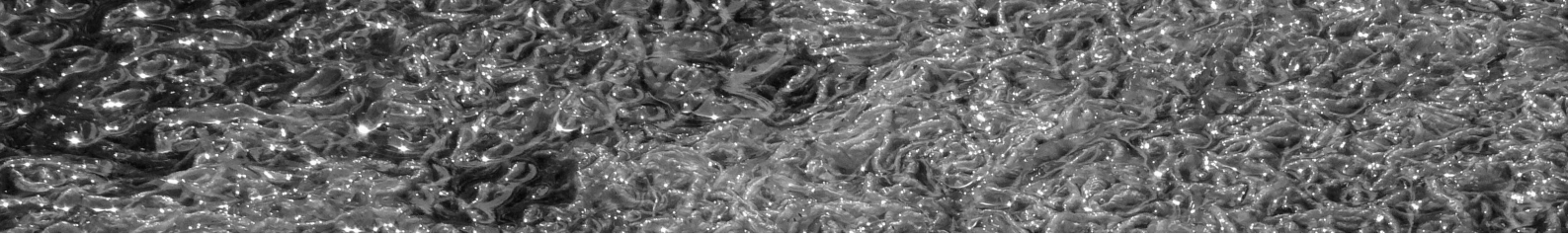


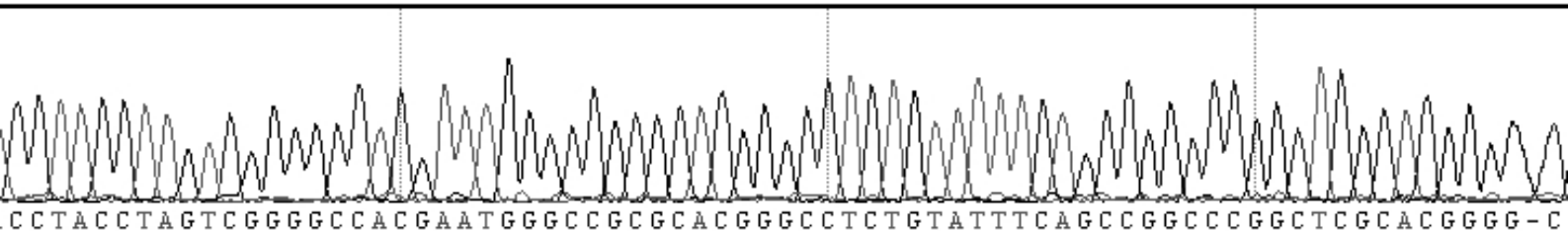
University of South Bohemia, Faculty of Science
Department of Botany, 2009

Combined morphological and molecular approach to the assessment of *Ulva* (Chlorophyta, Ulvophyceae) in the Czech Republic

Master thesis
by

Jan Mareš

Supervised by: RNDr. Jan Kaštovský, Ph. D.



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Annotation

This study was aimed at discovering taxonomical identity of freshwater populations of a prevailably marine green-algal genus *Ulva* in the Czech Republic. Furtherly, collected samples were compared to some relevant material from European herbaria.

The identity of collected specimens was successfully resolved by a combined methodology that involved classical and modern molecular techniques, and discussed in the framework of distribution and ecology of close European taxa.

Prohlašuji, že svoji diplomovou práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č.111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

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Jan Mareš

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Valuable herbarium specimens were kindly provided by following herbaria: BRNM (Moravian Museum, Brno, Czech Republic), WU (Universität Wien, Austria), L (Nationaal Herbarium Nederland, Leiden University branch, The Netherlands), PRC (Charles University in Prague, Czech Republic), E (Royal Botanic Garden Edinburgh, UK).

Contents

1. Introduction

1.1. General description of the genus

Species of the green algal genus *Ulva* (Chlorophyta, Ulvophyceae) are among the most common coastal seaweeds all over the world. They grow abundantly on all available substrates in the intertidal zone of seas and in brackish waters, tolerating wide range of salinity, temperature and water quality (Poole and Raven 1997). Some of the species are also present in continental waters with more or less increased salinity, such as salt marshes, springs or other waters with higher concentration of chlorides and sulphates (Mattern 1968, Conner et al. 1978, Polderman 1980, Wolgemuth et al. 1984, Lederer et al. 1998, Sitkowska 1999, Skácelová 2004, and others).

Ulva species usually grow in form of typical vividly green tube- or leaf-shaped thalli (see Plates 1-19 in Appendix), often also with various types of branches, attached to the substrate by rhizoids, or later as free-floating intestinoid clusters (Fig. 1). Within its life cycle, *Ulva* forms morphologically similar haploid and diploid thalli, both of which produce asexual zoospores by mitotic division of vegetative cells (van den Hoek and Mann 1996). *Ulva* is also often dispersed by fragmentation of thalli. During sexual reproduction, the opposite mating types (gametophytes) produce haploid isogametes which fuse to create a zygote (alternatively they can develop directly into adult thalli) (Bliding 1963).

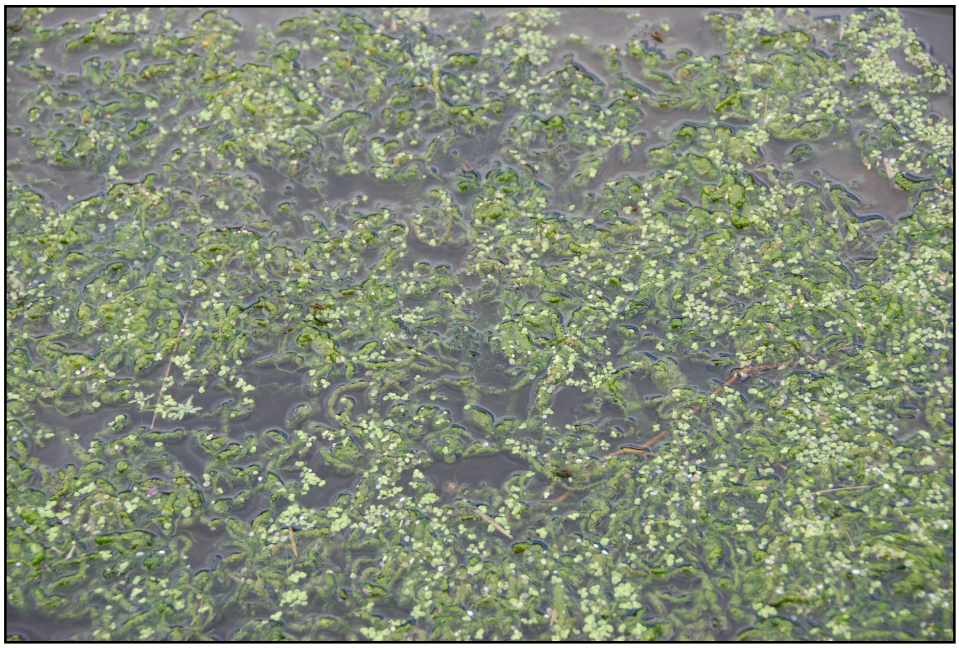

Figure 1. *Ulva* in form of free-floating intestinoid clusters. Hradec Králové, 2007.

Another genus called *Enteromorpha* used to be distinguished from *Ulva* on the basis of different morphology of the thallus. A typical specimen of *Ulva* is flat, two cell layers thick, appearing mostly like a lettuce while *Enteromorpha* should form one cell layer thick, more or less inflated tubes.

Interestingly, Linnaeus (1753) himself included both types in the genus *Ulva* that was only later divided into the two above mentioned genera by Link (1820) according to the macro-morphological features. In the last decade, studies using modern molecular methods (Blomster et al. 1999, Malta et al. 1999, Tan et al. 1999, Woolcott and King 1999, Hayden et al. 2003) revealed and repeatedly confirmed that the species of the separated genera did not form any monophyletic clusters that could be assigned to a certain general morphology, thus revalidating the original concept of *Ulva sensu lato* by Linnaeus. In this study we use both names as synonyms, ´*Enteromorpha*´ being reserved for citations of other authors´ work, historical specimens, etc.

It is important from the ecological point of view that in eutrophicated marine environments, *Ulva* forms the majority of so-called ´green tides´ (Fletcher 1996, Blomster et al. 2002). These harmful algal blooms, consisting of densely accumulated green-algal biomass, often cause changes in biogeochemical cycles, negatively affect seagrass beds (due to shading), habitats important for wading birds, invertebrates, and also disrupt human recreation and other activities (Fletcher 1996, Valiela et al. 1997, Raffaelli et al. 1998, Lopes et al. 2000). For example, according to Dion and LeBozec (1996), about 10^8 kg of seaweeds has to be removed only from French Atlantic recreational beaches every year.

Moreover, these algae have been considered as major marine fouling organisms, growing quickly on ship hulls and other subtrates (Fletcher 1980, Callow 1986). For a long time they also have been used as bioindicators of eutrophication along European Atlantic coast (Briand 1991, Schories and Reise 1993, Hernandez et al. 1997), and are known to tolerate marine pollution (Wallentinus 1979).

The ability of *Ulva* to create vast accumulations of biomass is not restricted only to marine and estuarine environments. Under favourable conditions, it is able to form troublesome blooms also in continental waters, especially those with high nutrient concentration and increased salinity. Up to now, the largest blooms in freshwaters were reported from Lake Michigan, USA, where *Ulva flexuosa* together with other green filamentous algae covered part of the shores in 2003 (Lougheed and Stevenson 2004). However, minor blooms have been observed also in ponds in Poland (Sitkowska 1999), Mecklenburg Lakes in Germany

(Gardavský in Skácelová 2004), and in the Czech Republic (e.g. Wolgemuth et al. 1984, Skácelová 2004).

1.2. Identification of species

A keystone taxonomic work concerning European taxa of the former *Enteromorpha* (Bliding 1963), which is cited in all relevant studies, includes 17 species and about 40 intraspecific units in 7 groups. Although it is not the only existing approach to *Ulva* taxonomy (see Burrows 1991), it has been accepted by most European authors and further revisions (Koeman and van den Hoek 1982 a, b, 1984; Blomster et al. 1998,1999; Brodie et al. 2007) were built on its basis. For these reasons we stick to this taxonomical system also in our study.

Despite the fact that there is available literature for determination of European *Ulva* species, identification of specimens collected from natural habitats has been considered extremely difficult or sometimes impossible even for specialized researchers. The major reason is the extensive variability in morphological traits (Bliding 1963, Blomster et al. 1998, 1999, 2002).

