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New records from the southern North Sea and first records from the Baltic Sea of Kornmannia leptoderma

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Abstract: Combined genetic, morphological and ontogenetic observations show that the circumarctic boreal green algal macrophyte Kornmannia leptoderma has expanded its distribution range into the Baltic Sea, on a German coastal section of 220 km length. The species is also again (or still) established at its former extreme southern distribution limit in the North Sea, the German island of Helgoland, where it has not been detected during the last four decades. Macroscopic visible sporophytes of K. leptoderma are nowadays present in the Baltic Sea and at Helgoland from February to September, while they were in the past only detected from February to May at Helgoland. This capacity for formation of sporophytes in summer correlates with the circumstance that K. leptoderma from the Baltic Sea can complete its life cycle at 15 °C while several studies conducted decades ago with material from Helgoland and from Pacific coasts consistently reported an inhibition of the algal gametogenesis at temperatures that exceed 12 °C. Possibly K. leptoderma has undergone adaptations that facilitate its spread into warmer environments, unless the Kornmannia present in the Baltic Sea and on Helgoland today represents a newly introduced cryptic species.

Keywords: Baltic Sea; Kornmannia; marine invader; range expansion; Ulvales.

Introduction

The SW Baltic Sea is an atidal brackish water environment that offers similar temperature conditions to the SE North Sea, although seasonal minimum and maximum temperatures are more extreme (Lennartz et al. 2014). Between the Danish Straits and the Darss Sill at the German island of Rügen, its mean salinity decreases over a distance of approximately 300 km from more than 20 to 8 (Meier and Kauker 2003). However, the surface salinities along this relatively steep gradient vary considerably both in time and space, due to changes in river runoff, periodic seawater inflow from the North Sea, stratification and upwelling. Many marine species reach their distribution limit within this salinity gradient, which is not only the reason for a decreased diversity (Schubert et al. 2011), but often also for significantly reduced growth (Russell 1988) and – especially in certain groups of macrophytes – for other morphological changes (Russell 1994, Ruuskanen and Kiirikki 2000).

Some green algal groups within the order Ulvales are notorious for their morphological variability. Salinity has repeatedly been reported to affect the morphology of Ulvales (Burrows 1959, Reed and Russell 1978, Sanders 1979) and members of this order exhibiting “unusual” morphologies have occasionally been reported from the Baltic Sea. For example, in Finland green tides of the usually tubular species Ulva intestinalis L. were observed that exhibited a sheet-like monostromatic morphology (Blomster et al. 2002). The variability of Ulvales often hampers their identification based upon morphological characteristics, which became apparent with the introduction of DNA barcoding techniques into the field (Hayden and Waaland 2002, Hayden et al. 2003). As a consequence, the potential for cryptic introductions and hidden extinctions of Ulvales appears relatively high.

We here report that our recently conducted revision of the species inventory of Ulvales along the German Baltic Sea coast resulted in the discovery of Kornmannia leptoderma (Kjellman) Bliding which was so far not known from this ecoregion. Kornmannia leptoderma was first found on Novaja Zemlja, and originally described...
as *Monostroma leptodermum* Kjellman (1877), due to its monostromatic sheet-like thallus that is composed of only one cell layer. Later studies revealed major differences among the life cycles of members of *Monostroma* (Kornmann and Sahling 1962, Tatewaki 1969) and, based on its heteromorphic life cycle with a monostromatic sporophyte and a disk-like gametophyte, *M. leptodermum* was redescribed as *K. leptoderma* (Bliding 1968). Specimens of the genus *Kornmannia* from the North Pacific (Washington) that lived primarily as epiphytes on seagrass and other macrophytes and exhibited slightly divergent morphologies were described as *K. zostericola* (Tilden) Bliding (Bliding 1968). A later comparative study concluded that the two species were indistinguishable with respect to morphology and ontogeny (Golden and Cole 1986). Since then *K. leptoderma* is regarded as the only taxon within the genus *Kornmannia*, but molecular comparisons of Atlantic and Pacific populations and type specimens are still missing.

