SYSTEMATICS AND PHYLOGENY

DNA barcoding of the German green supralittoral zone indicates the distribution and phenotypic plasticity of *Blidingia* species and reveals *Blidingia cornuta* sp. nov.

Sophie Steinhagen,[1,2] Luisa Düsedau[1] & Florian Weinberger[1]

1 *GEOMAR Helmholtz Centre for Ocean Research Kiel, Marine Ecology Department, Düsternbrooker Weg 20, 24105 Kiel, Germany*
2 *Department of Marine Sciences-Tjärnö, University of Gothenburg, 452 96 Strömstad, Sweden*
Address for correspondence: *Sophie Steinhagen, sophie.steinhagen@gu.se*

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Abstract In temperate and subarctic regions of the Northern Hemisphere, green algae of the genus *Blidingia* are a substantial and environment-shaping component of the upper and mid-supralittoral zones. However, taxonomic knowledge on these important green algae is still sparse. In the present study, the molecular diversity and distribution of *Blidingia* species in the German State of Schleswig-Holstein was examined for the first time, including Baltic Sea and Wadden Sea coasts and the off-shore island of Helgoland (Heligoland). In total, three entities were delimited by DNA barcoding, and their respective distributions were verified (in decreasing order of abundance: *Blidingia marginata*, *Blidingia cornuta* sp. nov. and *Blidingia minima*). Our molecular data revealed strong taxonomic discrepancies with historical species concepts, which were mainly based on morphological and ontogenetic characters. Using a combination of molecular, morphological and ontogenetic approaches, we were able to disentangle previous misidentifications of *B. minima* and demonstrate that the distribution of *B. minima* is more restricted than expected within the examined area. *Blidingia minima*, the type of the genus name *Blidingia*, is epitypified within this study by material collected at the type locality Helgoland. In contrast with *B. minima*, *B. marginata* shows a higher phenotypic plasticity and is more widely distributed in the study area than previously assumed. The third entity, *Blidingia cornuta* sp. nov., is clearly delimited from other described *Blidingia* species, due to unique characters in its ontogenetic development and morphology as well as by its *tufA* and *rbcL* sequences.

Keywords Baltic Sea; barcoding; Helgoland; Heligoland; phylogeography; ontogeny; *tufA*

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

Green algae of the genus *Blidingia* Kylin are a substantial component of the upper and mid-supralittoral zones of the Northern Hemisphere, where they can be found as dense mats on various natural and artificial surfaces and additionally as epiphytes on other macrophytobenthic organisms. *Blidingia* spp. can withstand extreme conditions such as periodic and sustained desiccation, heat waves or frost periods with snow cover, and marine flooding as well as extended freshwater immersion. Thus, representatives of the genus *Blidingia* often indicate the transition between the marine or estuarine zone and the terrestrial zone. *Blidingia* species shape these particular environments and give them a unique and important ecological character by providing habitats for small invertebrates. However, in-depth understanding of the genetic species diversity within the genus and hence about geographic species distribution is still sparse. The recognition of *Blidingia* spp. is largely based on morphological characters of mature thalli and ontogenetic stages, while molecular knowledge is limited.

Kylin (1949) proposed the new genus *Blidingia* for *Enteromorpha minima* Nägeli ex Kütz., based on observations made by Bliding (1938). One of the main characteristics that distinguishes *Blidingia* from the closely related and morphologically similar genus *Enteromorpha* Link (nowadays *Ulva* L.) is its small cells, with a diameter less than 10 μm (Kylin, 1949). Both genera include species with monostromatic, tubular thalli; however, their ontogenetic development was also found to differ significantly (Kylin, 1949). The motile swarmers of *Blidingia* have no eyespot, in contrast to the motile swarmers of *Ulva*. The settled *Blidingia* swarmer grows into an elongated tube that incorporates the intracellular spore contents, and the empty spore sleeve is separated by a transverse membrane. A prostrate disc develops, and from the expanded centre of this partly bi-layered disc, a monostromatic tube begins to emerge (Bliding, 1938; Kylin, 1949). While several studies

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supported these ontogenetic findings (Dangeard, 1961; Gayral, 1967), others emphasized variations in the ontogenetic development between different *Blidingia* species (Kornmann & Sahling, 1978; Tatewaki & Iima, 1984; Iima, 1989), particularly during spore sleeve formation (Bliding, 1963; Kornmann & Sahling, 1978). Bliding (1963) stated, "the germinating tube of the swarmer is mostly divided in a basal empty-cell and an upper cell containing all the cytoplasm", suggesting that differences in spore sleeve formation have been observed. Detailed observations of early ontogenetic developmental patterns on spore sleeve formation were made by Kornmann & Sahling (1978), who focused on the different *Blidingia* species from Helgoland (Heligoland). Whereas half of the observed entities (*B. minima* (Nägeli ex Kütz.) Kylin, *B. chadefaudii* (Feldmann) Bliding) cut off an empty spore sleeve (called embryospore), this pattern was not observed for *B. subsalsa* (Kjellm.) Kornmann & Sahling ex Scagel & al. and *B. marginata* (J.Agardh) P.J.L.Dang. ex Bliding. Thus, it was suggested that early ontogenetic stages were suitable criteria for species delimitation (Kornmann & Sahling, 1978).

Three different ontogenetic developmental patterns of prostrate discs were observed in studies focused on the sexual reproduction and early development of *Blidinga minima* from Japan (Tatewaki & Iima, 1984; Iima, 1989). The authors distinguished compact discs with short cells (D-type), open discs with longer cells (F-type) and an intermediate disc type (M-type) (Iima, 1989). Notably, European specimens that exhibited thallus discs similar to the F-type were assigned to a newly differentiated species, namely *B. chadefaudii* (Bliding, 1938; Chadefaud, 1957; Kornmann & Sahling, 1978). However, due to the interfertility of specimens forming the different disc-types identified by Iima (1989), it was suggested that *B. chadefaudii* should be considered as a variant of *B. minima* (Woolcott & al., 2000). Those authors undertook a molecular reassessment of the taxonomic affiliation of individuals exhibiting different disc-morphotypes based on nuclear rDNA ITS sequences. By showing that the allegedly ontogenetic criteria that were used for species delimitation encompass an integrated continuum of variation, together with the fact that molecular analysis and interbreeding-experiments did not demonstrate a species boundary between Japanese *B. minima* and *B. chadefaudii*, the authors concluded that the two taxa are conspecific. Thus, the observations of Woolcott & al. (2000) blur the boundary between *B. minima* and *B. chadefaudii* and underline that morphological characters within most Ulvales are highly variable and that molecular sequencing is needed to delimit species boundaries.

Currently, six *Blidingia* species are accepted taxonomically: *B. chadefaudii*, *B. dawsonii* (Hollenb. & I.A.Abbott) S.C.Lindstr. & al., *B. marginata*, *B. minima*, *B. subsalsa*, *B. tuberculosa* (P.J.L.Dang.) Benhissoune & al. (Guiry & Guiry, 2020). However, molecular knowledge of these species is sparse and most have primarily been distinguished by morphological and ontogenetic traits.

The present study aimed to examine the diversity of *Blidingia* species in the northern German State of Schleswig-Holstein. Schleswig-Holstein's coastline is of limited length, but is structurally very diverse, including a south-east section of the fully marine North Sea and a south-west section of the brackish Baltic Sea, as well as various estuaries and lagoons. The area also includes the North Sea island of Helgoland, the focus of phycological research in the mid-19th century (Reinke, 1889) and among the best-studied seaweed habitats in Europe (Bartsch & Kuhlenkamp, 2000). Long-term observations of the benthic flora of Helgoland have shown that the establishment of artificial substrata resulted in an increased abundance of *Blidingia* spp. (Bartsch & Kuhlenkamp, 2000). Subsequent to C.W. Nägeli collecting the holotype of *B. minima* on Helgoland around the mid-19th century (historically: *Enteromorpha minima*, suppl. Fig. S1), the first recordings of other *Blidingia* species for the island were made by Wollny (1881). More recently, Kornmann & Sahling (1978) documented the *Blidingia* species of Helgoland in a detailed synopsis, focusing on their developmental differences. The study emphasized the presence of four *Blidingia* species (*B. chadefaudii*, *B. marginata*, *B. minima*, *B. subsalsa*). The authors observed several – and often vague – micro-morphological differences between the four species, but also one commonly shared macro-morphological character: All species were characterized as unbranched tubes (Kornmann & Sahling, 1978).

According to species inventories, only *B. minima* and *B. marginata* have been recorded from Schleswig-Holstein's mainland coasts (Schories & al., 2009). However, due to the aforementioned taxonomic difficulties, historical records of *Blidingia* species require confirmation, and the first molecular assessment of the distribution of Ulvales and Ulotrichales in the region (Steinhagen & al., 2019a,b) has indicated that the present distribution of *Blidingia* spp. may not accord with recent inventories. However, there has not been a molecular characterization of the northern European *Blidingia* species or a review of their allegedly significant identification criteria undertaken until now.

A crucial point for molecular studies using DNA barcoding is always the choice of suitable marker genes that are on the one hand side variable enough to delimit species but also stable within the respective species group. A marker gene that has been widely used within molecular studies of green algae is the coding gene for the ribulose-bisphosphate carboxylase large subunit *rbcL* (Saunders & Kucera, 2010). Later, the plastid encoded *tufA* gene was promoted as the most suitable marker for delimitation of green algae, providing the highest amplification success and the largest barcode gaps for green macroalgae (Saunders & Kucera, 2010). Genetic databases such as GenBank include numerous sequences for both markers. Reference sequences, however, need to be selected with caution, as type specimens of Ulvales have rarely been successfully sequenced.

By combining *tufA* and *rbcL* sequencing with morphological and ontogenetic observations, we have assessed the diversity of *Blidingia* species within Schleswig-Holstein and have recognized an undescribed new species that is relatively abundant in the area. In addition, our study highlights past difficulties in distinguishing *Blidingia* species.

■ MATERIALS AND METHODS

Field collection and sample preparation. — Sites along the North Sea and Baltic Sea coasts of Schleswig-Holstein – including the island of Helgoland – were repeatedly visited in the years 2014–2017 (see also Steinhagen & al., 2019a). In 2016, sampling also covered the heavily trafficked Kiel Canal, which connects both sea areas (Fig. 1, sites 14–16, see also Steinhagen & al., 2019b). Upper littoral and supralittoral zones were checked for macroalgal growth with a focus on freshwater inflows (e.g., drainages, river inflows, beach showers). Several sites were re-visited in the years 2018 and 2019, to verify the presence of populations and obtain material for cultivation (Table 1). Altogether, *Blidingia* spp. were observed at 19 of the visited sites (Fig. 1; Table 1).