Koeman and van den Hoek (1982 a) created a set of characters to which should be paid attention for proper discrimination of species: (a) the macroscopic morphology of the plants (including colour and texture); (b) the structure of the basal region of the axis (and, if present, of branches); (c) the form and arrangement of the cells in surface view; (d) the structure of the tips of branchlets (if present); (e) the number of pyrenoids per cell; (f) the appearance of the chloroplast in surface view; (g) the cell size; (h) the mode of reproduction; (i) the morphology of germlings.

Unfortunately, such morphological approach seems to be insufficient. Even the authors of the taxonomic handbook (Koeman and van den Hoek 1982 a) state: ´All taxonomically valid criteria within *Enteromorpha* are of a quantitative nature, each criterion being represented by a graded series of variable expressions with overlaps between species´. Therefore they suggest using a combination of all available characters, emphasizing the macroscopic morphology, the morphology of filiform branchlets and of the basal parts of the plant as the most distinctive features.

Nevertheless, it has been repeatedly described, that *Ulva* species show extreme morphological plasticity which seems to be closely related to environmental conditions such as salinity (Burrows 1959, Reed and Russell 1978, Koeman and van den Hoek 1982 a, Young et al. 1987), seasonal changes (Blomster et al. 1999), or growth in green tides (Blomster et al.

2002). The species may actually switch between the tube-like and the leaf-like general morphology (Tan et al. 1999), and they can even form monostromatic blades resembling another genus *Monostroma* (Blomster et al. 2002), still inside the borders of a single species.

If a specimen growing under specific conditions and/or in a phase of its life cycle inappropriate for identification (e.g. young or free-floating thalli) is collected, its determination to the species level may easily become impossible. Usually, the attempts at identification lead only to a wider group of taxa with common anatomical traits (number of pyrenoids, structure of branchlets, etc.). Koeman and van den Hoek (1982 a, b, 1984) were able to confirm the attributes of their species thanks to devoted work on cultivation of the algae in various conditions and measuring of the morphological characters. Unfortunately, such method is too demanding and time-consuming for common sampling and determination in other type of studies than large-scale taxonomic investigations.

Where morphological characters fail in determination of taxa, methods based on various molecular markers have been increasingly emloyed. The ITS region of the rRNA gene (ITS1-5.8SrRNA-ITS2) is among the most widely used markers for discrimination of algae (Marks and Cummings 1996, Yeh and Chen 2004, Nuber et al. 2007, and others). For *Ulva* it was thoroughly characterized by Leskinen and Pamilo (1997), and since then it has been repeatedly used for identification of *Ulva* species with satisfactory results. For example, Blomster et al. (1998, 1999, 2000) were, in their studies based on the rRNA ITS marker, able to discriminate between the morphologically similar taxa *U. intestinalis* and *U. compressa*; they showed that *U. usneoides* was probably only an ecotype of *U. compressa*, and *U. clathrata* was a seasonal morphotype of *U. muscoides*. Similarly, Coat et al. (1998) applied the same marker for characterization of *U. armoricana* in Brittany; Tan et al. (1999) and Malta et al. (1999) used it in extensive phylogenetic research of the *Ulva*/*Enteromorpha* complex, and Leskinen et al. (2004) in characterization of the distribution and genetic variability of *U. intestinalis* and *U. compressa* in the Baltic Sea area.

Except rRNA ITS, other molecular markers have been used in phylogenetical and taxonomical studies concerning green algae, among which 18S rRNA and chloroplast *rbc*L genes have been very common (Manhart 1994, Hepperle et al. 2000, Fama et al. 2002, Rindi et al. 2007, etc.). Many researchers take an advantage of obtaining information from more than one marker to be able to create more accurate cladograms with better bootstrap support (rRNA ITS and *rbc*L in Ulvaceae e.g. Hayden and Waaland 2002, Hayden et al. 2003, Loughnane et al. 2008). Interestingly, in their recent study of the green algal order Prasiolales, Rindi et al. (2007) concluded that the *rbc*L gene had higher sequence divergence

than the 18S rRNA gene, and was therefore more useful for phylogenetic investigation at the ranks of genus and species.

Considering this, a combination of two DNA markers (ITS region of the rRNA gene and *rbc*L) seems to be most promising to solve taxonomical problems in *Ulva*. By putting together molecular data with classical morphological and anatomical description, it should be possible to classify the species more easily, reliably and comprehensibly than by any of these methods alone.

1.3. *Ulva* in the Czech Republic

In the Czech Republic, *Ulva* spp. has been commonly reported for a long time from two distinct areas – a salt marsh in the Soos National Nature Reserve in west Bohemia (Brabez 1941, Lederer et al. 1998) and from ponds and pools with increased salinity at several places in south Moravia (Nave 1863, Fischer 1920, Zapletálek 1932, Krist 1934, Heteša 1962, Ettl et al. 1973, Marvan and Sládeček 1974, Marvan and Komárek 1978, Skácelová 2004, and others).

Hansgirg (1892) reports in his monumental study on Czech algal flora only one species to be present in Bohemia, *Enteromorpha intestinalis* Linnaeus (Link) with two other varieties (var. *crispa* (Roth) Greville and var. *tubulosa* Kützing) defined only according to the macroscopic morphology of the thallus. Probably here began the tradition of identification of Czech specimens as *U. intestinalis* Linnaeus (or *E. intestinalis*) because of their tube-like appearance.

It is true that *U. intestinalis* is known to be a euryhaline species (Koeman and van den Hoek 1982 a) that may tolerate almost freshwater conditions, e.g. in estuaries where dilution of salts in the brackish water by riverine input is compensated by high nutrient levels of the river water (Kamer and Fong 2001, McAvoy and Klug 2005). On the other hand, different species such as *U. flexuosa* Wulfen or *U. prolifera* O. F. Müller can survive in waters with relatively low concentration of salts, and their range of macroscopical appearance includes intestinoid thalli, too (Koeman and van den Hoek 1982 a, 1984).

However, most of the authors have stuck to the traditional approach, and the specimens from Czech localities have been mostly identified as *E. intestinalis*, which is therefore regarded as an indigenous species. In contrary, isolated findings from the first half of the twentieth century were attributed to *E. prolifera* (O. F. Müller) Kützing (Fischer 1920, Zapletálek 1932), *E. tubulosa* (Kützing) Kützing (Zapletálek 1932) or *E. salina* Kützing (Brabez 1941). Some of these species were later reclassified, most importantly *E. tubulosa*

and *E. intestinalis* var. *tubulosa* were transferred to *U. flexuosa*, and *E. salina* to *U. prolifera* (Bliding 1963).

Recently there have been some reports of algae determined as *E. intestinalis* (Wolgemuth et al. 1984, Marvan et al.1997, Adamec pers. comm.), but also as *E. flexuosa* (Wulfen) J. Agardh (Lederer et al. 1998, Marvan et al. 1997) or *E. linza* (Linnaeus) J. Agardh (Heteša and Sukop 1998, 2001) from several localities throughout the country. Further occurrence of the genus, including local blooms of *Ulva* sp., was repeatedly observed by Janeček (pers. comm.) and Koza (pers. comm.) at various localities in northwest and one in east Bohemia (drainage dikes, eutrophicated streams, flooded abandoned lignite mine) – usually human-made or human-impacted localities with higher nutrient and salt levels caused by runoff from agriculture, industry, or by wastewater. All findings of *Ulva* from the Czech Republic known to the author are summarized in Tab. 1.

Unfortunately, all the specimens collected in the Czech Republic have been either determined only to the genus level, or assigned to a certain species inconsistently using various morphological characters according to different taxonomical handbooks, sometimes with a belief of *U. intestinalis* being the only taxon to occur in the area. At the same time, increasing numbers of present findings at commonly present habitats like small eutrophicated streams, dikes, and pond outlets leads us to a hypothesis that *Ulva* is probably more widely distributed at correspondent localities throughout the country.

For these reasons, we suggest that an examination of populations of *Ulva* from various localities in the Czech Republic is necessary to clear away present taxonomical confusion. Since the specimens often show few usable morphological characters, the addition of methods based on molecular markers (rRNA ITS, *rbc*L) would be helpful for separation of the species.

As it is possible (at least according to morphology) but uncertain that there is more than one species currently present in the Czech Republic, another question appears: Are these all closely related populations spreading from the two original areas, or have they come from abroad? Such question may get more realistic content if we consider that many of the recently observed populations tend to show some of the characteristic traits of primarily marine species *U. flexuosa* and *U. linza* (Lederer et al. 1998, Marvan et al. 1997, Heteša and Sukop 1998, 2001, Kaštovský et al. 2006). On the other hand, various species, including supposedly marine *U. prolifera* (also as *E. salina*) and *U. flexuosa* (as *E. tubulosa* and *E. intestinalis* var. *tubulosa*), have been occassionally reported since the nineteenth century.

Therefore, the answer is crucial for understanding not only the taxonomy, but also ecology of these algae in our country – in other words to assess if it is a native species expanding

to the newly established or human-influenced habitats with favourable conditions, or perhaps a potentially dangerous alien species coming from the European seashores.

Table 1. Summary of published findings of *Ulva* in the Czech Republic.

Locality		Source	Taxa
Name	**Description**		
Františkovy Lázně, Soos	salt marsh, mineral springs	continual reports since the 19th century (Hansgirg 1892, Brabez 1941, Lederer et al. 1998, etc.)	*Enteromorpha intestinalis*, *E. flexuosa*, *E. salina*
Břeclav region, southern Moravia	small fishponds, pools, dikes, mostly alluvium of the Dyje river	continual reports since the 19th century (Nave 1863, Krist 1934, Ettl et al. 1973, Heteša and Sukop 1998, 2001, Skácelová 2004, and many others)	*Enteromorpha intestinalis, E. flexuosa*, *E. linza, E. prolifera*, *E. tubulosa*, *E.* spp.
Třebíč region, Moravia	eutrophicated ponds	Wolgemuth et al. 1984	*Enteromorpha intestinalis*
Jihlava river	small tributary	Marvan et al. 1997, Adamec 2008	*Enteromorpha* cf. *flexuosa*, *Enteromorpha* sp.
Prague neighborhood	small fishponds in settlements on the southern edge of the Prague agglomeration	Skácelová 2004, Marvan et al. 1997	*Enteromorpha* cf. *flexuosa*
Studená	ponds in the Czech-Moravian Highlands	Skácelová 2004, Marvan et al. 1997	*Enteromorpha* cf. *flexuosa*
Poděbrady region	pools in alluvium of the Labe river	Frič and Vávra 1901	*Enteromorpha intestinalis*
Český Krumlov	small stream	Pascher 1903	*Enteromorpha intestinalis*
Jedovnice	fishpond near a village	Skácelová 2004, Marvan et al. 1997	*Enteromorpha* cf. *flexuosa*
Kopidlno	littoral of the Pilský fishpond	Adamec 2008	*Ulva sp.*
Hradec Králové	artificial stream channel in an industrial part of the city	Koza pers. comm.	*Ulva* sp.
Lukavec	artificial stream channel with wastewater input	Janeček pers. comm.	*Ulva* sp.
Malhostice	drainage channel near the Bílina river	Janeček pers. comm.	*Ulva* sp.
Chabařovice	recultivated lignite dump, small pools	Janeček pers. comm.	*Ulva* sp.
Srpina	small stream in agricultural landscape	Janeček pers. comm.	*Ulva* sp.
Teplice	Barbora - a flooded lignite mine, recreational reservoir	Janeček pers. comm.	*Ulva* sp.
Oleksovice	eutrophicated fishpond outlet	Geriš pers. comm.	*Ulva* sp.