*Kornmannia leptoderma* has a circumarctic-boreal distribution (Golden and Cole 1986), and in Europe its documented southern distribution limits are in Norway (Rueness et al. 2001) and at the Faroe Islands (Nielsen and Gunnarsson 2001), with the remarkable exception of the German North Sea island of Helgoland, at least 500 km to the South (Figure 1). At Helgoland *K. leptoderma* was probably observed for the first time in 1934 (Schmidt 1938) and thereafter each year from 1960 (Kornmann and Sahling 1962) to 1966 and three more times in the 1970s (Kornmann and Sahling 1983). The species disappeared after 1977 and has been considered as extinct in Germany since 1996 (Ludwig and Schnittler 1996).

**Materials and methods**

Sheet-like monostromatic Ulvales and Ulothrichales were collected during repeated samplings between February 2013 and September 2015 at 110 sites on the German Baltic Sea coast between Flensburg and Rerik and at the North Sea island Helgoland (Table 1). They underwent microscopic examination in the laboratory and parts were conserved for DNA barcoding. At 16 sites material was observed that could not be clearly assigned to *Monostroma grevillei* (Thuret) Wittrock – an abundant monostromatic species in the area – and was investigated further (Table 1, Figure 1). To observe the algal life cycle, pieces of approximately 1 cm² of the material collected at Mönkeberg were transferred into glass Petri dishes (diameter 9 cm) containing 40 ml of ¼ strength Provasoli Enriched...
Table 1: List of samples of *Kornmannia leptodema* identified by DNA barcoding with information on sampling sites.

<table>
<thead>
<tr>
<th>Date</th>
<th>Collector</th>
<th>Site no.</th>
<th>Site name</th>
<th>Environment</th>
<th>Coordinates</th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Exposure</th>
<th>Accession no.</th>
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<td>SST</td>
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<td>Kiekut Beach</td>
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<tr>
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<tr>
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<td>SST</td>
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<td>Strande Harbor</td>
<td>Harbor</td>
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<tr>
<td>18.08.2014</td>
<td>SST</td>
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<td>Kiel-Falkenstein Beach</td>
<td>Beach</td>
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<td>Protected</td>
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<td></td>
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<td>7</td>
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<td>Marina</td>
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<td>11.5</td>
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<td>16.9</td>
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<td>14.3</td>
<td>32.0</td>
<td>Protected</td>
<td></td>
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</table>

Collector: SST, Sophie Steinhagen; FW, Florian Weinberger; DA, Dmitry Afanasyev. Site no.: number in Figure 1. Temperature: temperature at collection time. Salinity: salinity at collection time. Accession no.: GenBank accession number for *tufA* gene sequence included in Figure 3. Two lines printed in bold indicate samples used for life cycle studies.
Seawater (salinity 17). The Petri dishes were maintained for 15 months at 15°C, with a light regime of 40 μmol photons m⁻² s⁻¹ ("cool white", 12L:12D) and replacement of the medium every 3 months. The same approach was repeated with identical cultivation conditions and material collected at Heiligenhafen Binnensee on 30th August 2017.

Genomic DNA was extracted from material dried in silica gel or from frozen fresh material, using the Invivisor Spin Plant Mini Kit (Stratec, Birkenfeld, Germany) and following the manufacturer’s instruction protocol. DNA-barcode fragments of the plastid encoded elongation factor Tu (tufA) were amplified by polymerase chain reaction (PCR), using the primers tufGF4 (Saunders and Kucera 2010) and tufAR (Famà et al. 2002). For amplification the following temperature profile was used: initial denaturation 4 min at 94°C, 38 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, final extension for 7 min at 72°C. Sequencing in both directions was provided by GATC biotech (Konstanz, Germany). Sequences were assembled, reciprocally edited in both directions was provided by GATC biotech (Konstanz, Germany). Sequences were assembled, reciprocally edited with Sequencher (v. 4.1A, Gene Codes Corporation, Ann Arbor, MI, USA) and aligned, using the multiple sequence alignment program MAFFT v. 7.311 (Katoh and Standley 2013).