Specimens were collected, placed into sealed plastic bags, and stored on ice until further processing in the lab. Most samples were preserved as herbarium vouchers (see Table 1) and lodged in the Natural History Museum Denmark, Copenhagen (C). A subsample was divided, with part of it stored at 4°C or −20°C for subsequent morphological observation and part of it stored in a microreaction tube at −80°C for genomic DNA extraction. Light microscopy observations were undertaken, and microscopic documentation was carried out on the remaining part, using a digital camera (Nikon DS-Vil) attached to a light microscope (Nikon Eclipse TS 100). Salinity was measured using a WTW portable conductivity meter (Xylem Analytics, Weilheim, Germany).

In addition to the field collected samples, herbarium specimens in the Herbarium of the Helgoland Biological Station of the Alfred Wegener Institute (BRM) were included in our analyses (barcode numbers: BRM007967 and BRM008079; see also Table 1). Several additional herbarium specimens were investigated, including the type specimen of *B. minima* (Naturalis Biodiversity Center, Leiden, Netherlands [L], barcode L 0054691; suppl. Fig. S1). However, they yielded insufficient amounts of DNA due to the vouchers being pre-treated with fixation solutions, or the amplification of marker genes was impossible due to impurities of the herbarium vouchers (diatoms, multi-algal vouchers, etc.). Thus, these herbarium vouchers were excluded from the molecular analyses of this study.

DNA extraction, amplification and sequencing. — Genomic DNA was isolated from the lyophilized algal tissue with an Invisorb Spin Plant Mini Kit (Stratec, Birkenfeld, Germany) following the manufacturer's protocol. Extracted DNA was stored at −80°C and used for amplification of the *rbcL* and *tufA* genes. PCR amplifications of the *rbcL* gene used the primer pairs rbcLstart and R750, as well as F650 and rbcLend (Shimada & al., 2003). The PCR reactions were performed as follows: 94°C for 1 min; 35 cycles at 94°C for 30 s, at 56.3°C for 30 s, and at 72°C for 1 min; and a final extension step at 72°C for 7 min. PCR amplification of the *tufA* gene followed the detailed description of Steinhagen & al. (2019a). PCR products were purified using the QUIAquick PCR Purification Kit (Quiagen, Hilden, Germany). Subsequent sequencing of the purified amplicons was provided by GATC Biotech (Konstanz, Germany). Forward and reverse sequence reads were assembled to produce contigs in Sequencher (v.4.1.4, GeneCodes, Ann Arbor, Michigan, U.S.A.), and a multiple sequence alignment was constructed for each gene region using MAFFT v.7.402 (Katoh & al., 2002) (for Alignments, see supplementary Appendices S1 and S2). Sequences obtained in this study are publicly available in GenBank (for accession numbers, see Table 1).

Phylogenetic analyses. — *RbcL* and *tufA* sequences were analysed in separate datasets. Newly generated sequences

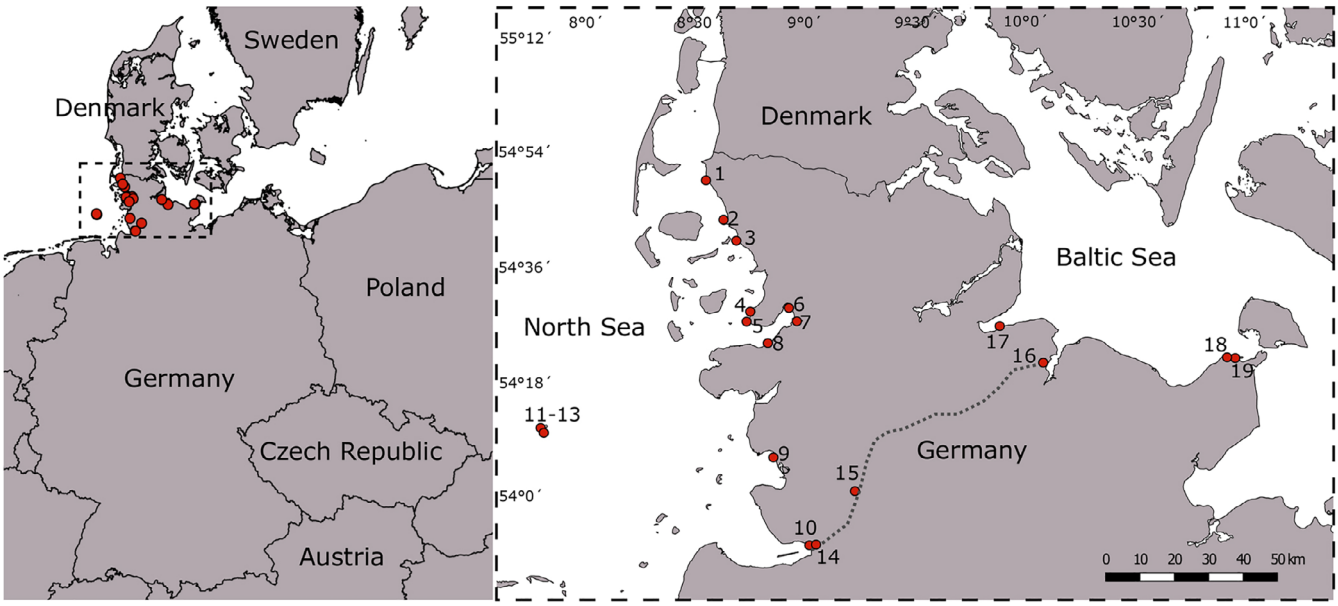


Fig. 1. Sites of the *Blidingia* samples in northern Germany processed in this study. Overview map about northern Germany with numbered sampling sites at the Wadden Sea (no. 1–10), on Helgoland (no. 11–13), within the Kiel Canal (no. 14–16) and in the Baltic Sea (no. 17–19).

Table 1. List of *Blidingia* samples collected and genetically assessed in 2014–2017 in northern Germany. Additionally, the herbarium (BRM) specimens included in this study are listed.

Species	Sample ID	Country	Region	Lat/Long	Station Fig. 1	Date	Collector	Voucher	*tufA* accession no.	*rbcL* accession no.
Blidingia marginata	S_129	Germany: Schleswig-Holstein, Schluettsiel	Wadden Sea	N 54.6813333 E 8.7544167	3	30 Jul 2014	*S. Steinhagen*	C-A-99366	MH538542	–
Blidingia marginata	S_147_A	Germany: Schleswig-Holstein, Pellworm	Wadden Sea	N 54.49882 E 8.8087	4	31 Jul 2014	*S. Steinhagen*	C-A-99378	**MH475464**	**MN258044**
Blidingia marginata	S_147_B	Germany: Schleswig-Holstein, Schobuell	Wadden Sea	N 54.5078167 E 8.9955667	6	31 Jul 2014	*S. Steinhagen*	C-A-99379	MH538543	–
Blidingia marginata	S_156	Germany: Schleswig-Holstein, Woehrden	Wadden Sea	N 54.1173167 E 8.9359333	9	05 Aug 2014	*S. Steinhagen*	C-A-99382	MH538548	–
Blidingia marginata	S_327	Germany: Schleswig-Holstein, Heiligenhafen	Baltic Sea	N 54.3765333 E 10.9800667	19	25 Aug 2014	*S. Steinhagen*	–	MH538549	–
Blidingia marginata	S_474	Germany: Schleswig-Holstein, Schluettsiel	Wadden Sea	N 54.68435 E 8.75385	3	12 Sep 2014	*S. Steinhagen*	C-A-99458	MH538546	–
Blidingia marginata	S_577	Germany: Schleswig-Holstein, Brunsbuettel estuary	Wadden Sea	N 53.889 E 9.101133	10	14 Apr 2015	*S. Steinhagen*	–	**MH475465**	**MN258045**
Blidingia marginata	S_661	Germany: Schleswig-Holstein, Nordstrand	Wadden Sea	N 54.4707167 E 8.8068333	5	21 Apr 2015	*S. Steinhagen*	–	MH538544	–
Blidingia marginata	S_708	Germany: Helgoland	Helgoland	N 54.1780333 E 7.8887167	11	23 Apr 2015	*S. Steinhagen*	–	MH538545	–
Blidingia marginata	S_737	Germany: Helgoland	Helgoland	N 54.1825 E 7.8906167	12	24 Apr 2015	*S. Steinhagen*	C-A-99417	MH538547	–
Blidingia marginata	S_911	Germany: Schleswig-Holstein, Aschau	Baltic Sea	N 54.4608 E 9.92665	17	26 Aug 2017	*S. Steinhagen*	–	**MN258036**	**MN258046**
Blidingia marginata	S_930	Germany: Schleswig-Holstein, Schobuell	Wadden Sea	N 54.50782 E 8.995567	7	27 Aug 2017	*S. Steinhagen*	C-A-99681	**MN258037**	**MN258047**
Blidingia marginata	S_941	Germany: Schleswig-Holstein, Finkhaushallig	Wadden Sea	N 54.41558 E 8.903633	8	29 Aug 2017	*S. Steinhagen*	–	**MN258038**	**MN258048**
Blidingia marginata	S_944	Germany: Schleswig-Holstein, Finkhaushallig	Wadden Sea	N 54.41558 E 8.903633	8	29 Aug 2017	*S. Steinhagen*	–	MN258039	–
Blidingia sp. 1	–	Germany: Schleswig-Holstein, Kiel Canal	Kiel Canal	N 54.031933 E 9.300167	15	May 2016	*S. Steinhagen*	–	MG797655	–
Blidingia sp. 1	–	Germany: Schleswig-Holstein, Kiel Canal	Kiel Canal	N 54.369100 E 10.124917	16	May 2016	*S. Steinhagen*	–	MG797656	–
Blidingia sp. 1	–	Germany: Schleswig-Holstein, Kiel Canal	Kiel Canal	N 53.888778 E 9.116567	14	May 2016	*S. Steinhagen*	–	MG797657	–
Blidingia sp. 1	S_21	Germany: Helgoland	Helgoland	N 54.1825 E 7.890617	12	23 Jul 2014	*S. Steinhagen*	C-A-99667	MH538693	–

(Continues)

Table 1. Continued.