1.4. Objectives

The main scope of our project is to investigate the taxonomical identity of chosen populations of *Ulva* spp. in the Czech Republic, including samples from localities with supposed native occurrence and from secondary localities. This will be achieved by a combined approach based on respected determination literature (as for morphology and anatomy) and sequencing of suitable molecular markers (rRNA gene ITS region and *rbc*L).

In addition, our findings will be compared to available data of other scientists, especially those concerning European samples of *Ulva* from both freshwater and marine habitats.

The results should be able to give us a more comprehensible picture of this taxonomically complicated genus in our country, and may even bring useful information about its phylogenetic relationship with congeneric algae from the closest continental and marine habitats, and consequently also about its ecology and expansive potential in Europe.

2. Methods

2.1. Samples and localities

This study includes 6 samples of *Ulva* collected personally by the author from diverse localities in the Czech Republic. Additional 18 samples were obtained from other scientists and from several European herbaria. All samples and information about respective localities, collectors, dates of collection, identification, source herbaria, and accomplished analyses are given in Tab. 2.

Collection of fresh specimen was carried out in August 2007, and between May and September 2008. A higher amount of the material was directly transferred into laboratory in suitable bottles filled by water from the sampling locality.

Morphology and anatomy of the thalli was assessed as soon as possible by examination of the fresh algae. The best plants were chosen for preparation of herbarium specimens by pressing and drying on herbarium sheets. Part of the material was preserved in absolute ethanol (and paralelly in silica-gel) for molecular analysis.

Water conductivity, temperature, and pH were measured by a HI 98129 (HANNA Instruments) pocket multimeter at the sampling place. About 250 mL of water from each of the five sampling localities were taken for chemical analysis and stored in dark at -20°C for 2-5 months. Chosen chemical variables, especially nutrient levels and concentration of basic ions, were assessed by the state-accredited chemical laboratory of the Vltava Watershed Agency (see Table 3. in Results for all acquired physical and chemical values). The sampling site Lednice (2007) was visited prior to the beginning of the project, thus no water was taken for analysis.

The studied herbarium specimens were all in form of exsiccates. The type specimens of *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne and *Ulva flexuosa* subsp. *flexuosa* Wulfen were kindly sent to the author on loan by respective herbariums (see Tab. 2).

The samples from Elina Leskinen (University of Helsinki) were microscopically analysed in her lab only in form of permanent microscopy slides (previously mounted by E. Leskinen using Kaiser´s glycerine gelatine (Merck Biosciences) according to Gray 1954).

The specimen of *Ulva* from theYthan estuary, Scotland, was examined during a visit in Jaanika Blomster´s lab (University of Helsinki) where it was borrowed from the herbarium of The Royal Botanic Garden Edinburgh (UK).

Only part of the material available in Helsinki was studied to unify our morphological terms and check the determination methods. A small piece of each of the E. Leskinen´s exsiccates (respective to the permanent slides) and the herbarium specimen of *Ulva* from the Ythan estuary, Scotland, were taken for molecular analysis.

The only specimen from Lodz, Poland was received from Małgorzata Sitkowska (University of Lodz), already fixed by ethanol and paralelly by formaldehyde, together with photographs of fresh plants.

Morphological and anatomical analysis of the remaining specimens (from the Herbarium of the Charles University in Prague) was realized in the algological laboratory of the Department of Botany, Faculty of Science, Charles University in Prague.

2.2. Analysis of morphology and anatomy

Classical determination of the algae according to morphological and anatomical characters was made after Bliding (1963) and Koeman and van den Hoek (1982 a, b, 1984).

The macroscopic morphology of the collected specimens was documented by taking photographs of fresh plants (*in situ*) and herbarium specimens. The rest of the herbarium specimens included in the study were also photographed.

Microscopic analysis involved taking several pieces of the fresh material to document the size and arrangement of cells in variable parts of the thallus. Special attention was paid to the structure of microscopic branchlets (if there were any) and number of pyrenoids in chloroplasts, the main anatomical features with taxonomic value. Neither asexual zoospores nor sexual process were observed in any of the studied samples.

As for the borrowed herbarium specimens, only a very small part of the valuable material (especially from the type specimens) was taken for microscopic analysis, and rehydrated for several minutes before examination to check as many characters as possible.

The permanent microscopy slides usually consisted of enough material of more or less satisfactory quality to conduct the examination in a similar way to the fresh specimens.

All microscoped samples were photographed during examination if the quality of material was good enough to show any relevant features.

Table 2. Summary of specimens of *Ulva* included in our study.

Taxon	Locality	Author (leg.) and Year	Herbarium	Morphology	ITS	*rbc*L
Ulva flexuosa subsp. *paradoxa* I (C. Agardh) M. J. Wynne	Soos salt marsh, Czech Republic	Mareš 2008	author´s personal herbarium	Y	Y	Y
Ulva flexuosa subsp. *pilifera* I (Kütz.) M. J. Wynne	stream, Hradec Králové, Czech Republic	Mareš 2007	author´s pers. herb.	Y	Y	Y
Ulva cf. *flexuosa* I	fishpond, Hlohovec, Czech Republic	Mareš 2008	author´s pers. herb.	Y	Y	Y
Ulva flexuosa subsp. *pilifera* II (Kütz.) M. J. Wynne	stream, Oleksovice, Czech Republic	Mareš 2008	author´s pers. herb.	Y	Y	Y
Ulva cf. *flexuosa* II	water tank, Nemilkov, Czech Republic	Mareš 2008	author´s pers. herb.	Y	Y	Y
Ulva flexuosa Wulfen	fishpond, Lednice, Czech Republic	Mareš 2007	author´s pers. herb.	Y	Y	Y
Ulva flexuosa subsp. *pilifera* III (Kütz.) M. J. Wynne	fishpond, Lodz, Poland	Sitkowska 2008	author´s pers. herb.	Y	Y	Y
Ulva flexuosa subsp. *pilifera* IV (Kütz.) M. J. Wynne	Žitava river alluvium, Slovakia	Palaticka 2008	BRNM (Moravian Museum, Brno, Czech Republic)	Y	N	N
Ulva flexuosa subsp. *flexuosa* Wulfen (as *Ulva flexuosa* Wulfen), holotypus	Adriatic coast, Duino, Italy	Wulfen	WU (Universität Wien, Austria)	Y	N	Y
Ulva flexuosa subsp. *pilifera* (Kütz.) M. J. Wynne, holotypus (as *Enteromorpha pilifera* Kützing)	fresh water, Tennstedt, Germany	Kützing	L (Nationaal Herbarium Nederland, Leiden University branch, The Netherlands)	Y	N	Y
Ulva cf. *flexuosa* III (as *Enteromorpha intestinalis* (Linnaeus) Nees)	fishpond, Vranovice, Czech Republic	Krist 1934	PRC (Charles University in Prague, Czech Republic)	Y	N	N
Ulva cf. *flexuosa* IV (as *Enteromorpha intestinalis* var. *crispa* (Roth) Greville)	stream, Budapest-Kossuthfalva, Hungary	Filárszky 19??	PRC (Charles University in Prague, Czech Republic)	Y	Y	N
Ulva flexuosa subsp. p*aradoxa* II (C. Agardh) M. J. Wynne *(*as *Enteromorpha intestinalis* var. *tubulosa* Kützing)	stagnant water, Larga, Romania	Teodorescu 19??	PRC (Charles University in Prague, Czech Republic)	Y	Y	N
Ulva cf. *flexuosa* V (as *Enteromorpha intestinalis* var. *cylindracea* J. Agardh*)*	dike, Lednice, Czech Republic	Zimmermann 19??	PRC (Charles University in Prague, Czech Republic)	Y	Y	N
Ulva flexuosa subsp. p*aradoxa* III (C. Agardh) M. J. Wynne *(*as *Enteromorpha intestinalis* var. *tubulosa* Kützing)	stream, Budapest-Kossuthfalva, Hungary	Filárzsky 19??	PRC (Charles University in Prague, Czech Republic)	Y	N	N
Ulva cf. f*lexuosa* VI	Ythan estuary, Scotland	Taylor 1997	E (Royal Botanic Garden Edinburgh, UK)	Y	Y *	N
Ulva flexuosa subsp. *pilifera* IV (Kütz.) M. J. Wynne	lake, Mälaren, Sweden	Leskinen 1996	author´s pers. herb.	Y	Y *	N
Ulva flexuosa subsp. *pilifera* (a) (Kütz.) M. J. Wynne	river Saxån, Skåne, Sweden	Leskinen 1997	author´s pers. herb.	Y *	Y *	N
Ulva flexuosa subsp. *pilifera* (b) (Kütz.) M. J. Wynne	river Saxån, Skåne, Sweden	Leskinen 1997	author´s pers. herb.	Y *	Y *	N
Ulva flexuosa subsp. *pilifera* (c) (Kütz.) M. J. Wynne	river Saxån, Skåne, Sweden	Leskinen 1997	author´s pers. herb.	Y *	Y *	Y
Ulva flexuosa subsp. *pilifera* (Kütz.) M. J. Wynne	lake, Mälaren, Sweden	Leskinen 1994	author´s pers. herb.	Y *	Y *	Y
Ulva flexuosa subsp. *paradoxa* IV (C. Agardh) M.J. Wynne	Baltic Sea,Krogarviken, Tvärminne, Finland	Leskinen 1994	author´s pers. herb.	Y *	Y *	N
Ulva flexuosa subsp. *paradoxa* (C. Agardh) M. J. Wynne	Baltic Sea, Trelleborg, Skåne, Sweden	Leskinen 1997	author´s pers. herb.	Y	Y *	N
Ulva flexuosa subsp. *paradoxa* (C. Agardh) M. J. Wynne	Oslofjord, Voll, Porsgrunn, Norway	Leskinen 1996	author´s pers. herb.	Y	Y *	N
Ulva flexuosa subsp. *flexuosa* Wulfen	sea (Skagerrak), Orust, Sweden	Leskinen 1995	author´s pers. herb.	Y	Y *	Y

* analyses performed by E. Leskinen (University of Helsinki)

2.3. Molecular analysis

2.3.1. DNA Extraction

Total genomic genomic DNA extraction from the algae collected in summer 2007 and 2008 (preserved in ethanol or silica-gel for several days or weeks) was executed by the Invisorb Spin Plant Mini Kit (Invitek) according to the manufacturer´s instructions.