Phylogenetic analysis

We analyzed the dataset using the Maximum likelihood (ML) approach. For a robust analysis we included several reference sequences downloaded from GenBank indicated by their accession numbers. Reference sequences of Bryopsis corticulans (accession number: HJ610243) and Prasiola stipitata (accession number: GWS004831) were used as outgroups. We constructed ML phylogenies with RAxML v. 8 (Stamatakis 2014). As nucleotide substitution model GTR + GAMMA was used. To test the robustness of the tree an iteration of 1000 pseudoreplicates was performed. DNA sequences of the tufA gene of Kornmannia leptoderma are available from GenBank (for accession numbers see Table 1).

Results

The first specimen was discovered in late winter (February) 2013. It drifted in very sheltered and shallow water at Mönkeberg in the Kiel Fjord. Cultivation of this specimen allowed for observation of the heteromorphic life cycle of Kornmannia. Within 3 months spores were released that formed monostromatic disks of characteristic morphology (Figure 2A and B). During seven more months the crusts increased in size until they reached a diameter of up to 1.5 mm (Figure 2C). At the same time pseudoparenchymatous growth also increased the thickness of the disks, in particular toward their center. A release of propagules was not directly observed, but in December 2014 – 10 months after the launch of the experiment – spores or gametes had been released that germinated into small filaments (Figure 2D). Some of these formed a new generation of disks, which gave rise to minute thalli with an erect tubular morphology (Figure 2C and D), that were closed at the tip. Within three more months these germings increased in size to a length of approximately 5 mm before they liberated their content as spores. Only the cell wall structures remained (Figure 2E). Attached material from Heiligenhafen Binnensee that had been collected in late summer (August 2017) developed considerably faster. Large numbers of tetrafagellate swarmers were immediately released from the thallus. As previously observed with the material from Mönkeberg, germination of attached swarmers resulted in a formation of disks, which increased in size and thickness. After 5–6 months some of the primary disks formed saccate thalli (usually one, in one case two, Figure 2F and G) that increased in length as previously observed on secondary disks with the material from Mönkeberg. At the same time biflagellate swarmers were also released by primary disks (Figure 2H) and after attachment they germinated into a second generation of filaments (Figure 2F–H) that eventually formed secondary disks.

DNA sequencing revealed that the tufA marker gene of the first parental sample from Mönkeberg had more than 99% identity with a reference sample of Kornmannia leptoderma from the Canadian west coast (Saunders and Kucera 2010). Twenty additional specimens were collected until April 2015 (Table 1) at 16 different locations along a Baltic Sea coastal section approximately 220 km long and at Helgoland (Figure 1). They were all – based upon fully or partially sequenced tufA marker genes – closely related and most probably conspecific with the specimen collected in Mönkeberg and with the reference sample (see Figure 3 for a phylogenetic tree with selected samples). All specimens were light green monostromatic sheets with central or basal attachment, in most cases with strongly ruffled margins (Figure 4). Occasionally multiple thalli arose from the same base. Thallus lengths of 5 cm were rarely – if ever – exceeded. Cells in the basal parts were always stretched and between 1.5 and 4.5 times longer than wide. Cells in the middle parts of thalli were much less stretched. They exhibited diameters of 14–26 × 8–19 μm and they typically clustered in groups of two or four.
Figure 2: Life cycle stages of *Kornmannia leptoderma*, raised from material collected in Mönkeberg (A–E) and Heiligenhafen (F–H). Primary thallus disks after (A) 3 months, (B) 4 months and (C) 10 months, in (C) surrounded by five secondary thallus disks. (D) secondary basal disk bearing a young erect tubular thallus on the left and newly germinated filaments on the right (after 12 months). (E) primary disk and two dead tubular thalli (after 15 months). (F) and (G) primary basal disk bearing two erect tubular thallus branches of different size and surrounded by early filamentous stages of secondary disks (5.5 months). (H) biflagellate swarmer. Arrows indicate flagella in (H) and tubular sporophytes in other images, the latter appear often blurred because images were taken with an inverted microscope through the bottom of the culture vessel. Length of scale bars: 50 μm in (A), (B), (D), (F) and (G), 1 mm in (C) and (E), 20 μm in (H).
Discussion