Species	Sample ID	Country	Region	Lat/Long	Station Fig. 1	Date	Collector	Voucher	*tufA* accession no.	*rbcL* accession no.
Blidingia sp. 1	S_93	Germany: Schleswig-Holstein, Aschau	Wadden Sea	N 54.4608 E 9.92665	17	24 Jul 2014	*S. Steinhagen*	–	MH538691	–
Blidingia sp. 1	S_179	Germany: Schleswig-Holstein, Brunsbuettel estuary	Wadden Sea	N 53.889 E 9.101133	10	06 Aug 2014	*S. Steinhagen*	C-A-99682	**MH475459**	**MN258049**
Blidingia sp. 1	S_578	Germany: Schleswig-Holstein, Brunsbuettel estuary	Wadden Sea	N 53.888778 E 9.116567	14	14 May 2015	*S. Steinhagen*	–	MN258043	–
Blidingia sp. 1	S_622	Germany: Schleswig-Holstein, Heiligenhafen	Baltic Sea	N 54.3787167 E 10.95545	18	16 Apr 2015	*S. Steinhagen*	–	MH538692	–
Blidingia sp. 1	S_813	Germany: Schleswig-Holstein, Friedrich-Wilhelm-Luebke-Koog	Wadden Sea	N 54.83735 E 8.6122	1	24 Jul 2017	*S. Steinhagen*	C-A-99677	**MH475458**	**MN258050**
Blidingia sp. 1	S_815	Germany: Schleswig-Holstein, Finkhaushallig	Wadden Sea	N 54.41558 E 8.903633	8	24 Jul 2017	*S. Steinhagen*	C-A-99678	**MH475457**	**MN258051**
Blidingia sp. 1	S_818	Germany: Schleswig-Holstein, Husum	Wadden Sea	N 54.47113 E 9.027917	7	24 Jul 2017	*S. Steinhagen*	C-A-99679	MH475456	–
Blidingia sp. 1	S_828	Germany: Schleswig-Holstein, Schobuell	Wadden Sea	N 54.50782 E 8.995567	6	24 Jul 2017	*S. Steinhagen*	C-A-99680	**MH475455**	**MN258052**
Blidingia sp. 2	Hel_59	Germany: Helgoland	Helgoland	–	ca. 11–13	25 Jul 1977	*P.-H. Sahling*	BRM008079	**MT076212**	**–**
Blidingia sp. 2	S_1	Germany: Helgoland	Helgoland	N 54.18367 E 7.888633	13	22 Jul 2014	*S. Steinhagen*	C-A-99660	MH475461	–
Blidingia sp. 2	S_34	Germany: Helgoland	Helgoland	N 54.18367 E 7.888633	13	23 Jul 2014	*S. Steinhagen*	C-A-99668	**MH475460**	**MN258053**
Blidingia sp. 2	S_39	Germany: Helgoland	Helgoland	N 54.1825 E 7.890617	12	23 Jul 2014	*S. Steinhagen*	C-A-99671	**MH475462**	**MN258054**
Blidingia sp. 2	S_124	Germany: Schleswig-Holstein, Dagebuell	Wadden Sea	N 54.73007 E 8.689167	2	30 Jul 2014	*S. Steinhagen*	C-A-99663	**MH475463**	**MN258055**
Blidingia sp. 2	S_949	Germany: Schleswig-Holstein, Nordstrand	Wadden Sea	N 54.4707167 E 8.8068333	5	29 Aug 2017	*S. Steinhagen*	–	MN258040	–
Blidingia sp. 2	S_951	Germany: Schleswig-Holstein, Nordstrand	Wadden Sea	N 54.4707167 E 8.8068333	5	29 Aug 2017	*S. Steinhagen*	–	**MN258041**	**MN258056**
Blidingia sp. 2	S_950	Germany: Schleswig-Holstein, Nordstrand	Wadden Sea	N 54.4707167 E 8.8068333	5	29 Aug 2017	*S. Steinhagen*	–	MN258042	–
Ulva intestinalis	Hel_27	Germany: Helgoland	Helgoland	–	ca. 11–13	29 Jul 1997	*I. Bartsch*	BRM007967	**MT076211**	**–**

Specimens used in phylogenetic analysis are indicated by accession numbers in bold; however, all individuals were included in the calculation of intra- and interspecific genetic divergence values.

were aligned with reference sequences downloaded from GenBank and used for further phylogenetic analysis. Particularly, sequences of specimens of the genera *Ulva* L., *Kornmannia* (Kjellm.) Bliding and *Monostroma* Thuret were included to assess relationships with closely related taxa, whereas sequences of *Protomonostroma undulatum* (Wittr.) K.L.Vinogr. (*rbcL*: HQ603387; *tufA*: HQ619275, MH475501) were chosen as outgroups. Preference for reference sequence selection was given to peer-reviewed sequences. The models that best fit our data were found under the Akaike information criterion by employing MrModeltest v.2.2. (Nylander, 2004). For both datasets, the optimal substitution model was determined and found to be GTR+Γ+I. Maximum likelihood (ML) analyses were then carried out using RAxML v.8 (Stamatakis, 2014), employing the chosen substitution model with 1000 bootstrap replicates for each alignment.

Cultivation. — Complete thalli of selected mature *Blidingia* specimens were washed thoroughly and repeatedly with sterile seawater (salinity of the respective collection site) to remove dirt and adhering impurities and were isolated into cultures. Clean thalli were transferred into polystyrene 24-well plates and were incubated in sterile artificial seawater adjusted to the salinity of the respective sites at 15°C under a photon flux density of 40–70 µmol m^{-2} s^{-1} and a 17 : 7 h light : dark photo regime. To prevent the growth of diatoms, 1 mg l^{-1} GeO_2 was added. The thalli were examined daily for sporulation events. After sporulation had taken place, adult thalli were removed from the wells, and spore development was observed with an inverted microscope (Nikon Eclipse TS 100) and photographed (Nikon DS-Vil).

■ RESULTS

Phylogeny. — The phylogenetic analyses performed on datasets of the *rbcL* and *tufA* markers resulted in comparable and nearly identical results, and almost equivalent evolutionary relationships of both investigated marker genes were discovered (Fig. 2). The *rbcL* alignment consisted of a total of 695 positions (suppl. Appendix S1), whereas the *tufA* gene dataset was 771 basepairs long (suppl. Appendix S2).

The node separating the genus *Blidingia* from closely related and outgroup taxa received full bootstrap support for both marker genes and unequivocably confirmed the taxonomic position of *Blidingia* as a separate genus. Whereas the *tufA* dataset reveals *Ulva* as sister clade to *Blidingia* (bootstrap support: 99), the *rbcL* phylogram displays a reference sequence of *Kornmannia leptoderma* (AF499677) as the closest relative of *Blidingia* before *Ulva*. Both analyses resolve the German *Blidingia* samples from the examined area in three clades (*Blidingia marginata*, *Blidingia* sp. 1, *Blidingia* sp. 2) with high (>85) to full bootstrap support (Fig. 2). The clades delimiting *B. marginata* showed low intraspecific genetic variability (*tufA*: 0%–0.3%; *rbcL*: 0%–0.2%) (see also Table 2) and could be resolved with reference sequences (*rbcL*: HQ603379, Canada; *tufA*: HQ610237, Canada). A reference sequence that seemed to be incorrectly assigned to *B. minima* (MG721599) clustered within the clade representing *B. marginata*.

The clusters representing *Blidingia* sp. 1 and *Blidingia* sp. 2 could not be matched to any GenBank sequence entries. However, both clades are clearly separated from other *Blidingia* species and receive full bootstrap support (Fig. 2). *Blidingia* sp. 1 finds its next relative in a well-delimited cluster of unidentified *Blidingia* specimens from North America (HQ610241, MF124265) (genetic dissimilarity of *Blidingia* sp. 1 and *Blidingia* sp.: *tufA* 4.8%; *rbcL* n.a.), whereas the next closest relative of *Blidingia* sp. 2 is *B. marginata* (genetic dissimilarity of *B. marginata* and *Blidingia* sp. 2: *tufA* 8.6%–9.4%; *rbcL* 3.2%–3.7%) (see also Table 2).

The phylograms of both the *tufA* (KT290281, HQ610239) and *rbcL* genes (AF499676, MF90430, MF90429) include clades of downloaded reference sequences from GenBank that were supposed to represent *Blidingia minima* (Fig. 2). However, the topology of these clades and thus their placement within the trees is not congruent. One of the specimens assigned to the clade representing *B. minima* within the *tufA* phylogram originates from a site 30 km to the east in the neighbouring German State of Mecklenburg-Vorpommern, Wohlenberg, which was observed by us in a previous study (Steinhagen & al., 2019a).

Notably, GenBank entries of the *rbcL* and *tufA* genes of *Blidingia minima* showed significant differences in their nucleotide reads (indicating different species are combined in GenBank under this species name). Since most of the uploaded sequences contain several ambiguous bases and are rather short (250–500 bp), we decided to exclude them from the displayed results. However, within this study, we were not able to validate a genotype representing any of the genotypes associated with *B. minima* in GenBank, not even at the type locality of *B. minima* on Helgoland. Intra- and interspecific divergence values of the here investigated *Blidingia* entities are summarized and listed in Table 2.

Both herbarium specimens from Helgoland (Table 1) showed no agreement between their previous identification based on morphological traits and their molecular identification by DNA barcoding. Within our phylogenetic analysis of the *tufA* gene, voucher BRM008079 (morphologically identified as *Blidingia minima*, GenBank accession no.: MT076212) clustered within the clade representing *Blidingia* sp. 2, and voucher BRM007967 (morphological identity *B. marginata*, GenBank accession no.: MT076211) was assigned to the clade of *Ulva intestinalis* (Fig. 2).

Morphological characterization, habitat and distribution. — In the following section the *Blidingia* species present in the study area of northern Germany are described in more detail. Macro- and micromorphological observations including respective ontogenetic features, as well as ecological and distribution information, are presented for each species:

Blidingia marginata (J.Agardh) P.J.L.Dang. ex Bliding in Opera Bot. 8(3) [Crit. Surv. Eur. Taxa Ulvales 1]: 32. 1963 ≡ *Enteromorpha marginata* J.Agardh., Alg. Mar.

Medit.: 16. 1842 ≡ *Enteromorpha nana* var. *marginata* (J.Agardh) V.J.Chapm. in J. Linn. Soc. London, Bot. 55: 416. 1956 – Lectotype: FRANCE. Nice, Alpes-Maritimes, France, 1842 (LD Herb. Agardh No. 14161).