DNA from the remaining silica-gel-preserved or otherwise dried samples was isolated using the CTAB method (Doyle and Dickson 1987), modified for 10-20 mg of starting material, with an initial rehydration step following Hayden et al. (2003).

Unfortunately, significant part of the genomic DNA of some of the old herbarium specimens was fragmented (Fig. 2), probably due to the long-term desiccation, and resisted amplification. Attempts were made to separate the remains of the undamaged DNA (plus large fragments) from the highly fragmented part, as follows.

Two alternative methods, each successful in some of the samples, were employed:

a) 20 μL of the extracted total genomic DNA stained by SYBR Green Nucleic Acid Dye (Sigma-Aldrich) was run on 1.5 % agarose gel (45 min, 70V). Then the gel was visualized on a Herolab UVT-20 BE (Herolab) blue-light (wavelenght 420-500 nm) transilluminator, and a band of DNA just below the loading well was cut out.

   DNA was isolated from the agarose slice using the freeze-squeeze method (Tautz and Renz 1983) – briefly, the structure of the agarose gel was broken by deep freezing for several minutes in liquid nitrogen, and the solution containing DNA was squeezed from it by centrifugation through a glass-wool (Serva) filter, and finally precipitated by ethanol and resuspended in sterile distilled water;

b) 20μL of the stained DNA was run on 1.5% low melting temperature agarose gel (Nu Sieve GTG Agarose, Cambrex Bio Science), visualized by blue light and cut out similarly as in (a). The agarose slice was melted at 65°C for 10 min prior to the polymerase chain reaction (PCR). Finally, a suitable amount (1-3 μL) of the melted agarose with DNA was added directly to the PCR reaction.

2.3.2. Polymerase Chain Reaction (PCR)

Two different parts of the genome were amplified: the ITS1-5.8S-ITS2 part of the rRNA gene area and the chloroplast *rbc*L gene. Primers used for amplification and sequencing are listed in Tab. 4.

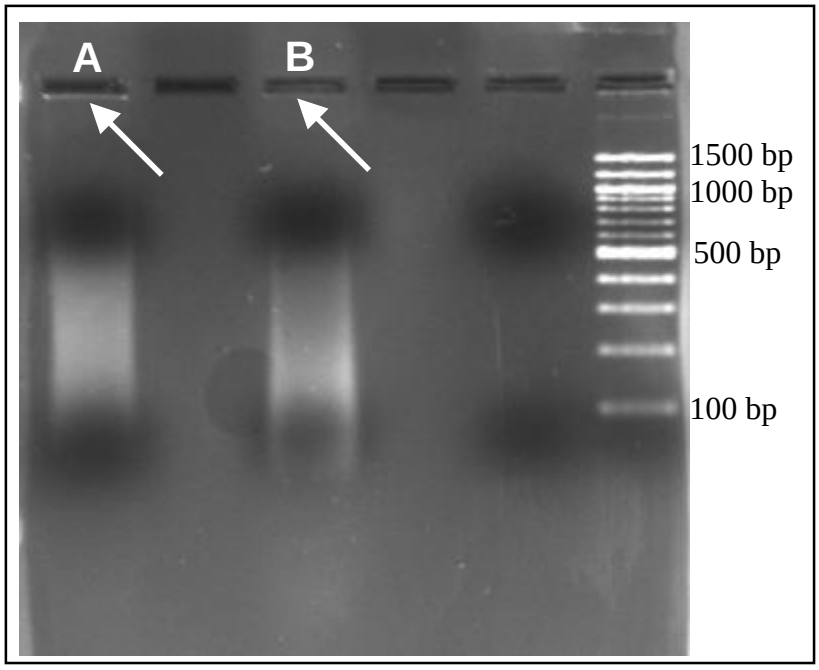

Figure 2. 1.5 % agarose gel with total genomic DNA isolated from the holotypes of *U. flexuosa* subsp. *flexuosa* (A) and *U. flexuosa* subsp. *pilifera* (B), stained by SYBR Green and run at 80 V for 60 min. Most of the DNA is fragmented to 100-500 bp. Small part of unfragmented DNA can be seen at the loading wells (arrows).

Total genomic DNA (5-20 ng) was added to PCR reactions each containing 12.5 μL of the Plain PP Master Mix (2x conc., Top-Bio), 6.25 pmol of each primer, and PCR Water (Top-Bio) up to the final 25 μL.

Samples U1-U14 and P2-4 were amplified at least twice in separate PCR reactions to avoid sequence errors resulting from DNA polymerase mistakes. PCR reactions of *rbc*L genes of E. Leskinen‘ s samples and the type specimen of *Ulva flexuosa* subsp. *flexuosa* Wulfen were completed only once due to problems with amplification of the partly damaged DNA. The work on amplification is still in progress in order to validate the results.

Table 4. Primers used for DNA amplification and sequencing.

Primer	Sequence	Target	Direction
ITS1[1]	5´ TCCGTAGGTGAACCTGCGG 3´	ITS	Forward
ITS4[1]	5´ TCCTCCGCTTATTGATATGC 3´	ITS	Reverse
RH1[1]	5´ ATGTCACCACAAACAGAAACTAAAGC 3´	*rbc*L	Forward
1385r[1]	5´ AATTCAAATTTAATTTCTTTCC 3´	*rbc*L	Reverse
rbc571[2]	5´ TGTTTACGAGGTGGTCTTGA 3´	*rbc*L	Forward
rbc590[2]	5´ TCAAGACCACCTCGTAAACA 3´	*rbc*L	Reverse

[1] – amplification and sequencing; [2] – only sequencing.

PCR amplification was carried out in the Biometra T3000 (Whatman Biometra) and TC-XP Cycler (BIOER Technology) thermo-cyclers.

The genome area containing ITS1, ITS2 and the 5.8S ribosomal subunit was amplified with primers ITS1 and ITS4. The reaction sequence consisted of an initial denaturation step (5 min at 95°C); followed by 1 min at 94°C, 1 min at 52°C and 1 min at 72°C for 35 cycles; and a final 10 min extension at 72°C.

The *rbc*L gene was amplified using primers RH1 and 1385r after Manhart (1994). These primers amplify only the first 1357 bp (95%) of the gene (without primers), excluding the variable 3´ terminus. The reaction profile included initial denaturation at 94°C for 3 min; followed by 1 min at 94°C, 2 min at 45°C and 3 min at 65°C for 35 cycles; and finished by extension for 10 min at 65°C.

Aliquots (2μL) of the PCR products were checked on 1.5 % agarose gel, and the remaining volume was purified using the JETQUICK PCR Purification Spin Kit (Genomed) according to the accompanying manual.

As for the ITS region, most of the products contained non-specific amplified DNA. In such cases, the PCR products of expected length were separated on 1.5 % agarose gel stained by SYBR Green, visualized in blue-light, and cut out from the gel. The DNA was purified from gel by the JETQUICK Gel Extraction Spin Kit (Genomed) according to the manual.

2.3.3. DNA Sequencing

The PCR-amplified products were directly sequenced using primers listed in Tab. 4. The analysis was provided by Macrogen, Inc. and by The Laboratory of Genomics, Biology Centre of the Academy of Sciences of the Czech Republic on ABI 3730 XL (Applied Biosystems) or ABI PRISM 3130 XL (Applied Biosystems) automated sequencers. All products were sequenced on both strands, more than once in independent runs.

2.4. Molecular data analysis

2.4.1. Preparation of DNA sequence data sets

Raw data from the DNA sequencers were analysed and assembled into final nucleotide sequences using the SeqMan 5.06 (Burland 1999) computer programme.

ITS1-5.8S-ITS2 region sequences of the samples of *Ulva flexuosa* from Sweden and Finland (Tab. 2) were kindly provided by E. Leskinen (University of Helsinki).

Additional sequences for phylogenetic calculations were downloaded from the GenBank on-line database (http://www.ncbi.nlm.nih.gov). Suitable items were chosen

from the nucleotide collection using the BLAST alignment algorithm available at the same website, providing several of our original sequences as input data. Too short and too similar sequences (e.g. repeated sequences of a few isolates from one population) were omitted, so that the final dataset included about 50 sequences of each gene from the closest 100 found by BLAST.

For combined analysis of both genes, a new dataset was created only from specimens for that nucleotide sequences of both genes were available. The GenBank accession numbers of them were inferred from published papers (Hayden et al. 2003, Hayden and Waaland 2004), and they are described in Results.

Outgroup taxa were derived from previous studies by Malta et al. 1999, Tan et al. 1999, Hayden and Waaland 2002, Hayden et al. 2003 and Hayden and Waaland 2004.

All GenBank sequences included in this study are listed in Tab. 5.

2.4.2. Alignment

Alignments included in this study were created using five different datasets:

The first two were constructed from the ITS or *rbc*L sequences obtained in this study, sent by E. Leskinen, and from respective sequences chosen from BLAST results (65 ingroup taxa + 2 outgroups for ITS, 61 ingroup taxa + 10 outgroups for *rbc*L).

The following three alignments consisted of our original and E. Leskinen´s sequences, together with taxa with available sequences of both genes (33 ingroup taxa and 3 outgroups). The ITS and *rbc*L sequences were first aligned separately, and then merged into a combined data matrix. Prior to merging, separate phylogenetic analyses of the partial matrices were executed to assess if they can be combined into a single dataset.

The alignments of the analysed nucleotide sets were obtained using Clustal W (included in BioEdit v. 7.0.9.0 – Hall 1999) with default settings.

Initially, the ITS sequence alignments were too variable, and contained a lot of gaps. Thus, they were improved by the MAFFT v. 6 on-line algorithm (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) with default parameters.

Consequently, regions with too many gaps were removed using GBlocks v. 0.91b internet application (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), allowing small gaps to be included in final blocks.

Eventual adjustments of the resulting alignments (deletion of ambiguous sites, setting the correct reading frame of coding sequences) were carried out in BioEdit.

