete ITS2 region were amplified using the primer pair ITS3-ITS4 (White *et al*. 1990) at an annealing temperature of 53°C. The ITS2 sequence of *C. amphibia* resulting from direct sequencing contained several ambiguous positions, therefore the PCR product was cloned into the pCR®II vector using the TOPO® TA Cloning® Kit (Invitrogen Ltd., Carlsbad, USA) according to the manufacturer's protocol and re-sequenced.

Results

The material from Oregon (specimens L0793284 and L0793286) consisted of a buried prostrate system with erect branches penetrating the surface of the mud and covering algae (Figs. 3-4). The creeping main axes were densely branched with upright shoots (Figs. 3-5) and produced few rhizoidal filaments (Fig. 5). Reproductive structures were not observed, except one instance of possible beginning akinete formation (Fig. 5). The erect shoots were poorly branched, and were more densely pigmented than the buried prostrate filaments, especially the apical cells that penetrated the mud surface (Figs. 6-8). Erect branches developed with delayed cross-wall formation at more or less right angles to the prostrate supporting filament (Fig. 8). Typically, filaments ranged from 48 µm to 80 µm in diameter, but rarely they were only 28 µm. The material was morphologically identified as *Cladophora amphibia* Collins.

The long-term culture of *Wittrockiella paradoxa* obtained from the CCMP culture collection (strain 1674) contained only rare erect branches and did not show the pronounced differentiation of a prostrate and an upright system. Instead many cells produced outgrowths that were reduced in diameter and were not cut off by cross walls. Occasionally pseudodichotomies were formed. Furthermore, many akinetes and aplanosporangia (Fig. 9) were present. Although quite different from these cultures, the material from Oregon was similar to field collections of *W. paradoxa* from the same location as the CCMP culture (South 1981).

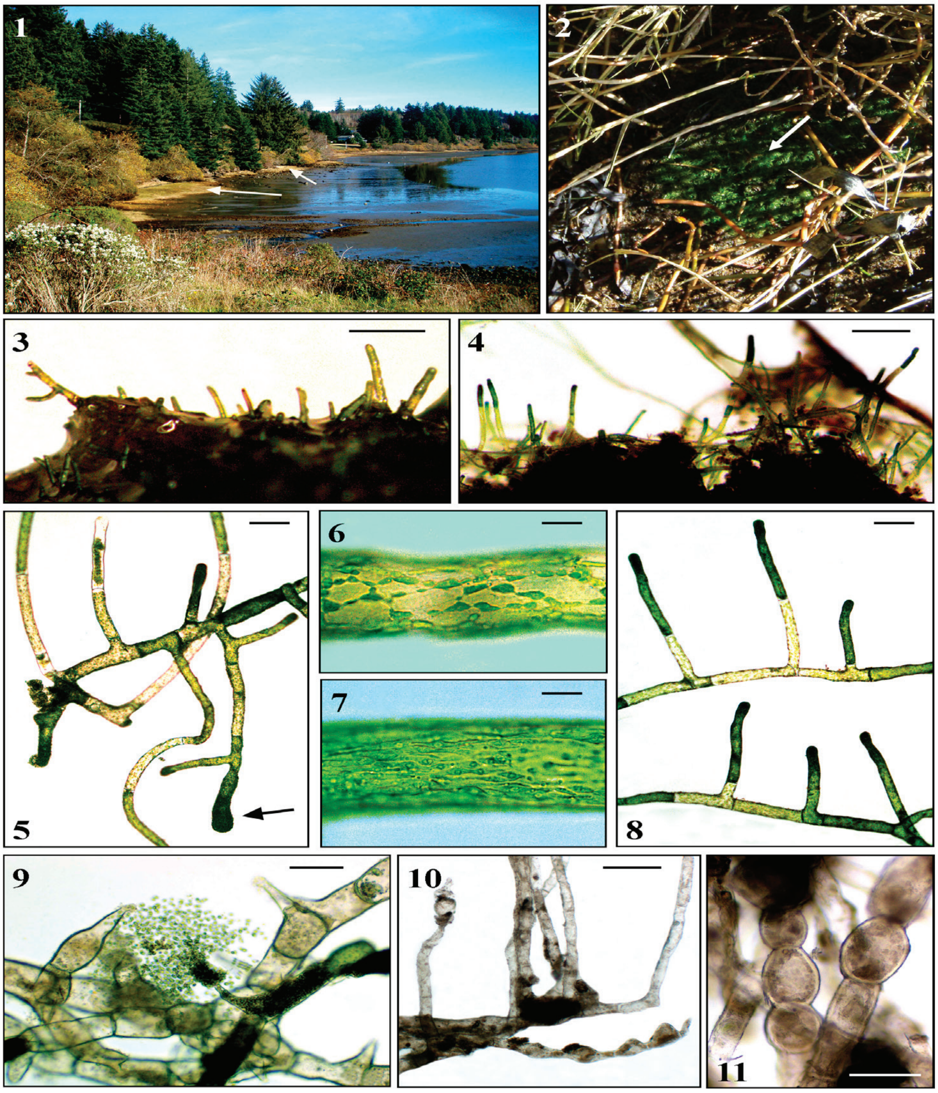
The reconstituted type material of *Cladophora amphibia* consisted of heterotrichous filaments similar to the material from Oregon. Erect branches were formed at right angles and cut off by cross-walls at some distance from the base (Fig. 10). Akinetes up to 95 µm in diameter were present singly and in short chains (Fig. 11).