Habitat, *seasonality and distribution*. – *Blidingia marginata* is the most common *Blidingia* species within the study area and has the widest distribution. It is abundant on Baltic Sea and Wadden Sea coasts and at Helgoland (Table 1). This species can be found in remote as well as anthropogenically strongly impacted habitats (see also Steinhagen & al., 2019a,b) and inhabits fully marine and brackish water ecosystems. However, *B. marginata* was seldomly observed in water bodies

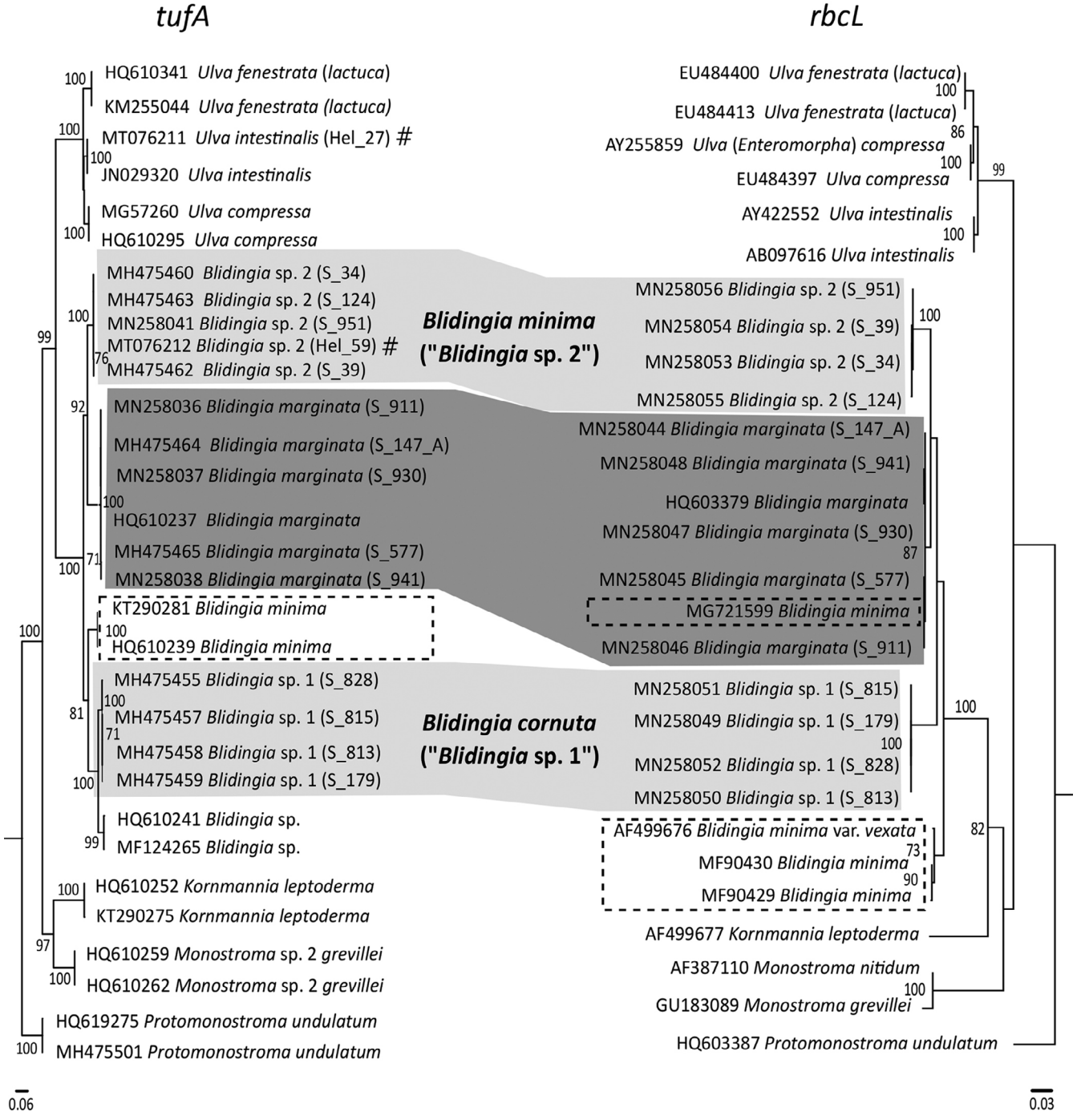


Fig. 2. Comparative maximum likelihood phylograms of *tufA* and *rbcL* sequences from taxa of *Blidingia* from northern Germany. The grey-shaded boxes indicate clades that were present in the study area and connects the respective clades of species in both phylogenetic analyses for direct comparison. Dashed boxes indicate the ambiguous use of the historic construct of "*Blidingia minima*" and highlight reference sequences identified as such. Numbers at nodes indicate bootstrap values. Poorly supported nodes (<70% bootstrap support) are not labelled. Branch lengths are proportional to sequence divergence. # symbol marks herbarium samples (see also Table 1).

below 5 PSU, and it was not detected in the Kiel Canal. As is typical for the genus, *B. marginata* was observed growing as dense turf mats in the upper and middle intertidal zone (Fig. 3A–F). Adults of *B. marginata* can be found throughout the year, but their peak abundance was observed during early and mid-summer (May–July). In late summer and fall (August–October), dense stands began to bleach and populations shrank in size, so that in winter and spring only a few smaller individuals were encountered. Individuals were always found attached to the substratum and only observed detached after extreme weather events. Especially in the North Sea areas, *B. marginata* was the most abundant alga in the intertidal zone. It occupied a variety of natural and artificial hard substrata (stones, cobble, breakwaters, wooden piles, etc.) and was often found growing epiphytically on other macrophytes (e.g., *Fucus* spp.) and higher plants (*Phragmites* sp.) in the intertidal (Fig. 3B,C). The same distribution patterns were encountered in the Baltic Sea. Here, however, *B. marginata* was found in mixed stands with *Ulva intestinalis*, and in some cases, young individuals of *U. intestinalis* were difficult to distinguish from *B. marginata* by morphological characters alone. Individuals of *B. marginata* could resist strong UV-radiation and desiccation in summer as well as snow cover and long frost periods in winter (Fig. 3F).

Morphology. – Individuals of *Blidingia marginata* exhibited all the morphological features described for this species (Bliding, 1963), but also some differences. The long (1–12 cm) and narrow thallus was tubular and usually formed dense mats (Fig. 3D–F). Broader thalli were often wrinkled and twisted and resembled *Ulva intestinalis* (Fig. 3G–I). Contrary to the observations of Bliding (1963), who stated that specimens of *B. marginata* exhibited rare small proliferations, 80% of the individuals of the investigated material had microscopically visible branches or branchlet-like appendages (Fig. 3H). These branchlets were often uni- or biseriate with a single apical cell. Macroscopic branching in the middle or apical thallus parts was rarely observed, whereas some specimens had macroscopic branches in the rhizoidal zone. In young, narrow thalli, the cells were arranged in distinct rows (Fig. 3J,K); however, mature or broader thalli exhibited only short cell rows or no cell organization. Cells were quadratic to rectangular, sometimes of amorphous shape, 4–10 µm long and 2–9 µm wide in surface view. The chloroplast filled the whole cell with one central pyrenoid (Fig. 3J,K).

Ontogeny. – As also described by Kornmann & Sahling (1978), the quadriflagellate spore settled on the substratum and immediately developed a germination tube without cutting off an empty cell (Fig. 4A,B). The first mitotic cell divisions resulted in the formation of a prostrate disc (Fig. 4C,D), which then in most of the cases became 2-layered (Fig. 4E). The tubus typically started to develop from lateral initial cells and was rarely observed to develop form the disc's centre.

Blidingia sp. 1

Habitat and distribution. – Specimens of this species were also found on Baltic Sea and Wadden Sea coasts and on Helgoland (Table 1). However, dense, turf-like populations of *Blidingia* sp. 1 were not as frequent and abundant as those of *B. marginata*, and they were more clearly restricted to the upper supralittoral zone. Notably, most of the observed populations of *Blidingia* sp. 1 grew in the direct vicinity of freshwater inflows (drain pipes, beach showers, stream run-off, etc.; Fig. 5A). As a consequence – and in contrast with *B. marginata* and *Blidingia* sp. 2 – specimens of *Blidingia* sp. 1 were rarely found desiccated during the summer months, despite their location in the upper supralittoral. When freshwater inflows were more located towards the medio- or infralittoral, *Ulva intestinalis* was the prevailing species, and *Blidingia* sp. 1 was absent. The species was also absent from more inland freshwater inflows that had no direct connection to the sea. *Blidingia* sp. 1 was present throughout all seasons; however, it was more common during July to August.

Table 2. Overview on intra- and interspecific divergence values of the *Blidingia* entities investigated within this study.

	Interaction	*tufA* [% difference]	*rbcL* [% difference]
Intraspecific	*Blidingia marginata*	0–0.3	0–0.2
	Blidingia sp. 1	0–0.6	0
	Blidingia sp. 2	0–0.2	0–0.2
Interspecific	*Blidingia marginata* : *Blidingia* sp. 2	8.6–9.4	3.2–3.7
	Blidingia marginata : *Blidingia* sp. 1	15.6–17.9	3.2–5.6
	Blidingia sp. 1 : *Blidingia* sp. 2	11.8–13.1	6.4–6.8
	Blidingia marginata : "*Blidingia minima*"	13.7–15.4	4.1–4.4
	Blidingia sp. 1 : "*Blidingia minima*"	10.7–12.1	5.8–6.0
	Blidingia sp. 2 : "*Blidingia minima*"	10–11.1	4.7–5.7
	Blidingia sp. 1 : *Blidingia* sp. (MF124265, HQ610241)	4.8	n.a.

Values for interspecific comparison of "*Blidingia minima*" were calculated on the respective historically mis-identified clades represented in Fig. 2 (dashed box *tufA*; *rbcL* lower dashed box). Therefore, they are framed by double quotation marks within this table.