The proportion of parsimony-informative sites, sequence distances and other parameters were evaluated using MEGA v. 4.0.2 (Tamura et al. 2007).

Table 5. Summary of used GenBank sequences.

Taxon	Source	GenBank Accession Numbers
Ulva armoricana	Shimada et al. 2003	AB097630
Ulva californica	Tan et al. 1999	AJ234315
Ulva californica	Hayden et al. 2003	AY260560
Ulva californica	Hayden and Waaland 2004	AY422518
Ulva californica	Hayden and Waaland 2002	AF499667
Ulva californica	Hayden et al. 2003	AY255866
Ulva californica	Hayden and Waaland 2004	AY422555
Ulva californica	Loughnane et al. 2008	EU484415
Ulva compressa	Blomster et al. 1998	AF035350, AY255859
Ulva compressa	Loughnane et al. 2008	EU484397
Ulva curvata	Sherwood et al. 2000	AF189071
Ulva fasciata	Hayden et al. 2003	AY260561, AY255867
Ulva fasciata	Shimada et al. 2003	AB097634
Ulva fasciata	Loughnane et al. 2008	EU484418
Ulva fasciata	Hayden and Waaland 2004	AY422565
Ulva fenestrata	Tan et al. 1999	AJ234316
Ulva fenestrata	Hayden et al. 2003	AY260562, AF499668
Ulva flexuosa	Shimada et al. 2003	AB097644
Ulva flexuosa	Shimada et al. 2003	AB097618
Ulva intestinalis	Tan et al. 1999	AJ000212
Ulva intestinalis	Tan et al. 1999	AJ234299
Ulva intestinalis	Hayden and Waaland 2004	AY422508, AY422552
Ulva intestinalis	Sherwood et al. 2000	AF189070
Ulva intestinalis	Hayden and Waaland 2002	AF499671
Ulva intestinalis	Blomster et al. 1998	AF035342, AY255860
Ulva intestinalis	Leskinen et al. 2004	AJ550760
Ulva intestinaloides	Tan et al. 1999	AJ234303
Ulva lactuca	Tan et al. 1999	AJ234311
Ulva lactuca	Hayden and Waaland 2004	AY422542
Ulva linza	Shimada et al. 2003	AB097648
Ulva linza	Shimada et al. 2007	AB298633
Ulva linza	Blomster et al. 2000	AF185944
Ulva linza	Tan et al. 1999	AJ000203
Ulva linza	Tan et al. 1999	AJ000204
Ulva linza	Hayden et al. 2003	AY260557, AY255861
Ulva linza	Zheng et al. 2008	EU888138
Ulva linza	Ying et al. 2008	DQ813496
Ulva linza	Shimada et al. 2003	AB097620

Table 5. Continued.

Ulva lobata	Hayden et al. 2003	AY260563, AY255868
Ulva lobata	Hayden and Waaland 2004	AY422505
Ulva muscoides	Hayden and Waaland 2004	AY422563
Ulva muscoides	Blomster et al. 1999	AF127170, AY255862
Ulva ohnoi	Hiraoka et al. 2004	AB116035
Ulva pertusa	Shimada et al. 2003	AB097658
Ulva pertusa	Tan et al. 1999	AJ234321
Ulva pertusa	Hayden and Waaland 2004	AY422504
Ulva procera	Hayden and Waaland 2004	AY422521, AY422562
Ulva procera	Hayden et al. 2003	AY260558, AY255863
Ulva procera (as *U. ahlneriana*)	Leskinen and Pamilo 1997	AJ012276
Ulva prolifera	Wang et al. 2008	FJ002301
Ulva prolifera	Zhang et al. 2008	FJ026732
Ulva prolifera	Shimada et al. 2007	AB298309
Ulva prolifera	Tan et al. 1999	AJ234305
Ulva prolifera	Hayden and Waaland 2004	AY422510
Ulva prolifera	Hayden and Waaland 2002	AF499670
Ulva pertusa	Hayden and Waaland 2004	AY422549
Ulva prolifera	Hayden and Waaland 2004	AY422554
Ulva prolifera	Tan et al. 1999	AJ234304, AY255864
Ulva pseudocurvata	Tan et al. 1999	AJ234312, AY255869
Ulva pseudocurvata	Tan et al. 1999	AJ234314
Ulva pseudocurvata	Hayden and Waaland 2004	AY422509, AY422553
Ulva reticulata	Shimada et al. 2003	AB097635
Ulva reticulata	Hayden and Waaland 2004	AY422568
Ulva rigida	Loughnane et al. 2008	EU484395
Ulva rigida	Tan et al. 1999	AJ234319
Ulva rigida	Hayden and Waaland 2004	AY422522, AY422563
Ulva rotundata	Loughnane et al. 2008	EU484406
Ulva scandinavica	Loughnane et al. 2008	EU484412
Ulva scandinavica	Tan et al. 1999	AJ234317, AY255870
Ulva scandinavica	Loughnane et al. 2008	EU484416
Ulva scandinavica	Shimada et al. 2003	AB097629
Ulva spinulosa	Shimada et al. 2003	AB097636
Ulva stenophylla	Hayden et al. 2003	AY260569, AY255874
Ulva taeniata	Hayden and Waaland 2004	AY422566
Ulva taeniata	Hayden et al. 2003	AY262335, AY255875
Ulva taeniata	Tan et al. 1999	AJ234320
Ulva tanneri	Woolcott and King 2000	AY016309
Ulva tanneri (*Chloropelta caespitosa*)	Hayden et al. 2003	AY260556, AY255858
Ulva tanneri	Hayden and Waaland 2004	AY422519

Table 5. Continued.

Ulva tanneri (*Chloropelta caespitosa*)	Hayden and Waaland 2002	AF499672
Ulva sp.	Shimada et al. 2007	AB298464
Ulva sp.	Shimada et al. 2007	AB298465
Ulva sp. (Ythan estuary)	Tan et al. 1999	AJ234308
Ulva sp.	Tan et al. 1999	AJ234323
Ulva sp.	Hayden et al. 2003	AY260559, AY255865
Ulva sp.	Hayden and Waaland 2004	AY422520, AY422561
Ulva sp.	Zheng et al. 2008	FJ374286
Ulva sp.	Zheng et al. 2008	FJ374872
Ulva sp.	Lu et al. 2008	FJ94955
Ulva sp.	Lu et al. 2008	FJ94956
Ulva sp.	Hayden et al. 2003	AY260566, AY255871
Ulva sp.	Hayden et al. 2003	AY260568, AY255873
Ulva sp.	Hayden et al. 2003	AY422515, AY422558
Ulva sp.	Hayden et al. 2003	AY255872
Ulva sp.	Lu et al. 2008	FJ94958
Chlorella vulgaris	Hayden and Waaland 2002	AF499684
Gloeotilopsis planctonica	Friedl and Zeltner 1994	Z28970
Gloeotilopsis planctonica	Hayden and Waaland 2002	AF499681
Kornmannia leptoderma	Hayden and Waaland 2002	AF499677
Monostroma arcticum	Su et al. 2001	AF415171
Myrmecia biatorellae	Hayden and Waaland 2002	AF499685
Percursaria percursa	Hayden et al. 2003	AY260570, AF499674
Pseudendoclonium fucicola	Hayden and Waaland 2002	AF499678
Pseudoneochloris marina	Hayden and Waaland 2002	AF499682
Tellamia contorta	Hayden and Waaland 2002	AF499679
Ulothrix zonata	Hayden and Waaland 2002	AF499683
Ulvaria obscura var. *blyttii*	Hayden et al. 2003	AY260571, AF499673
Umbraulva olivascens	Hayden et al. 2003	AY260564, AY255876

2.4.3. Construction of phylogenetic trees

Phylogenetic analysis was performed using the maximal parsimony (MP), maximal likelihood (ML), and Bayesian methods. MP trees were calculated using MEGA v. 4.0.2, ML trees were obtained by PHYML v. 2.4.4 (Guindon and Gascuel 2003), and Bayesian trees were constructed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). All alignments were analysed by all the three methods.

MP analyses were executed using close-neighbour-interchange (CNI) branch-swapping algorithm with search level 1 (default setting in MEGA). One hundred replicate searches with

randomized initial trees were conducted to avoid local optima of most parsimonous trees. To compare relative support for branches, 1000 bootstrap replications with default parameters were performed. Gaps were coded as missing data, and all bases and base changes were weighted equally.

ML trees were constructed using the general time-reversible substitution model (GTR) with discrete gamma distribution in 6 categories. Gamma shape parameter α as well as proportion of invariable sites were estimated from the dataset (GTR + I + G model), and 1000 bootstrap replications were executed to evaluate the relative support of branches.

Bayesian analyses were performed with a default likelihood model (without weighing of bases or base changes). Two independent Markov chains were run for 1-5 million generations, until the average standard deviation of split frequencies was lower than 0.01 and the potential scale reduction factor (PSRF) of all parameters reached a value between 1.00 and 1.01. Burn-in of ten percent generations allowed stabilisation of the likelihood value, and a 50 % majority rule consensus tree with posterior probabilities of branches was constructed.

Phylogenetic trees were drawn and edited using TreeView v. 1.6.6 (Page 1996).

3. Results

3.1. Chemical and physical analysis of water

Several parameters of water from five sampling localities visited in 2008 were assessed. Their values are shown in Tab. 3.

Table 3. Chemical and physical parameters of water from Czech sampling sites (2008).

Sampling Site	Conductivity [$\mu S.m^{-2}$]	pH	t [℃]	$ANC_{4.5}$ [$mmol.L^{-1}$]	TP [$mg.L^{-1}$]	PO_4-P [$mg.L^{-1}$]	TN [$mg.L^{-1}$]	NH_4-N [$mg.L^{-1}$]
Soos	N/A	N/A	N/A	16.20	0.61	0.47	<0.50	0.05
Hradec Králové	830	8.02	23.50	4.20	1.41	1.26	0.50	0.07
Hlohovec	1550	8.75	22.50	3.83	0.35	0.20	0.80	0.07
Oleksovice	1005	7.57	15.40	5.46	0.06	0.03	<0.50	0.16
Nemilkov	2705	7.24	22.30	3.81	0.14	0.09	<0.50	0.92
Sampling Site	**NO_2-N [$mg.L^{-1}$]**	**NO_3-N [$mg.L^{-1}$]**	**Cl^- [$mg.L^{-1}$]**	**SO_4^- [$mg.L^{-1}$]**	**Ca [$mg.L^{-1}$]**	**Mg [$mg.L^{-1}$]**	**Na [$mg.L^{-1}$]**	**K [$mg.L^{-1}$]**
Soos	0.01	<0.01	448.00	1300.00	39.50	19.60	63.50	21.20
Hradec Králové	0.06	0.70	48.80	127.00	96.90	18.10	41.20	8.60
Hlohovec	0.02	<0.01	122.00	542.00	111.00	119.50	116.00	19.60
Oleksovice	0.02	0.05	84.00	194.00	67.50	75.80	34.20	6.60
Nemilkov	0.01	<0.01	270.00	788.00	121.00	95.60	202.00	142.00

3.2. Morphological studies

All six samples from the Czech Republic, formalin-fixed samples from Poland and Slovakia, and some of the gathered dry herbarium specimens (if needed) were examined, determined and documented as follows. Plates with photographic documentation are given in Appendix.

i. *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne – specimen I (Pl. 1)

Locality : Small, artificially adjusted stream with increased concentration of phosphorus and salts; industrial part of the city of Hradec Králové, Czech Republic; whole season, most abundant in September (2007, 2008).