The SSU and LSU sequences of *C. amphibia* from Oregon and of *W. paradoxa* (CCMP1674) from Washington were identical. In the 364 bp-long ITS2 region, seven point mutations were found between the two specimens, corresponding to less than two percent pairwise differences.

In addition to their morphological similarity, the low sequence divergence in this variable marker indicates that the two species should be considered conspecific. This requires the new combination *Wittrockiella amphibia* (Collins) comb. nov., since the older name *Cladophora amphibia* Collins 1907 has priority over *W. paradoxa* Wille (1909). The holotype of *W. paradoxa* (Lyngør, Norway, Leg. N. Wille, 1907, in L) remains the type specimen for the genus *Wittrockiella*.

***Wittrockiella amphibia* (Collins) Boedeker et Hansen**

Isotypes: Alameda, San Francisco Bay, California, USA, Leg. W.J.V. Osterhout and N.L. Gardner, 26 September 1903, in L, voucher numbers L1284 and L2989, L1284 distributed



Figs 1-11. Habitat, heterotrichous mats and morphological features of *Wittrockiella amphibia* (Collins) comb. nov. from the Pacific. **Figs. 1-2.** Habitat of *Wittrockiella amphibia* (Collins) comb. nov. **1** Estuarine habitat: Sally's Bend, Yaquina Bay, Oregon, USA. Arrows indicate collection sites. **2** *Chaetomorpha* mat (ca. 10 cm in diameter, arrow) between saltmarsh plants covering *W. amphibia* mats. **Figs. 3-11.** Habit and morphological features of *W. amphibia* (Collins) comb. nov. from the northwestern Pacific. **Figs. 3-8.** Material from Sally's Bend, Yaquina Bay, Oregon, USA, November 2009 (specimen L0793284). **3** Prostrate system buried in mud, upright branches penetrating the surface. Scalebar = 500 µm. **4** *W. amphibia*-mat with most of the sediment washed away, showing erect branches with densely pigmented apical cells and parts of the prostrate system. Scalebar = 500 µm. **5** Rhizome-like filament of the prostrate system with upright branches, a rhizoidal filament, and possibly beginning akinete-formation (arrow). Scalebar = 200 µm. **6** Sparsely pigmented cell of the prostrate system, showing the reticulate chloroplast. Scalebar = 10 µm. **7** Densely pigmented apical cell of an erect branch, pyrenoids visible. Scalebar = 20 µm. **8** Upright branches with densely pigmented apical cells, sprouting at right angles and cut off at some distance from the base. Scalebar = 200 µm. **Fig. 9.** Cultured filaments (as *W. paradoxa* (CCMP1674)) showing a sporangium releasing numerous aplanospores through an elongated exit tube. Scalebar = 100 µm. **Figs. 10-11.** Type material of *C. amphibia*, from Alameda, San Francisco Bay, California, USA, 26 September 1903 (voucher number in Leiden herbarium L1284). **10** Heterotrichous growth with erect branches sprouting at right angles from the prostrate filaments. Note one prostrate filament with beginning branch formation ("bumps"). Scalebar = 200 µm. **11** Round and ellipsoid akinetes arranged singly and in short chains. Scalebar = 100 µm.

as Exsiccatae Phycotheca Boreali-Americana (PB-A) No. 1284 (Collins *et al.* 1905). Additional isotypes distributed as No. 1284 of the PB-A to AHFH, BM, FH, PC, TRH, UC and WIS (visit http://sweetgum.nybg.org/ih) for herbarium acronyms), among others. We select the specimen L1284 as the lectotype.

Basionym: *Cladophora amphibia* Collins 1907: 200.
Synonym: *Wittrockiella paradoxa* Wille 1909: 220-221.

Discussion

The initial purpose of our study was to identify the partially buried, heterotrichous mats of green cladophoroid filaments from Oregon. We were able to morphologically compare these filaments with the type description and type specimens of *Cladophora amphibia* from California and with published reports and cultured material of *Wittrockiella paradoxa*. In addition to showing morphological similarity between all of these specimens, we generated molecular data that showed little variation between *C. amphibia* and *W. paradoxa*, leading to the synonymy of these species and to the new combination *Wittrockiella amphibia*.