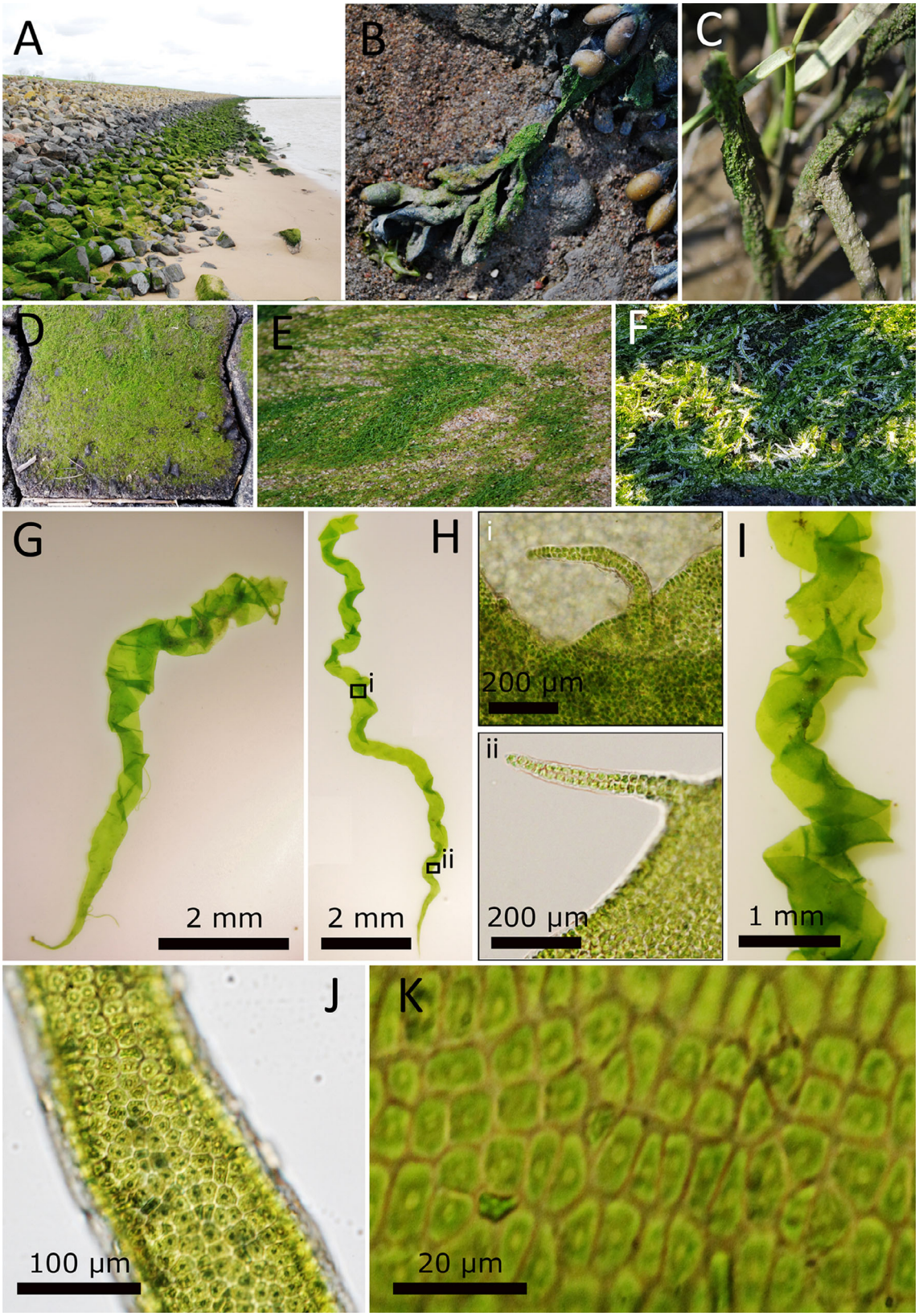


Fig. 3. Morphology of *Blidingia marginata* specimens from Germany. Population of *B. marginata* growing on a breakwater (**A**), epiphytic on *Fucus vesiculosus* (**B**) and *Phragmites* (**C**), on cobbles (**D**) and on sand (**E**). As a species of the upper intertidal zone, it can manage frost periods (**F**). Individuals mostly exhibit no macromorphological branches (**G** & **H**) but often have microscopic branchlets (**H i** & **ii**) and can be of cork-screw-like morphology (**I**). Cells usually exhibit one central pyrenoid and are arranged in longitudinal rows (**J** & **K**). — Photos by S. Steinhagen.

Morphology. – The morphology of *Blidingia* sp. 1 shows similarities with the description of Bliding's (1963) "*Blidingia minima* var. *ramifera*" (not validly published, type not indicated), which was later "raised to species rank" by Garbary & Barkhouse (1987; as "*Blidingia ramifera*"). "*Blidingia ramifera*" is currently regarded as a synonym of *B. marginata* (Guiry & Guiry, 2019). However, there are distinct differences:

The small, tubular and branched thalli were compact and reached 1–10 mm in length (rarely taller) (Fig. 5B,E) whereas thalli of "*Blidingia ramifera*" were distinctly larger (Bliding, 1963; Garbary & Barkhouse, 1987). The width of the thallus increased as it proceeded from the rhizoidal zone to the tip (0.08–0.8 mm wide). Branches were antler shaped, uni- to multiseriate and present across the whole thallus (Fig. 5B,C). Unbranched individuals were rarely encountered. In addition to the clearly formed branches, spine-like microscopic appendages (10–60 µm) were frequently found across the whole thallus (Fig. 5E,G). Branches and microscopic appendices were blunt-ended. Thalli grew in tufts that formed dense stands, and several individuals were connected by their rhizoidal zones. Cells formed clear, longitudinal rows (Fig. 5F) that sometimes blurred in broader thallus areas of the apical region (Fig. 5G), whereas no distinct cell arrangement was observed in mature individuals of "*B. ramifera*" (Bliding, 1963; Garbary & Barkhouse, 1987). Individuals with unordered cell arrangements were observed infrequently. The cells were quadratic, rectangular, often polygonal with blunt to rounded corners and 3–8 µm long and 2–8 µm broad. The chloroplast filled the cell or was rarely parietal, with 1 (rarely 2) central pyrenoid(s) (Fig. 5F,H).

Ontogeny. – After the quadriflagellate spore had settled, a germination-tube began to form (Fig. 4F). The cell content migrated through the germination-tube and formed a germinating cell, detaching the initial spore sleeve and in most cases also parts of the germination tube (Fig. 4G,H). By mitotic cell divisions, a monostromatic, relatively open disc developed, and its cells were not as dense as in other entities (Fig. 4I,J). After becoming distromatic, the disc bulged out, and an erect tube formed.

Molecular analysis. – The sequence divergence of the clade representing *Blidingia* sp. 1 from other species within the genus, in combination with morphological differences, indicates that *Blidingia* sp. 1 is genetically and

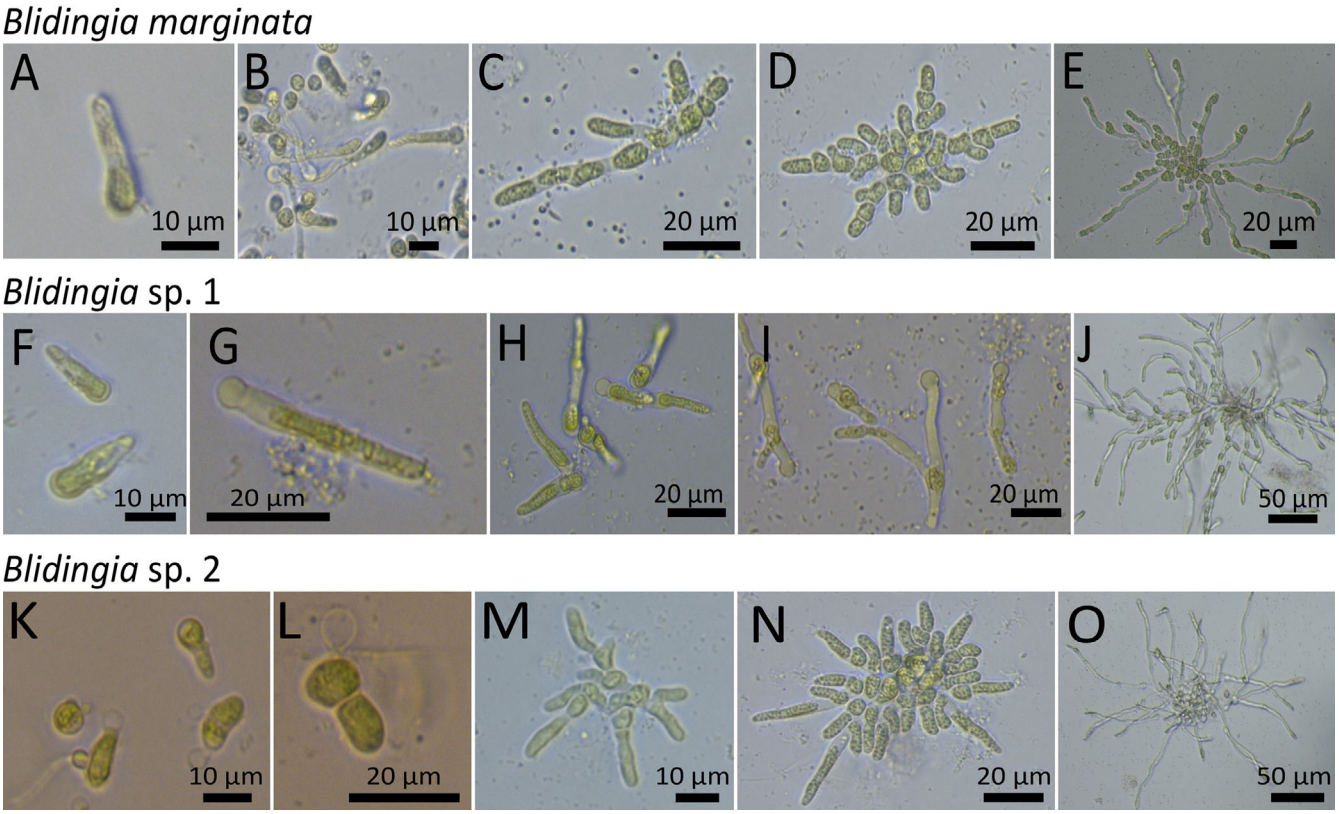


Fig. 4. Ontogenetic development of German *Blidingia* spp. After the spore of *B. marginata* had settled, a germination-tube began to form (**A**). No cutting-off of an empty cell was observed (**B**). A prostrate disc began to form (**C–E**) with densly packed cells in its centre (**E**). The early development of *Blidingia* sp. 1 started with the formation of a germination-tube after the spore had settled (**F**). The spores cell content migrated through the germination tube and formed a germinating cell while detaching the empty initial spore sleeve (**G** & **H**). Open discs were formed (**I** & **J**). The settled spore of *Blidingia* sp. 2 formed a small germination tube (**K**) that then divided into an empty initial cell and a cell containing the cell content (**L**). A monostromatic disc with dense cell arrangement formed (**M**) that in its centre became distromatic (**N** & **O**). — Photos by L. Düsedau.

morphologically distinct from other previously described species within the genus *Blidingia*. We here describe this new species as *Blidingia cornuta*:

Blidingia cornuta S.Steinhagen & F.Weinberger, **sp. nov.** – Holotype: GERMANY. Brunsbüttel harbour, Schleswig-Holstein, N 53.889° E 9.101133°, 6 Aug 2014, *S. Steinhagen S_179* (C barcode C-A-99682).