Collected by author.

Description: Thallus is vividly green, tubular, the main axis is usually inflated and tapering towards basis, 5-20 cm (or more) long and 0.5-3 cm wide, irregularly curved and wrinkled. Sometimes, intensive branching occurs in the basal region of the thallus (Pl. 1 a), microscopic proliferations are present on the whole plant.

Cells in young branchlets are a little elongate, 25-38 x 15-20 μm, arranged in parallel longitudinal rows (Pl. 1 b). In central parts of broader branches and the main axis, most of the cells are rounded-polygonal, 10-20 (25) μm in diameter, arranged irregularly, in short straight or curved rows, or even in small, almost radial groups, sometimes with 2-4 cells within the wall of a common mother cell (Pl. 1 c, e). Chloroplasts are parietal, girdle-shaped, covering most of the cell wall, often forming a hollow cylinder (without bases), with irregular margin (Pl. 1 d). Mostly 2-4 pyrenoids occur in a cell (Pl. 1 e).

The microscopic branches are uniseriate or narrowed towards uniseriate ends, apical cell is globular and usually somewhat bigger than previous cells (Pl. 1. f).

Found floating on the water surface, entangled among macrophytes (together with *Lemna minor*), occasionally forming dense accumulations.

ii. *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne – specimen II (Pl. 2)

Locality: Small, artificially adjusted shallow stream, outlet from an enriched fishpond, increased concentration of salts; near Oleksovice, southern Moravia, Czech Republic; July 2008.

Collected by author.

Description: Thallus is vividly green, tubular, somewhat tapering towards the basis, 10-30 cm (or more) long and 0.1-1 cm wide, irregularly waved and wrinkled, but generally straight (Pl. 2 a). Microscopic branches are abundant on the whole plant, occasional bigger ramifications occur in the basal region (Pl. 2 a).

Cells in smaller branches are more or less rectangular (16-24 x 9-12 μm), arranged in longitudinal rows (Pl. 2 b). In most of the thallus, the cells are polygonal or rounded-polygonal, only 7-16 (22) μm in diameter, arranged irregularly, in short rows, or in small radial-like groups (Pl. 2 c). Chloroplasts are parietal, girdle-shaped, covering major part of the cell wall, often forming a hollow cylinder, with irregular margin (Pl. 2 b). Usually, 2-3 pyrenoids are present in each cell (Pl 2. c).

The microscopic branchlets are usually very thin or uniseriate, ending with a single row of cells, apical cell is widely rounded (Pl. 2 d).

The specimens grew attached to a solid substrate, in clusters, together with emergent macrophytes and *Lemna minor*.

iii. *Ulva flexuosa* Wulfen (Pl. 3)

Locality: Fishpond (Zámecký rybník), Lednice, south Moravia, Czech Republic; September 2007.

Collected by author.

Description: Thallus is vividly green, tubular, 5-10 cm long and 1 cm wide, a little inflated and wrinkled, with mineral incrustations. Neither macroscopic nor microscopic branches are present.

Cells are rounded-polygonal, having 10-18 (22) μm in diameter, arranged irregularly, in short rows, or in almost radial groups (Pl. 3 a). Chloroplasts are parietal, usually forming a hollow cylinder around the inner side of the cell wall, with 2-3 pyrenoids (Pl. 3 b).

Only a small part of this alga (5-10 cm long) was found floating among the littoral vegetation, and the whole specimen was used for microscopic analyses and extraction of DNA.

Similar to *Ulva flexuosa* subsp. *pilifera* I, but no branching was observed, perhaps due to lack of material.

iv. *Ulva* cf. *flexuosa* – specimen I (Pl. 4)

Locality: Fishpond (Hlohovecký rybník) with high concentrations of phosphorus and salts, near Hlohovec, south Moravia, Czech Republic; July 2008.

Collected by author.

Description: Thallus is light to vividly green, tubular in young algae and basal region (Pl. 4 a, d), later unfolding into a leaf-like morphology (Pl. 4 a). Tubes are 10 or more cm long and 0.25-1 cm wide, irregularly waved and wrinkled, narrowing towards basis. Leaf-shaped thalli are 10-15 cm long and 3-10 cm wide, flat but undulated, wrinkled, with curly margin, very fragile, intensively mineral-incrusted (Pl. 4 c). Neither macroscopic nor microscopic branches were observed.

Cells are rounded polygonal or round, 9-18 cm in diameter, organised in longitudinal rows in narrow parts of the tubular thalli, but mostly arranged irregularly, in short curved rows or radial-like groups (Pl. 4 b). Chloroplasts are parietal and cover most of the cell wall. Exact average number of pyrenoids could not be assessed from available material due to intensive granulation of the chloroplast. However, more than one (2-3) body appearing like pyrenoid was observed in some cells.

The algae grew in masses in littoral of the pond and at the boundary of water and wet mud (Pl. 4 e).

v. *Ulva* cf. *flexuosa* – specimen II (Pl. 5)

Locality: Water tank, inside a village, heavily impacted by wastewater, high concentration of salts, Nemilkov, northern Bohemia, Czech Republic; August 2008.

Collected by author.

Description: Thallus is vividly green, leaf-shaped, unbranched, 10-30 cm long and 3-15 cm wide, but occasionally narrowed and wound-up almost into a tube (<1cm wide), inflated, undulated, with irregular and sometimes curly margin (Pl. 5 a), intensively mineral-incrusted (Pl. 5 c).

Cells are rounded or rounded-polygonal, 10-20 cm in diameter, they grow irregularly, in short curved rows, or even in radially arranged groups (Pl. 5 b). Chloroplasts are parietal, at least sometimes forming a hollow cylinder. Usually, 2 pyrenoids are present in a single cell, but sometimes also 1 or 3 pyrenoids per cell were found (Pl. 5 b).

The algae were found floating freely in a small water tank, covering a significant part of its surface (the rest was covered by *Lemna minor*) – Pl. 5 d.

vi. *Ulva flexuosa* subsp. *paradoxa* (C. Agardh) M. J. Wynne – specimen I (Pl. 6)

Locality: Shallow, slowly flowing water, salt marsh Soos, near Františkovy Lázně, western Bohemia, Czech Republic; June 2008.

Collected by author.

Description: Thallus is light green and translucent, tubular, 10 cm and more long and 0.1-0.5 cm wide, more or less irregularly arcuated (Pl. 6 a). Broader branches sometimes occur near to the basis, microscopic branching is abundant throughout the thallus.

Cells in narrow branches are rectangular or quadrungular (11-22 x 10-18 μm), arranged in long rows (Pl. 6 b). In central areas of the thallus, the cells get rather polygonal or rounded-polygonal shape (14-22 μm in diameter), and they are arranged in shorter rows or irregularly (Pl. 6 c). Chloroplasts are parietal, usually cover majority of the cell wall, and possess 2-4 pyrenoids (in each cell) – Pl. 6 b.

The microscopic branchlets of variable size (poly- or uniseriate) are very abundant, narrowed into a uniseriate end, and terminated by an elongated (sometimes conical) cell, approximately 20-25 μm long and 10 μm wide (Pl. 6 d).

The algae grew in dense intestinoid clusters, loosely attached to the bed of the marsh, alone or among macrophytes. The material is very similar to *Ulva flexuosa* subsp. *pilifera* III from Hungary by both macroscopic and microscopic features.

vii. *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne – specimen III (Pl. 7)

Locality: Fishpond (Arturowek), enriched water, Lodz, Poland (2008).

Collected by M. Sitkowska (University of Lodz).

Description: Thallus is vividly green, tubular, 20-30 cm long and 1-3 cm wide, somewhat inflated and tapering towards basis, irregularly curved and wrinkled, minerally incrusted (Pl. 7 a, c). Macroscopic branching is scarce, some parts are unbranched, but usually, incountable microscopic proliferations are present throughout the thallus.

In narrower and marginal parts, cells are rectangular or square (14-24 x 12-20 μm), organized in rows (Pl. 7 d). In central areas of the thalli, they gain rounded-polygonal shape

(10-20 (24) μm in diameter), and the arrangement becomes rather irregular (short curved rows or completely disordered cells) - Pl. 7 b. Chloroplasts are girdle-shaped, with irregular margin, cover most of the cell wall, and possess (1) 2-3 (4) pyrenoids per cell (Pl. 7 b).

The microscopic branches are small, uniseriate, or with several rows of cells, mostly ended by a short row of cells, with an almost spherical or widely rounded terminal cell (Pl. 7 e).

Densely agglomerated specimens of this alga were found free-floating at the sampling locality, covering up to 30 % of the fishpond area. The material is morphologically identical with *Ulva flexuosa* subsp. *pilifera* I.

viii. *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne – specimen IV (Pl. 8)

Locality: Žitava river alluvium, Slovakia; May 2008.

Collected by A. Palaticka.

Description: Thallus is green, tube-shaped, 5 cm long and 0.5 cm wide, irregularly curved, mineral-icrusted (Pl. 8 a). Both macroscopic and microscopic branches are present.

Cells are mostly rounded-polygonal to quadrungular, 9-20 μm in diameter, arranged in rows (in narrower parts – Pl. 8 b) or irregularly, sometimes also in radial-like groups (Pl. 8 c). Small groups of cells within a common mother cell wall occasionally occur. Chloroplasts are girdle-shaped, and usually cover majority of the inner cell wall. 2-4 pyrenoids occur in each cell (Pl. 8 d).

The microscopic branchlets are almost exclusively polyseriate, ended by a single widely rounded or globose apical cell (Pl. 8 e).