Our combination of genetic, ontogenetic and morphological observations allows for a relatively unambiguous identification of the examined material as *Kornmannia*. A unique character of the genus is its heteromorphic life cycle that combines a discoidal or sometimes filamentous gametophyte and an erect, first tubular, then saccate and finally monostromatic sporophyte that emerges from an initial discoidal stage ("Disk-sac ontogeny"; Golden and Cole 1986). This life cycle has been described in detail for *Kornmannia leptoderma* from Helgoland (Kornmann and Sahling 1962) and Norway (Bliding 1968) and for *Kornmannia zostericola* from Japan (Yamada and Kanda 1941, Tatewaki 1969) and British Columbia (Golden and Cole 1986). Despite small variations, the life cycle traits of both taxa appeared similar, and this was an important argument in support of the view that the two taxa are synonyms (Golden and Cole 1986). In our study tetraflagellate spores released by monostromatic thalli from the Baltic Sea gave rise to a primary discoidal life stage that appeared morphologically identical to the discoidal gametophytes described by the authors mentioned above. We also observed a release of biflagellate swarmers from these disks, which is again in agreement with earlier observations of biflagellate gametes in *Kornmannia*. We could not directly observe a fusion of these swarmers as described by Tatewaki (1969), but such formation of...
zygotes probably happened, since the subsequent development of our cultures was again in accordance with earlier reports (Kornmann and Sahling 1962, Bliding 1968, Tatewaki 1969, Golden and Cole 1986): the propagules that had been released germinated into short filaments. These filaments formed secondary disks, which gave rise to tubular erect sporophytes that were closed at the apex, as previously described for K. leptoderma (Kornmann and Sahling 1962, Bliding 1968, Tatewaki 1969, Golden and Cole 1986). We were unable to observe the final development into monostromatic thalli because the sporophytes released zoospores and thereby emptied their cells before the necessary size had been reached. Nonetheless, the observed ontogeny – including the morphological traits of both generations – was fully in agreement with that of K. leptoderma. Tubular germlings were observed in culture, while monostromatic sheets were observed in nature. We did not observe the intermediate saccate morphologies in the Baltic Sea, but absence of saccate forms has also been observed elsewhere (Golden and Cole 1986). In our second experiment tubular erect sporophytes were already formed by primary disks. Such asexual reproduction of K. leptoderma sporophytes has also previously been reported, for example by Kornmann and Sahling (1962) with material from Helgoland and by Yamada and Kanda (1941) with material from Japan.

Further evidence for the identity of our material with Kornmannia comes from the DNA barcoding approach. The tufA marker gene indicated that our material is genetically more close to the only reference sample of the genus Kornmannia that has so far been published than to any other green algal genus that forms monostromatic blades. The reference sample in question represents a specimen of K. leptoderma from British Columbia (Saunders and Kucera 1986).