Figures 4F–J & 5A–H.

Species description. – Thalli tubular, light to dark green, compressed, bearing antler-like uni- and multiseriate branches across the whole thallus (rarely unbranched), with rounded

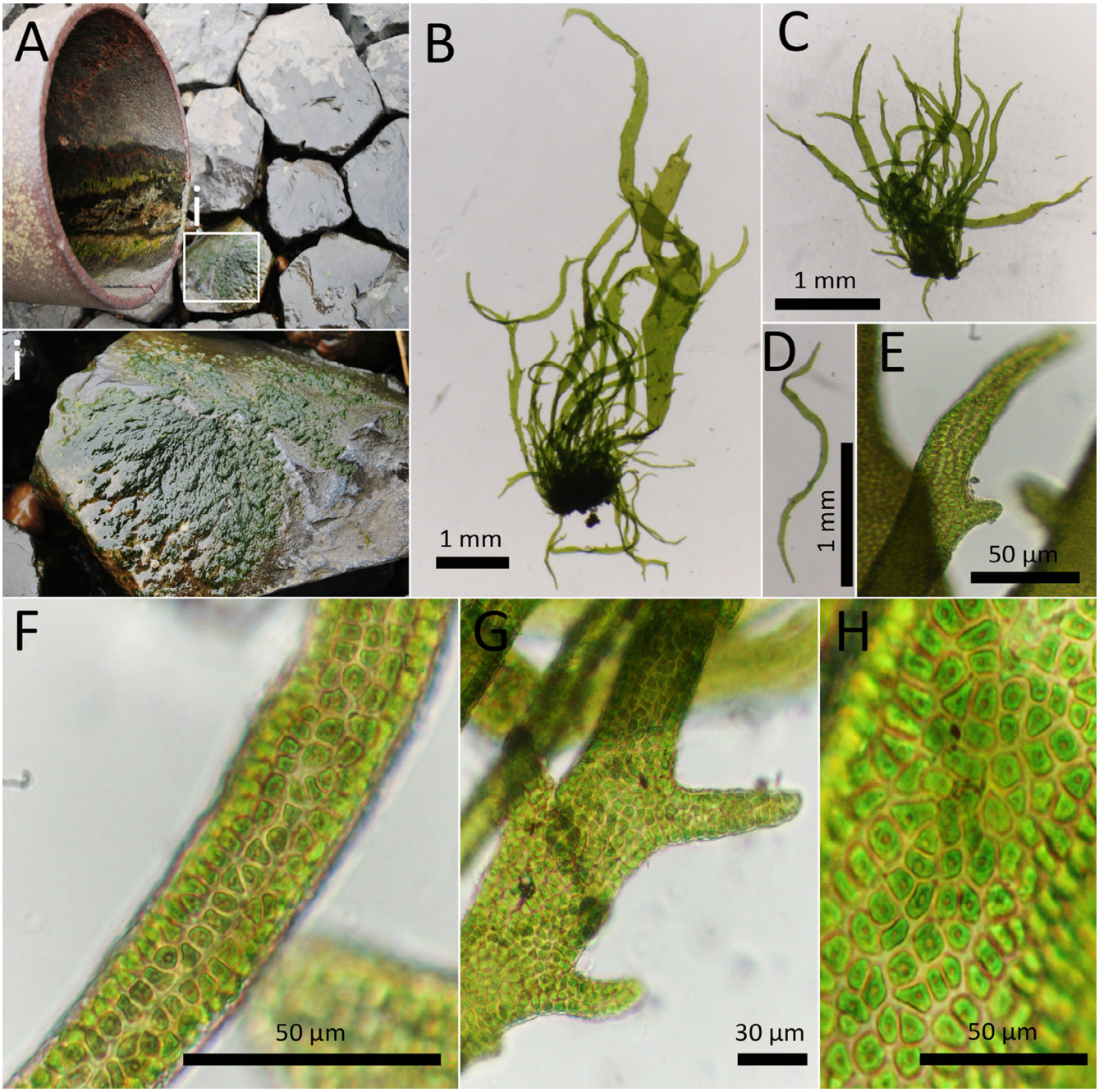


Fig. 5. Morphology of the holotype of *Blidingia cornuta* sp. nov. ("*Blidingia* sp. 1"). (**A**) Type locality of *B. cornuta* at the Elbe estuary in Brunsbüttel, Germany. The thalli are growing on a stone (**i**) directly underneath a water drainage pipe permanently releasing freshwater. The individuals exhibited small, tubular and antler-like branched thalli (**B** & **C**), and also microscopic, blunt-ended, spine-like appendages were frequently observed across the whole blade (**D** & **E**). The cells formed clear, longitudinal rows (**F**) that sometimes blurred in broader thallus areas of the apical region (**G**). The cells were quadratic, rectangular, often polygonal with blunt to rounded corners, and the chloroplast filled the cell (rarely parietal) and contained 1 (rarely 2) central pyrenoid(s) (**F** & **H**). — Photos by S. Steinhagen.

intact branch apices, attached by rhizoids to substratum, 1–10 mm (mean ± 6 mm; rarely >1 cm) long, 0.08–0.8 mm (mean ± 0.3 mm; rarely >1 mm) broad. Main axis increasing in breadth from 10–35 µm (rhizoidal zone) to 0.3 mm (middle and apical thallus). Middle thallus often with spine-like appendices (20–35 µm in length), these appendices shorter than real branches. Cells in surface view in clear longitudinal rows in basal and middle thallus parts, short longitudinal rows in apical regions, square, rectangular or polygonal with blunt to round corners, most commonly 3–8 µm long and 2–8 µm broad. The chloroplast is cell-filling (rarely parietal covering most of the cell wall) and cells containing 1 (rarely 2) central pyrenoid(s). Reproduction by quadriflagellate spores. From the spore a germination-tube arises, and the cell contents migrate to a germinating cell, detaching the initial spore sleeve and germination-tube. By mitotic cell divisions a monostromatic disc develops, and after becoming distromatic, the disc bulges out and an erect tube is formed.

Etymology. – The species epithet *cornuta* ("horned" in Latin) refers to the antler-like morphology of branches.

GenBank accessions. – MH475459 represents the sequence of the *tufA* marker gene, and MN258049 is the respective *rbcL* sequence.

Type locality. – Brunsbüttel harbour, Schleswig-Holstein, Germany (N 53.889° E 9.101133°). Thalli were growing as dense turf in the high intertidal zone directly under a drainage with constant freshwater seepage from land on a bulkhead (Fig. 5A). The site is part of the Elbe estuary, close to the outlet of the Kiel Canal.

Other selected specimens examined (paratypes). – Aschau, Schleswig-Holstein, Germany (N 54.4608°; E 9.92665°), 24 Jul 2014, *S. Steinhagen S_93*, GenBank MH 538691 (*tufA*), Baltic Sea; Heiligenhafen, Schleswig-Holstein, Germany (N 54.3787167°; E 10.95545°), 16 Apr 2015, *S. Steinhagen S_622*, GenBank MH538692 (*tufA*), Baltic Sea; Helgoland, Germany (N 54.1825°; E 7890617°), 23 Jul 2014, *S. Steinhagen S_21*, GenBank MH538693 (*tufA*), North Sea; Schobuell, Schleswig-Holstein, Germany (N 54.50782°; E 8.995567°), 24 Jul 2017, *S. Steinhagen S_828*, GenBank MH475455 (*tufA*) and MN258052 (*rbcL*), Wadden Sea; Husum, Schleswig-Holstein, Germany (N 54.47113°; E 9.027917°), 24 Jul 2017, *S. Steinhagen S_818*, GenBank MH475456 (*tufA*), Wadden Sea; Finkhaushallig, Schleswig-Holstein, Germany (N 54.41558°; E 8.903633°), 24 Jul 2017, *S. Steinhagen S_815*, GenBank MH475457 (*tufA*) and MN258051 (*rbcL*), Wadden Sea; Friedrich-Wilhelm-Luebke-Koog, Schleswig-Holstein, Germany (N 54.83735°; E 8.6122°), 24 Jul 2017, *S. Steinhagen S_813*, GenBank MH475458 (*tufA*) and MN258050 (*rbcL*), Wadden Sea; Brunsbuettel estuary, Schleswig-Holstein, Germany (N 53.893567°; E 9.141733°), 14 May 2015, *S. Steinhagen S_578*, GenBank MN258043 (*tufA*), Wadden Sea/river Elbe.

Blidingia sp. 2

Habitat and distribution. – This entity was only observed on Helgoland and in the northeastern Wadden Sea (Table 1) and was not present in the Baltic Sea. It inhabited the upper supralittoral zone and was found growing as turfs, but more often it was observed as small patches on stones, concrete, wooden piles or other hard substrates (Fig. 6A,B). When growing epiphytic on macrophytobenthic species (e.g., *Fucus* spp.), *Blidingia* sp. 2 did not cover the host like *B. marginata*. Instead, single individuals were found to be scattered across the host plants.

Morphology. – The morphology of *Blidingia* sp. 2 shows strong similarities with the description of Kützing's (1849) *Enteromorpha minima*, which was later raised to the type of *Blidingia* (as *B. minima*) by Kylin (1949). Striking morphological congruities among *Blidingia* sp. 2 and *B. chadefaudii* from Helgoland (Kornmann & Sahling, 1978) are also obvious.

The thalli of *Blidingia* sp. 2 were mostly only few millimetres long (rarely taller than 1 cm) and 50–300 µm wide (single individuals had broader thalli up to 700 µm) (Fig. 6C–E). No branches in the middle or apical thallus parts were observed, however the base sometimes exhibited branches (Fig. 6D,E). Thalli were most often compressed, but inflated individuals were also present. Whereas cells form clear and distinct longitudinal rows in the basal thallus parts (Fig. 6F), the arrangement of cells is less organised in the middle and apical thallus parts (Fig. 6G). Cells were of various shapes, quadratic to polygonal with rounded corners, 4–8 µm long and 4–6 µm wide in surface view. No thickened cell walls, nor any lamellar internal structures were observed. The chloroplast was parietal or filled the cell, with one central pyrenoid (Fig. 6H).