Only small part of the thallus fixed by formaldehyde was obtained from O. Skácelová (Moravian Museum, Brno) for microscopic analysis. The alga is very similar to our specimens of *Ulva flexuosa* subsp. *pilifera* I. It differs only by absence of small uniseriate proliferations, which might be due to lack of material.

ix. *Ulva* cf. *flexuosa* – specimen III (Pl. 9)

Locality: Fishpond, Vranovice, southern Moravia, Czech Republic; September 1934.

Collected by V. Krist.

Description: Thallus is vividly green, first tubular, 10-20 cm (or more) or long and 0.1-1 cm wide, then unfolding into a leaf-like morphology in apical parts (2-5 cm wide), irregularly

curved and wrinkled, minerally incrusted (Pl. 9 a). Microscopic branches are present but not very frequent.

Cells are mostly polygonal or rounded-polygonal, 10-16 µm in diameter, arranged in rows or irregularly, especially in central parts of the thallus, where they also form radial-like groups (Pl. 9 d). Chloroplasts girdle-shaped, parietal, covering majority of the cell wall, sometimes forming a hollow cylinder (Pl. 9 b). Pyrenoids were not saved in the herbarium material.

Several not very well preserved uniseriate microscopic branchlets were observed (Pl. 9 c). Although the specimen was originally identified as *Enteromorpha intestinalis*, the cell morphology and arrangement together with presence of branching lead us rather to a determiantion as *Ulva flexuosa*. The alga is very similar to our specimens *Ulva* cf. *flexuosa* I and II.

x. *Ulva* cf. *flexuosa* – specimen IV (Pl. 10)

Locality: Stream, Budapest-Kossuthfalva, Hungary, 19??.

Collected by F. Filárszky.

Description: Thallus is vividly green, tubular to saccate, 5-10 cm (or more) long and 1-3 cm wide, inflated, intensively mineral-incrusted, wrinkled (Pl. 10 a). Microscopic branches are very abundant throughout the thallus.

Cells are polygonal, 10-18 µm in diameter, arranged mostly in irregular rows or groups (Pl. 10 b), more ordered in branchlets. Chloroplasts are parietal, girdle-shaped, and cover most of the cell wall. Pyrenoids were not preserved in the dried specimen.

The microscopic ramuli are thin, often only uniseriate. The exact morphology of apical cells was not certain as they were damaged by desiccation, but they mostly appeared rounded or a little narrowed (Pl. 10 c).

Found by the collector, free-floating among aquatic plants.

The specimen was originally attributed to *Enteromorpha intestinalis* var. *crispa*. In our opinion, it is rather similar to *U. flexuosa* by the intensive branching and the morphology of cells.

xi. *Ulva flexuosa* subsp. *paradoxa* (C. Agardh) M. J. Wynne – specimen II (Pl. 11)

Locality: Stagnant water, near Larga, Romania; August 19??.

Collected by E. Teodorescu.

Description: Thallus is light green, tubular, filiform, 15 cm or more long and 0.1 cm wide, intensively ramified, with differentiated basis (Pl. 11 a, b), minerally incrusted. Branches of variable size are frequent throughout the thallus.

Cells are mostly polygonal or rounded-polygonal, sometimes rectangular, about 10-20 µm in diameter, arranged mostly in longer rows (Pl. 11 c). In central parts of wider thalli, the rows of cells occasionally get somewhat disordered (Pl. 11 d). Chloroplasts are parietal and cover most of the cell wall. Pyrenoids were not found in the dried material.

The specimen is typical with intensive branching up to long uniseriate branchlets that are narrowed into a rather conical apex. It is very similar to the specimen of *U. flexuosa* subsp. *paradoxa* III from Baltic Sea.

The alga was originally determined as *Enteromorpha intestinalis* var. *tubulosa* Kützing. This taxon was later synonymised with *E. flexuosa* (Wulfen ex Roth) J. Agardh by Bliding (1963).

xii. *Ulva* cf. *flexuosa* – specimen V (Pl. 12)

Locality: Flooded dike, near Lednice, south Moravia, Czech Republic; September 19??.

Collected by H. Zimmermann.

Description: Thallus is vividly green, tubular, inflated to saccate, 5-10 cm long and 0.3-1 cm wide, curved and wrinkled, mineral-incrusted (Pl. 12 a, b). The thalli sometimes seem to divide into wide macroscopic branches (Pl. 12 a), microscopic proliferation was not observed.

Cells are mostly polygonal or rounded-polygonal, having 10-18 µm in diameter. They are organized in more or less curved rows in marginal parts of the thallus, but they soon attain irregular arrangement in central parts (Pl. 12 b). Chloroplasts are parietal, covering major part of the cell wall; pyrenoids were not preserved in the desiccated specimen.

This material was originally identified as *Enteromorpha intestinalis* var. *cylindracea* J. Agardh. Our observations of cell morphology and arrangement, and similarity with previous samples lead us to determination as *U.* cf. *flexuosa*.

xiii. *Ulva flexuosa* subsp. *paradoxa* (C. Agardh) M. J. Wynne – specimen III (Pl. 13)

Locality: Stream, Budapest-Kossuthfalva, Hungary; June 19??.

Collected by F. Filárszky.

Description: Thallus is vividly green, tubular, slender, up to several decimeter long and 0.05-0.5 cm wide, occasionally ramified (Pl. 13 a). Microscopic branches are frequent throughout the thallus.

Cells are mostly polygonal or elongated, approximately 9-15 µm in diameter, in narrower parts prolonged and rather rectangular (10-20 x 7-12 µm), arranged mostly in longer rows (Pl. 13 b). In central parts of broader thalli, the rows of cells occasionally get somewhat disordered (Pl. 13 c). Chloroplasts are parietal and cover most of the cell wall. Pyrenoids were not found in the dried material.

The microscopic branchlets (poly- or uniseriate) occur abundantly, ending as narrowing uniseriate rows of prolonged cells with an elongated, roundedly conical apical cell (Pl. 13 d).

The specimen is very similar to *U. flexuosa* subsp. *paradoxa* I. Interestingly, it was found free-floating and attached to macrophytes at the same place as the distinctly separate morphotype *Ulva* cf. *flexuosa* IV.

The alga was originally determined as *Enteromorpha intestinalis* var. *tubulosa* Kützing. This taxon was later synonymised with *E. flexuosa* (Wulfen ex Roth) J. Agardh by Bliding (1963).

xiv. *Ulva* cf. *flexuosa* – specimen VI (Pl. 14)

Locality: Ythan estuary, close to the mouth, Scotland, UK; August 1997.

Collected by R. Taylor.

Description: Thallus is vividly green, tubular, 20-30 cm long and 0.1-0.3 cm wide, irregularly waved, rather smooth, with infrequent ramifications (Pl. 14 a). Microscopic branches are scarcely present.

Cells are mostly polygonal, 9-16 µm in diameter, arranged irregularly or in short rows (Pl. 14 b). Chloroplasts are girdle-shaped, their exact morphology and number of pyrenoids could not be assessed from the dried material.

The microscopic branches are short, ended by a uniseriate row of cells and a rather rounded or rounded-conical apical cell (Pl. 14 c).

The population was free-floating in brackish water of the river estuary. The morphology and arrangement of cells, together with presence of branches, lead us to identification as *Ulva* cf. *flexuosa*.

The following three described specimens represent three different subspecies of *Ulva flexuosa* Wulfen that were checked for comparison at E. Leskinen´s personal herbarium at the University of Helsinki. Only permanent microscopy slides were analysed. Therefore, only data about microscopic morphology of part of the thalli was obtained.

xv. *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne – specimen IV (Pl. 15)

Locality: Lake, Mälaren, Sweden, 1996.

Collected by E. Leskinen.

Description: Thallus is tubular, microscopic branching occurs frequently.
Cells are mostly polygonal or rounded-polygonal, approximately 8-16 μm in diameter, arranged in rows in branches and marginal parts of the thallus, but mostly in shorter curved rows, or disordered (Pl. 15 a). Chloroplasts are girdle-shaped, parietal, and cover most of the cell wall. Number of pyrenoids could not be assessed.
The microscopic branches of variable size (even uniseriate) are ended by a single cell or a uniseriate row, with a widely rounded apical cell (Pl. 15 b).
This material was determined by E. Leskinen, and its anatomy is similar to our specimens of *Ulva flexuosa* subsp. *pilifera*.

xvi. *Ulva flexuosa* subsp. *paradoxa* (C. Agardh) M. J. Wynne – specimen IV (Pl. 16)

Locality: Baltic Sea, Trelleborg, Skåne, Sweden, 1997.

Collected by E. Leskinen.

Description: Thallus is tubular, filiform (about 0.05 cm wide), intensively ramified. The branches of variable size are very abundant throughout the thallus (Pl. 16 a).
Cells are rectangular or quadrungular (9-20 μm in diameter) in narrow branches, but mostly rounded-polygonal, arranged in long rows that become disordered in central parts of broader thalli (Pl. 16 b). Chloroplasts are girdle-shaped, parietal, covering most of the cell wall. Number of pyrenoids could not be assessed.

The microscopic branchlets are poly- or uniseriate, end with a narrowing uniseriate row, and they are terminated by a rounded-conical (sometimes prolonged) apical cell (Pl. 16 c).

This material was determined by E. Leskinen and its anatomy is very similar to our specimen *U. flexuosa* subsp. *paradoxa* II, and generally also to *U. flexuosa* subsp. *paradoxa* I and III that have conspicuously wider and less ramified main axis of the thallus.

xvii. *Ulva flexuosa* subsp. *flexuosa* Wulfen (Pl. 17)

Locality: Sea (Skagerrak), Orust, Sweden, 1995.

Collected by E. Leskinen.

Description: Thallus is tubular, filiform, up to 0.05 cm wide. Branches are present (Pl. 17 a), but not as frequent as in *U. flexuosa* subsp. *paradoxa* III.

Cells are mostly rectangular and elongated (18-30 x 12-20 μm), sometimes polygonal, organised in long parallell longitudinal rows, only occasionally disordered (Pl. 17 b). Chloroplasts are girdle-shaped, parietal, but usually not big enough to form a complete hollow cylinder. Number of pyrenoids per cell could not be assessed.

The branches are mostly microscopic but polyseriate, ended by a uniseriate row of cells with a narrowed, roundedly conical apex (pl. 17 c).

This specimen was determined by E. Leskinen.