The current concept of *Wittrockiella paradoxa* already differed substantially from the original description of the species, due to later findings of a greater range in morphology and growth forms. In Wille's description (1909), *W. paradoxa* had no cylindrical cells, no upright system and was very poorly branched. The typical right angled branches and the delayed cell wall formation had not yet been observed. Fritsch (1935) commented on the 'apparent lack of a prostrate system', which was only later discovered to develop in culture (Polderman 1976) and in the field (South 1981). Other later morphological findings included the ability of the thallus to convert in culture from the rhizoidal to the cladophoroid form (Polderman 1976, South 1981), and the discovery of heterotrichous mats in the field (South 1981). These findings, together with the morphological match of *Cladophora amphibia* (Collins 1907) and the cladophoroid form of *W. paradoxa*, and the high genetic similarity between *C. amphibia*-like plants and *W. paradoxa*, make us consider them conspecific. Molecular sequences are increasingly leading to the discovery of taxonomic synonymy of separately described taxa (*e.g.,* Leliaert *et al.* 2008, West & Zuccarello 2009). It had previously been speculated by Silva (1979) that *Cladophora amphibia* may represent a species of *Spongomorpha*, but the present study shows an affiliation with the genus *Wittrockiella* instead.

Since all available sequences originate from the Pacific, there is the possibility that *Wittrockiella amphibia* represents a separate cryptic species of *Wittrockiella* in the Pacifc, with *W. paradoxa* only occurring in the Atlantic. The new name *W. amphibia* would still remain stable for the Pacific species in that case. The existence of one additional undescribed species of *Wittrockiella* that only occurs endophytically in *Zostera* leaves has been mentioned for the northeastern Pacific (Gabrielson *et al.* 2006), and preliminary molecular data suggest that it is a distinct species, not *W. amphibia* (C. O'Kelly - pers. comm.).

Unfortunately, no Atlantic specimens could be obtained for our molecular analyses. The sequence divergence in the ITS2 region between *Wittrockiella amphibia*, *W. salina* and *W. lyallii* is about 20-30%, but the sequences are difficult to align (Boedeker, unpublished data). Thus, the seven point mutations in the ca. 360 bp long ITS2 sequences between material from Washington and Oregon are regarded as low intraspecific variation. The synynomy of the Californian *Cladophora amphibia* with *W. paradoxa* extends the distributional range of the species in the North Pacific by approximately 1000 km further to the south. The species is possibly more widespread in temperate regions of the Northern Hemisphere than is currently known.

The densely pigmented assimilatory cells that penetrate upward through the mud and the covering mats of filamentous green algae contrast greatly with the weakly pigmented cells of the prostrate system. This heterotrichous growth form in mud might be an ecological adaptation to unstable environmental conditions in the estuarine habitat. Burial in soft sediment would help to retain moisture during low tides. Earlier authors have suggested that the species also has other strategies for protection against desiccation and fluctuating salinities. These include the formation of a thick gelatinous cover produced by swelling of the outer cell wall layers (Wille 1909), an endophytic lifestyle (Polderman 1976), and the presence of haematochrome/oil droplets (Wille 1909).

The rhizoidal growth form has been found both as endo- and epiphytic plants and in crusts on the sediment (Wille 1909, Polderman 1976, South 1989). Whether the rhizoidal or the cladophoroid form occurs or dominates could be either habitat-specific, or induced by physical environmental factors, such as different seasons. The few studies on the species provide only limited information on the vegetative or reproductive seasonality. In northwestern Europe, *Wittrockiella paradoxa* was reported to show no obvious seasonality in the epiphytic growth form while the soil growth form was most abundant in spring (Polderman 1976). In saltmarshes in northwestern Spain, it was found in winter, spring and summer but not in autumn. (Calvo *et al*. 1999). On the Atlantic coast of northeastern North America, aplanosporangia occurred in June and July, but not in May or August (South 1989). In Oregon, it was found in February and November, but no reproductive structures were found, except possibly one instance of akinete formation (Fig. 5). However, since this species is so easily overlooked, it could be present year-round in most locations. The thick-walled akinetes would allow it to persist as propagule banks in the sediment during unfavourable conditions.

Further sampling in estuarine environments and investigation of saltmarsh vegetation for epi-and endophytes will likely lead to more findings of *Wittrockiella amphibia*, and possibly further extend the distributional range in the temperate Northern Hemisphere. Additional molecular sequences will help to clarify the relation between Atlantic and Pacific populations of *W. amphibia*, and also provide insights into the relationships with the morphologically similar, subtropical genus *Cladophorella*.

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