Ontogeny. – The main ontogenetic patterns discovered in this study agree with the developmental descriptions of *Blidingia chadefaudii* (rather than *B. minima*) made by Kornmann & Sahling (1978). From the attached quadriflagellate spore, a small germination tube was formed (Fig. 4K). The tube divided into an empty initial cell and an upper cell containing the cell contents (Fig. 4L). A monostromatic disc with a dense cell arrangement was formed (Fig. 4M,N), which became distromatic in its centre (Fig. 4N,O) and gave rise to an erect tube.

Molecular analysis. – Our molecular analysis demonstrates that *Blidingia* sp. 2 is distinct from other *Blidingia* species represented in GenBank. However, as described above, morphological (Kützing, 1849; Kylin, 1949; Kornmann & Sahling, 1978) as well as ontogenetic (Kornmann & Sahling, 1978) patterns are in accordance with previously described entities.

■ DISCUSSION

We here provide a strongly revised picture of the diversity, distribution and morphological variability of *Blidingia* spp. in Schleswig-Holstein, Germany, a study area that includes coastal sections of two important ecosystems in northern Europe, the North and Baltic Seas (Fig. 1). In total, 30 populations of *Blidingia* spp. at 19 sites were analysed to cover the full diversity in the study area. However, whereas older studies and recent species inventories mention four *Blidingia* species as abundantly present along the coastlines of Schleswig-

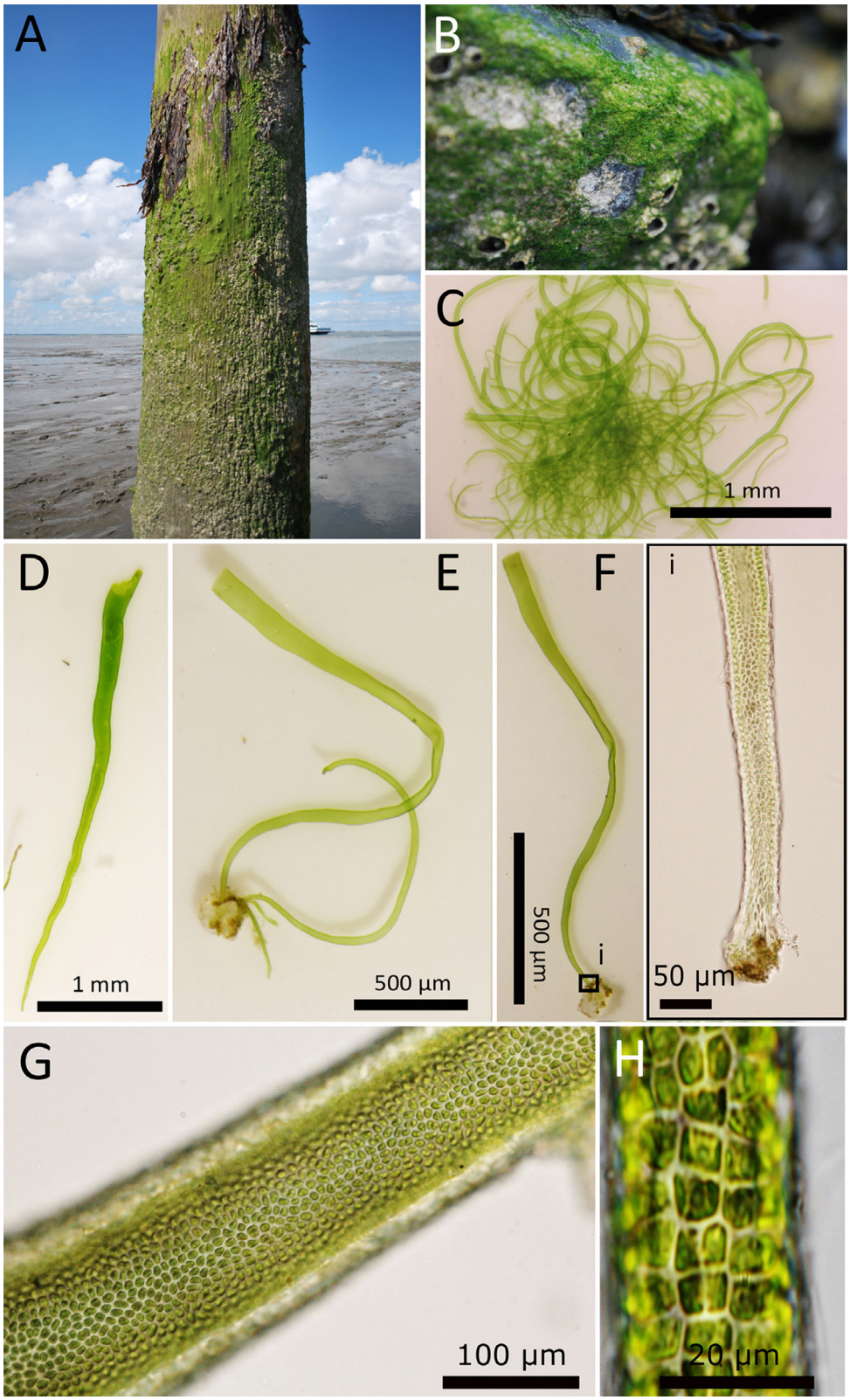


Fig. 6. Morphology of *Blidingia* sp. 2. Typical habitat of *Blidingia* sp. 2 at the German peninsula Nordstrand (**A** & **B**). Individuals were found growing in patches and turfs on wooden piles (**A**) and stones of breakwaters (**B**). The thalli were unbranched in the middle and apical thallus region (**C** & **D**); however, branching in the basal parts was observed infrequently (**E**). Cells of the basal part proceeded in clear and distinct longitudinal rows (**F**), but the arrangement of cells is disturbed in the middle and apical thallus parts, and no clear structure was observed (**G**). Cells were quadratic to polygonal with rounded corners, and the chloroplast was found to be parietal or cell filling and one central pyrenoid was observed (**H**). — Photos by S. Steinhagen.

Holstein (Kornmann & Sahling, 1978; Schories & al., 2009), we only discovered three. Our findings suggest that this discrepancy results from the exclusive use of morphological traits and ontogenetic developmental characters as identification criteria in the past. These criteria were probably invalid in part, due to morphological variability within species. That morphological identification criteria for *Blidingia* species are not in concomitance with more clear-cut molecular identification criteria should be discussed in detail.

Of the four *Blidingia* species listed for Schleswig-Holstein, only *B. marginata* was frequently observed. *Blidingia marginata* was found to be the dominant species within the intertidal zone, and it was frequently observed at Baltic Sea and Wadden Sea coasts and on Helgoland. The species is represented by a well-delimited clade with low intraspecific variation (0%–0.3% *tufA* and 0%–0.2% *rbcL*) within both provided phylogenetic trees (Fig. 2) and was well resolved to the species level by including several peer-reviewed reference sequences (GenBank accessions HQ610237 *tufA* and HQ603379 *rbcL*). Its closest relative was *Blidingia* sp. 2, and both entities had a sequence dissimilarity of 8.6%–9.4% within the *tufA* gene and 3.2%–3.7% in their *rbcL* sequences (Table 2). However, the combination of morphological and molecular techniques revealed that *B. marginata* exhibited a larger morphological variability than expected (Fig. 3).

Individuals of *Blidingia marginata* exhibited all the morphological features described for this species (Bliding, 1963), and our ontogenetic observations (Fig. 4) are in accordance with previous findings (Bliding, 1963; Kornmann & Sahling, 1978). The tubular thalli where either thin and elongated or had a corkscrew-like morphology, and their cells were arranged in clear longitudinal rows. It should be noted that mature thalli are morphologically similar to small *Ulva intestinalis* and could easily be confused with that species. Correspondingly, a herbarium sample from Helgoland that was in concordance with the morphological characters described for *B. marginata* (Biological Station Helgoland Herbarium BRM007967) was identified as *U. intestinalis* by DNA barcoding (Fig. 2).

Contrary to the observations of Bliding (1963), who stated that specimens exhibited rare small proliferations, more than 80% of the individuals of the examined material had microscopic branches or branchlet-like appendages (Fig. 3H). Bliding (1963) assigned specimens with branchlet-like structures to *Blidingia marginata* subsp. *subsalsa* (Kjellm.) Bliding. Later, Scagel & al. (1989) raised *B. marginata* subsp. *subsalsa* to species level, as already suggested by Kornmann & Sahling (1978). Our observations indicate that *B. marginata* can exhibit various morphologies, including those assigned to *B. subsalsa*; however, such morphological differences were not reflected by molecular differences of the marker genes *tufA* or *rbcL*, and no delimitations of the different morphotypes within our phylogenetic analyses were observed (Fig. 2).

Kornmann & Sahling (1978) observed that cultivated swarmers, released by material identified as *Blidingia subsalsa*, did not grow into the "naturally looking wildtype forms" of *B. subsalsa* and that their first-generation offspring were macro-morphologically rather identical with individuals of *B. marginata*. Based on our results we conclude that the morphological spectrum of *B. marginata* is broader than previously expected, as it includes the morphologies assigned to *B. subsalsa*.

Based on literature (Kornmann & Sahling, 1978; Pankow, 1990; Schories & al., 2009), *Blidingia minima* was expected to have the widest distribution in northern Germany. However, our study revealed that this species is restricted to Helgoland and some other North Sea locations. Past records, in particular those from Baltic Sea locations, may represent misidentifications due to flawed historic species concepts.

This view is strongly supported by our analysis of available barcoding sequences. Screening of databases such as GenBank for sequences of *Blidingia minima* provides several hits for *tufA* and *rbcL* sequences of various length. However, within our phylogenetic analyses (Fig. 2), different sequences from around the globe and identified as *B. minima* fell in several well delimited clusters (Fig. 2, dashed boxes) that are not necessarily closely related or even cluster with *B. marginata*. This unequivocally confirms that the recent species concept of *B. minima* is ambiguous, combines genetically distinct entities, and needs clarification.

Even though sampling sites were chosen in a way that distances between the sites did not exceed 25 km (see also Steinhagen & al., 2019a,b), no genotypes in accordance with any GenBank entries for *Blidingia minima* were observed in the investigated area. One of the reference sequences allegedly representing *B. minima* (KT290281) originates from Wohlenberg, 30 km to the east in the neighbouring German State of Mecklenburg-Vorpommern and was observed in a previous study. However, sequences from the type locality of *B. minima*, Helgoland – several hundred kilometres away from Wohlenberg –, were so far missing, which gains importance when we consider that this island was extensively studied within our survey (Fig. 1). A frequently found entity on Helgoland that could not be resolved to species level due to the absence of any similar GenBank entries was *Blidingia* sp. 2 (Fig. 2). *Blidingia* sp. 2 delimits in a unique clade and is the next closest relative to *B. marginata* (Fig. 2, Table 2).