Figure 4: Sporophytes of Kornmannia leptoderma.
(A) Living specimens from Heiligenhafen-Graswarder (22.8.2014); herbarium specimens from (B) Redentin (19.7.2013), (C) Heiligenhafen (20.7.2013, epiphytic on Fucus vesiculosus) and (D) Gollwitz (19.7.2013); natural assemblages at (E) Wulfen (27.9.2014) and (F) Redentin (19.7.2013). Scale bars in B-D = 2 cm.
2010). Unfortunately the authors did not mention how their specimen was recognized as *K. leptoderma*, possibly it was found in the characteristic epiphytic association with *Zostera* or *Phyllospadix* that is often observed on the North American Pacific coast and allows for a relatively reliable identification based upon morphological traits (Golden and Cole 1986). Together with our samples from the Baltic Sea and Helgoland, the reference sample of *K. leptoderma* clearly formed one distinct cluster (Figure 3). The identities between pairs of sequences within this branch were always larger than 99.2% and often complete. For example, no base pair divergence was detected between the reference sequence from British Columbia, two samples from Helgoland and two of the sequences from the Baltic Sea (sites Aschau and Hohen-Wieschendorf). All these specimens apparently belonged not only to the same genus, but to the same species. Thus, our data confirm that the same *Kornmannia* species is present at Atlantic and Pacific coasts, as previously suggested (Golden and Cole 1986). We were unable to compare our data to DNA sequences obtained from type material, but most probably the species is *K. leptoderma*, given that the only other species described within the genus (*K. zostericola*) is considered as a synonym. An in-depth comparison of the genetic structure of different Atlantic and Pacific populations of *Kornmannia* might provide an answer to the question whether the genus harbors more than one species or not. However, this was beyond the scope of the present work. Not only our DNA barcoding data and our observations of the ontogenetic development, but also our morphological observations of field-collected sporophytes largely correspond with descriptions of *K. leptoderma* (Bliding 1968, Kornmann and Sahling 1983). We therefore conclude that *K. leptoderma* is currently present not only at Helgoland – where the species has not been observed for four decades – but also in the SW Baltic Sea, where it has not previously been recorded (Nielsen et al. 1995, Schories et al. 2009).

Interestingly, *Kornmannia leptoderma* from the Baltic Sea differs from populations from Helgoland and Hokkaido that were investigated half a century ago by its capacity for life cycle completion at a temperature of 15°C. The optimal water temperature for life cycle completion of material from Hokkaido was approximately 5°C and gametophytes could only become fertile at temperatures below 10–12°C (Tatewaki 1969). Also in *K. leptoderma* from Helgoland a temperature of 15°C inhibited the formation of sporophytes completely and caused parthenogenetic multiplication and malformedness of gametophytes (Kornmann and Sahling 1962). In contrast, a continuous temperature of 15°C could not prevent the formation of sporophytes in our experiments and also no malformation of gametophytes was observed. Instead, the full life cycle was completed within 15 months and a parthenogenetic reproduction of sporophytes was observed within 5.5 months, which clearly contrasts with the above mentioned studies.

In addition to temperature, daylength often affects algal life cycles and this was also reported for *Kornmannia leptoderma*. At temperatures of 10°C or less and a daylength of 16 h, Golden and Cole (1986) observed formation of branched filaments instead of gametophytic disks and no reproduction of sporophytes. However, a development of gametophytic disks and reproduction of sporophytes already at an early stage was observed by the same authors at a daylength of 8 h. This behavior in short day conditions was very similar to the development of *K. leptoderma* from the Baltic Sea at a daylength of 12 h. We did not test the effect of long day conditions on our material, therefore we cannot exclude that such conditions would also inhibit the formation of sporophytes in specimens from the Baltic Sea. However, we frequently detected *K. leptoderma* sporophytes in summer between July and August (Table 1), when water temperatures may easily reach 20°C (Table 1, see also Lennartz et al. 2014) and days are longer than 12 h. This is in agreement with our observations of life cycle completion at temperatures above 10°C, but it contrasts with past observations by Kornmann and Sahling (1983), who explicitly described *K. leptoderma* from Helgoland as a “spring alga”. Also at Helgoland we recently discovered sporophytes not only in spring, but also in September, although mean sea surface temperatures of 12°C or less occur at Helgoland only from November to May (Table 1, see also Wiltshire et al. 2009). Clearly, the formation of sporophytes on Helgoland and in the SW Baltic is not restricted to spring or to the cool season.