In addition to the above-mentioned molecular differences of *Blidingia* sp. 2 and *B. marginata*, distinctive morphological delimitations of the two entities were also observed. *Blidingia* sp. 2 differed from specimens of *B. marginata* (Fig. 3) (Bliding, 1963; Kornmann & Sahling, 1978) in generally exhibiting smaller thalli and being mostly unbranched (Fig. 6). Only few individuals exhibited macroscopic branching in the basal thallus parts, and no microscopic branches were observed (Fig. 6). Concurrently, the morphological features of adult *Blidingia* sp. 2 (Fig. 6) showed high similarity with both the type description of *B. minima* (Kützing, 1849; Kylin, 1949) and traits described for *B. chadefaudii* (Kornmann & Sahling, 1978) from Helgoland.

Based on the results obtained within our study we conclude: (1) The type locality of *Blidingia minima* (as

Enteromorpha minima) is Helgoland (Kützing, 1849; Kylin, 1949); however, none of the entries in GenBank assigned to *B. minima* could be verified on Helgoland. (2) The morphology (Fig. 6) of the well-delineated clade representing *Blidingia* sp. 2 (Fig. 2) resembles the morphology described for *B. minima* (Kützing, 1849; Kylin, 1949; Kornmann & Sahling, 1978). (3) The molecular identification of a specimen within our study (Fig. 2, accession no.: MT076212) that was identified as *B. minima* by P. Kornmann in 1977 (Table 1, voucher number Herbarium Helgoland: BRM008079) is in congruence with sequences of the entity representing *Blidingia* sp. 2. (4) The ontogenetic development of *Blidingia* sp. 2 (Fig. 4) is similar with that of *B. chadefaudii* described in Kornmann & Sahling (1978). (5) We concur with several authors who have highlighted the strong overlap of morphological and ontogenetic traits of *B. minima* and *B. chadefaudii* and thus suggested their conspecificity (Woolcott & al., 2000).

Based on these findings, the holotype of *Blidingia minima* cannot be critically identified for purposes of the precise application of the name, and we suggest designating a specimen identified as *Blidingia* sp. 2 in our study as epitype of *B. minima*. *Blidingia minima*, as we epitypify it here, has been fully characterized molecularly (see Fig. 2), phenotypically (see Results and Fig. 6), and ontogenetically (see Results and Fig. 4) on the basis of isolates from the type locality of this species, Helgoland (Kützing, 1849; Kylin, 1949).

■ EPITYPIFICATION OF *BLIDINGIA MINIMA*

The holotype of *Blidingia minima* (L barcode L 0054691) was collected at Helgoland, Schleswig-Holstein, Germany by C.W. Nägeli. The exact date of the collection is not noted on the herbarium voucher (suppl. Fig. S1). However, since the work of Kützing (1849) refers to the herbarium voucher prepared by Nägeli it can be expected that the holotype of *B. minima* was sampled around the mid-19th century, which is also in agreement with the biographical data of Nägeli (1817–1891). The type specimen was first identified as *Enteromorpha minima* and has been transferred to *Blidingia* by Kylin in 1949 (Guiry & Guiry, 2019). Since no cultures ex-type are available, it was re-collected at Helgoland and cultivated (Figs. 4, 6). This collection is designated as epitype below. The described epitype is housed at C (C-A-99668).

Blidingia minima (Nägeli ex Kütz.) Kylin in Förh. Kungl. Fysiogr. Sällsk. Lund. 17: 181. 1949 ≡ *Enteromorpha minima* Nägeli ex Kütz., Sp. Alg.: 482. 1849 ≡ *Enteromorpha compressa* var. *minima* (Nägli ex Hauck) Hamel in Rev. Algol. 6(1): 65. 1931 ≡ *Enteromorpha nana* var. *minima* (Nägeli ex Hauck) Sjøstedt in Svensk Bot. Tidskr. 33: 38. 1939 – Holotype: GERMANY. Helgoland, Schleswig-Holstein, exact collecting date unknown, *Nägeli s.n.* (L barcode L 0054691!) – **Epitype (designated here):** GERMANY. Helgoland, Schleswig-Holstein, N 54.18367° E 7.888633°, 23 Jul 2014, *S. Steinhagen S_34* (C barcode C-A-99668).

Known geographical range. – This species was distributed on the German off-shore island Helgoland and at two sites located at the German Wadden Sea (see Table 1).

Selected specimens examined. – Nordstrand, Schleswig-Holstein, Germany (N 54.4707167°; E 8.8068333°), 29 Aug 2017, *S. Steinhagen S_949*, GenBank MN258040 (*tufA*), Wadden Sea; Helgoland, Germany (N 54.17195°; E 7.8993°), 23 Jul 2014, *S. Steinhagen S_34*, GenBank MH475460 (*tufA*) and MN258053 (*rbcL*); Helgoland, Germany (N 54.18367°; E 7.888633°), 22 Jul 2014, *S. Steinhagen S_1*, GenBank MH475461 (*tufA*); Helgoland, Germany (N 54.1825°; E 7.890617°), 23 Jul 2014, *S. Steinhagen S_39*, GenBank MH475462 (*tufA*) and MN258054 (*rbcL*); Dagebuell, Schleswig-Holstein, Germany (N 54.1825°; E 7.890617°), 30 Jul 2014, *S. Steinhagen S_124*, GenBank MH475463 (*tufA*) and MN258055 (*rbcL*).

Although individuals from Helgoland could be identified as *Blidingia chadefaudii* (Kornmann & Sahling, 1978), we do not include *B. chadefaudii* as a heterotypic synonym of *B. minima*. With the displayed results, we cannot rule out the existence of *B. chadefaudii*, a species exhibiting unique traits like a thickened inner cell wall that presents a lamellar structure of parallel arranged riffles (Chadefaud, 1957). Also, based on the multiple sequence entries in GenBank, it can be assumed that the genus *Blidingia* harbours even more species than we are aware of today. However, there is currently no evidence of the presence in Germany of *B. chadefaudii* in the past or today. The only existing records were based on material that did not show the characteristic traits of *B. chadefaudii*, such as cell wall thickenings (Kornmann & Sahling, 1978), and were apparently due to a misinterpretation of life cycle traits that are variable within *B. minima*.

Furthermore, *Blidingia cornuta* sp. nov. can be clearly distinguished from other described species of *Blidingia* by a unique combination of characters: Our phylogenetic analyses of the *tufA* and *rbcL* marker genes clearly distinguished *B. cornuta* from its closest genetic relatives. Within the *tufA* tree, a clade that contained sequences of an undescribed entity originating from Manitoba, Canada (HQ610241) and from Alaska, U.S.A. (MF124265) was found to be the next known closest relative to *B. cornuta*. Due to significant sequence dissimilarities of 4.8%, the independent status of *B. cornuta* can be confirmed (Fig. 2, Table 2). The clade representing *B. cornuta* is also supported with maximum bootstrap values (Fig. 2). Additionally, ontogenetic and morphological features separate the newly proposed species from already existing species (Fig. 4).

A unique pattern of ontogenetic development was observed in individuals of *Blidingia cornuta*: After a clear germination-tube had begun to form (Fig. 4F), the cell contents migrated through the germination-tube and formed a germinating cell, detaching the initial spore sleeve and germination-tube (Fig. 4G,H). This kind of pattern has not been observed in any other described *Blidingia* species

(Kylin, 1949; Bliding, 1963; Kornmann & Sahling, 1978). As another unique trait, thalli exhibited a specific antler-like branching pattern, together with a cell arrangement in longitudinal rows that has not previously been observed in any other *Blidingia* species. It should be mentioned, however, that "*Blidingia minima* var. *ramifera*" (Bliding, 1963) exhibits some similarities – but also significant differences – with the newly described *B. cornuta*. The thalli described by Bliding (1963) were distinctly taller in size and reached a length of up to 50 cm, whereas thalli of *B. cornuta* are less than 1 cm in length (rarely taller). The branches of "*B. minima* var. *ramifera*" were described as elongate, while the branching pattern in *B. cornuta* is antler-like, and the respective branches are compact rather than elongate. Bliding (1963) reported that cells of "*B. minima* var. *ramifera*" were predominantly unarranged or only arranged in short rows, whereas most specimens of *B. cornuta* have clearly visible longitudinal cell rows that are evident throughout the thallus. Garbary & Barkhouse (1987) treated "*B. minima* subsp. *ramifera*" at species level. Their description of "*B. ramifera*" encompassed the same striking differences from *B. cornuta* as previously described for "*B. minima* subsp. *ramifera*". Hence, branched specimens of "*B. ramifera*" were regarded as morphotypes of *B. marginata* (Burrows, 1991; Guiry & Guiry, 2019). However, the genetic distinction of *B. cornuta* seems indeed reflected in a unique branching pattern. In this light, the status of "*B. ramifera*" should be re-evaluated based upon support with genetic markers. Several unidentified *Blidingia* sequences are available via GenBank that hint a hidden diversity that is still to be discovered. Our study indicates once again that it can be rewarding to reassess the exact taxonomic relationships of allegedly well-known species groups within the Ulvophyceae based on molecular markers and subsequent phylogenetic techniques to reveal exact species relationships, potential morphological plasticity, and species-specific ecological traits.

We can conclude that the historical species concepts for several *Blidingia* spp. are flawed and problematic for species occurring in northern Germany. Misinterpretation of phenotypic plasticity in mature thalli, and to some degree also in ontogenetic developmental stages, has led to misidentifications in the past, and species delimitation based on morphological traits is often impossible. Thus, our findings support the use of molecular methods for correct and clear species identification and devalue the use of morphological characters alone.

■ AUTHOR CONTRIBUTIONS

SSt: original concept, experimental design, fieldwork and algae collection, laboratory work, macro- and microscopic observation, phylogenetic analysis, drafting and editing manuscript; LD: algae collection, laboratory work and algae cultivation; FW: algae collection, original concept, drafting and editing manuscript. — SSt, https://orcid.org/0000-0001-8410-9932; LD, https://orcid.org/0000-0002-2750-6437; FW, https://orcid.org/0000-0003-3366-6880

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