These field observations – together with our ontogenetic observations, that were obtained under temperature controlled conditions – strongly suggest that the Baltic Sea and Helgoland have been reached by an ecotype of *Kornmannia leptoderma* that is adapted to elevated temperatures. Our attempts to isolate DNA from existing historical samples of *K. leptoderma* from Helgoland so far failed. It is for this reason currently not possible to examine the genetic similarity between recent specimens and those that were present in Germany half a century ago. Thus, we are unable to decide whether recent German populations are descendants of the Helgoland population that existed five decades ago. Possibly this population never really became extinct, adapted to warm summer conditions and expanded its distribution range into the Baltic Sea,
which is at a distance of less than 200 km from Helgoland through the Kiel Canal (Figure 1).

Alternatively, all recent populations in Germany could result from introductions of more resistant individuals. For example, two different populations of *Kornmannia leptoderma* with overlapping geographical distribution have been distinguished in the NE Pacific (Golden and Cole 1986). One of these ("KZ") had a more southerly distribution limit than the second and was not only discovered in British Columbia, but also in California. This more southern population was reportedly sometimes sexual when adjacent stands of the second population were asexual. The authors compared the ontogenetic development of both populations under controlled conditions at 5°C and 10°C (but not at higher temperatures) and found no important differences. The authors therefore concluded that both populations probably belong to the same species, but they nonetheless suggested that *K. leptoderma* as a species might currently undergo radiation (Golden and Cole 1986). In this light, the question obviously arises whether recent southern North Sea and Baltic Sea populations in Germany are derived from southern Pacific populations of *K. leptoderma*. Also a range expansion into the Baltic Sea of populations in Northern Europe cannot be excluded. Such a scenario would be reminiscent of the southward range expansion by *Fucus evanescens*. C. Agardh into the Baltic Sea that happened approximately 25 years ago (Schueller and Peters 1994) and it would perhaps mirror the development of southern and northern populations of *K. leptoderma* in the Pacific that was proposed by Golden and Cole (1986). However, the genetic similarities or dissimilarities between Northern and Southern populations on Pacific and Atlantic coasts – as well as between the putative synonyms *K. leptoderma* and *Kornmannia zostericola* – have so far not been explored, and it is for this reason not possible to decide with certainty whether the *Kornmannia* populations that are present in our study area today and that were also reported from British Columbia by Saunders and Kucera (2010) represent the same species as *Kornmannia* populations that were present in our study area 40 years ago.

Interestingly, *Kornmannia leptoderma* was not found on a section of nearly 100 km between the Danish border and Kiekut (Figure 1). This observation could suggest that *K. leptoderma* did not reach the German Baltic Sea area by continuous southward migration from the Kattegat area through the Danish Belt, but rather by long distance transport, followed by a point introduction. At the same time, the apparent absence of *K. leptoderma* from the northern coastal section could indicate that its spread into the area is incomplete and perhaps relatively recent.

### Conclusion

The increased performance of *Kornmannia leptoderma* at elevated temperature in the Baltic Sea suggests that its establishment may be less transient than that of *K. leptoderma* on Helgoland 50 years ago. A synopsis of the environmental conditions at all 16 confirmed collection sites of *K. leptoderma* in the SW Baltic Sea (Table 1) suggests that the species mostly occurs in locations that are relatively protected from waves and very shallow (<50 cm below mean sea surface level). In such environments the species typically grows on stones or epiphytically on the bladder wrack *Fucus vesiculosus* L. Salinities down to at least 10 are tolerated. Given the general adaptation of *K. leptoderma* to boreal environments, its spread northward and eastward into the Baltic Sea seems very probable, if the species can tolerate salinities below 10 that predominate east of the Darss Sill. On the other hand, a further spread in the southern North Sea may also be expected, given the tolerance of this species to corresponding temperature conditions.

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