The microscopic analysis of holotypes of *Ulva flexuosa* subsp. *pilifera* (Kütz.) M. J. Wynne and *U. flexuosa* subsp. *flexuosa* Wulfen did not bring satisfactory results – the old herbarium material suffered serious damage during the long time of desiccation, cells and chloroplasts were misshaped and shrunken. Better photographs and descriptions of the same material are available in the work of Bliding (1963). Macroscopic photographs of the specimens are given in Pl. 18 and 19.

3.3. Molecular and phylogenetic analysis

3.3.1. DNA sequences

During our study, ITS region sequences of 10 different samples and *rbc*L sequences of 12 samples of *Ulva* were obtained as listed in Tab. 1 in Introduction.

After assembly, the ITS sequences were between 546-610 bp long without primers, and the coding *rbc*L sequences were all 1320 bp long.

3.3.2. Analysis of ITS data

Published data obtained from GenBank (BLAST) and our sequences were combined to give a 444-bp alignment (after deletion of ambiguously aligned regions) with 146 parsimony-informative sites and 235 invariable positions. The 5.8S rRNA gene sequences of our samples of *Ulva* (cf.) *flexuosa* included in Tab. 2 were identical as for coded polypeptides, and they differed in 5-6 coded amino-acids from the outgroups and in 1-2 amino-acids from several GenBank sequences of more distant ingroup taxa. On the other hand, the ITS1 and ITS2 non-coding regions were variable among different taxa.

The MP analysis resulted into 239 most parsimonous trees (length [l] = 478, consistency index [CI] = 0.638, retention index [RI] = 0.850, rescaled consistency index [RCI] = 0.542) that were merged into a 50% majority rule consensus tree. A single ML tree was found (-lnL = 2829.442), and the Bayesian analysis produced a 50% majority consensus of two trees from the independent runs.

All phylogenetic analyses produced trees of a roughly similar topology. The Bayesian tree with mapped consensual branches from the MP and ML trees is shown in Fig. 3.

The tree is rooted with *Gloeotilopsis planctonica*, and a second outgroup is *Monostroma arcticum*.

In the MP and Bayesian analysis, all our samples of *Ulva* (cf.) *flexuosa* fell into one clade with 57 % bootstrap (BP) and 0.63 Bayesian support (BI). This clade divides into a highly supported subclade of all *U. flexuosa* subsp. *paradoxa* samples (MP BP = 94 %, BI = 0.95) and two additional sister (MP BP = 55%, BI = 0.94) subclades: one includes all *U. flexuosa* subsp. *pilifera* specimens together with several samples of *U.* (cf.) *flexuosa*, and the second consists of *U. flexuosa* subsp. *flexuosa* and *U.* cf. *flexuosa* VI from Scotland. This organisation is also strongly supported (MP BP $\geq$ 89 %, BI $\geq$ 0.92).

Interestingly, in the ML tree, the clade of *U. flexuosa* subsp. *paradoxa* fell into a sister lineage to the resting *U. flexuosa*. However, this topology was relatively poorly supported (ML BP = 46 %).

The rest of the tree includes taxa in various positions, with a sister clade to *U. flexuosa* consisting mainly of GenBank sequences labelled as *U. linza*, *U. prolifera* and *U. californica*. This clade also contains one sequence labelled *´Ulva flexuosa´* from the Ryukyu Islands in Japan. More distant branches involve e.g. *U. intestinalis*, *U. lactuca*, *U. lobata*, *U. pertusa*, etc.

A matrix of sequence distances was calculated from the alignment. Pairwise distances of individual sequences from the *U. flexuosa* clade are shown in Tab. 6.

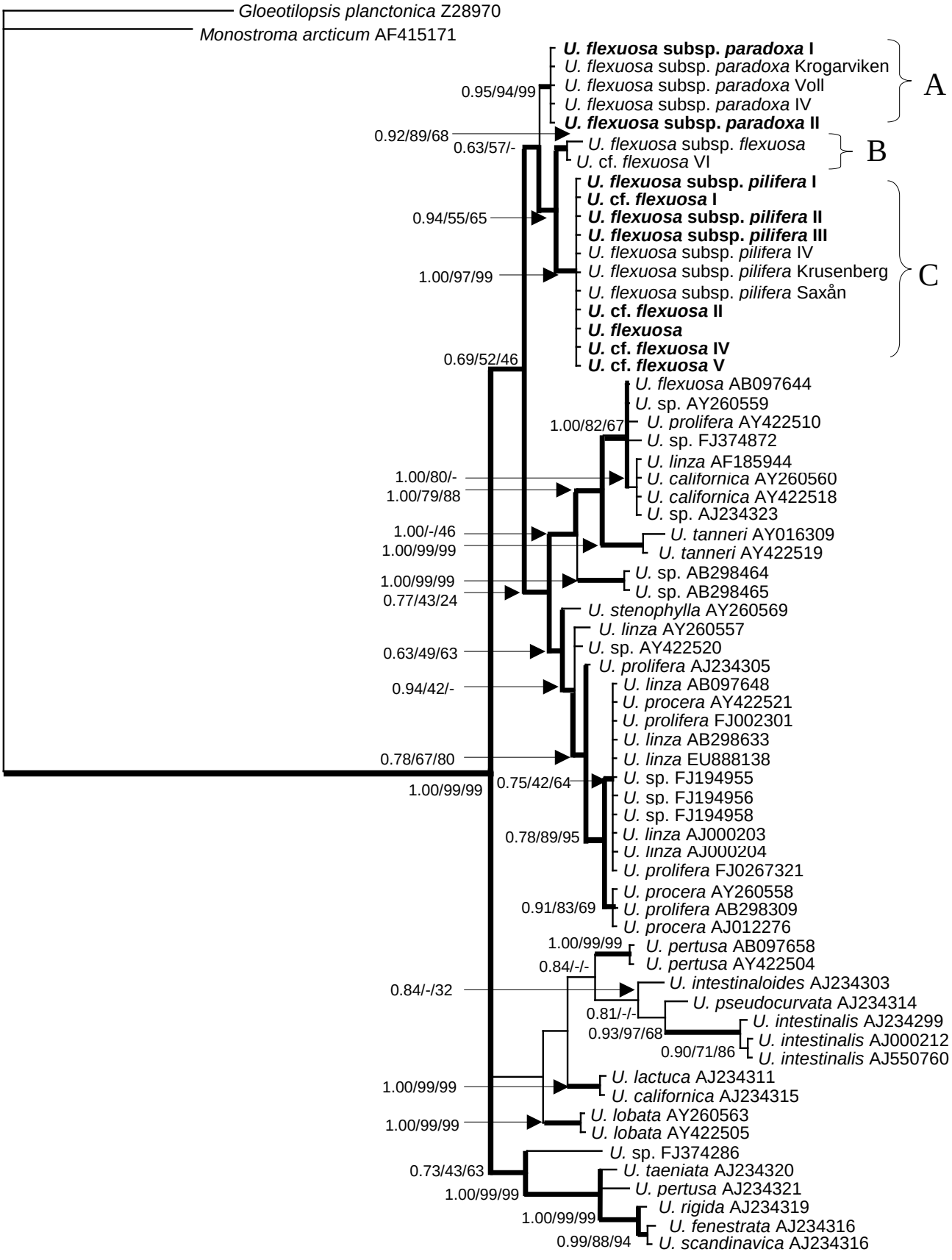

Figure 3. Bayesian tree based on ITS sequences. Bayesian probabilities, MP and ML bootstrap values (1000 replications in all analyses) are given at the nodes in this shape: Bayes/MP/ML. Our original sequences are written in bold font. Sequences are labelled with taxon name and GenBank accession number (if available). Branches present in Bayesian, MP and ML tree are drawn boldly (except final single-species branches). The tree is rooted by *Gloeotilopsis planctonica*, and branch lengths are proportional to sequence change. Clade A includes all *Ulva flexuosa* subsp. *paradoxa* samples, clade B consists of *U. flexuosa* subsp. *flexuosa* and *U.* cf. f*lexuosa* VI. All *U. flexuosa* subsp. *pilifera* samples are clustered in clade C together with further *U.* (cf.) *flexuosa* samples.

3.3.3. Analysis of *rbc*L data

Our original *rbc*L data were combined with a set of close sequences from GenBank (BLAST). The resulting alignment was 1320 bp long, with 341 parsimony-informative sites and 847 conserved positions.

The aligned region did not involve any indels. In our *Ulva flexuosa* sequences, there were no differences as for the coded polypeptide amino-acid sequences. However, in terms of the whole alignment, 22 % of the amino-acid sites were variable.

In MP analysis, 53 equally parsimonous trees were found (l = 1213, CI = 0.519, RI = 0.704, RCI = 0.366) and a 50 % majority rule consensual tree was created. ML analysis produced a single tree (-lnL = 7689.739), and two trees from the Bayesian independent runs were merged using the 50 % majority consensus rule.

Topology of the trees obtained by different algorithms was generally congruent. The Bayesian tree with mapped consensual branches from the MP and ML analyses is depicted in Fig. 4.

Table 6. Sequence distances among *U.* (cf.) *flexuosa* ITS sequences.

		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	***Ulva flexuosa* subsp. *paradoxa* I**	0.000	0.000	0.000	0.000	0.021	0.013	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
2	***Ulva flexuosa* subsp. *paradoxa* Krogarviken**		0.000	0.000	0.000	0.021	0.013	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
3	***Ulva flexuosa* subsp. *paradoxa* VoII**			0.000	0.000	0.021	0.013	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
4	***Ulva flexuosa* subsp. *paradoxa* III**				0.000	0.021	0.013	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
5	***Ulva flexuosa* subsp. *paradoxa* II**					0.021	0.013	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
6	***Ulva flexuosa* subsp. *flexuosa***						0.011	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021
7	***Ulva* cf. *flexuosa* VI**							0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011
8	***Ulva flexuosa* subsp. *pilifera* I**								0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	***Ulva* cf. *flexuosa* I**									0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10	***Ulva flexuosa* subsp. *pilifera* II**										0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
11	***Ulva flexuosa* subsp. *pilifera* III**											0.000	0.000	0.000	0.000	0.000	0.000	0.000
12	***Ulva flexuosa* subsp. *pilifera* IV**												0.000	0.000	0.000	0.000	0.000	0.000
13	***Ulva flexuosa* subsp. *pilifera* Krusenberg**													0.000	0.000	0.000	0.000	0.000
14	***Ulva flexuosa* subsp. *pilifera* Saxån**														0.000	0.000	0.000	0.000
15	***Ulva* cf. *flexuosa* II**															0.000	0.000	0.000
16	***Ulva flexuosa***																0.000	0.000
17	***Ulva* cf. *flexuosa* IV**																	0.000
18	***Ulva* cf. *flexuosa* V**																	

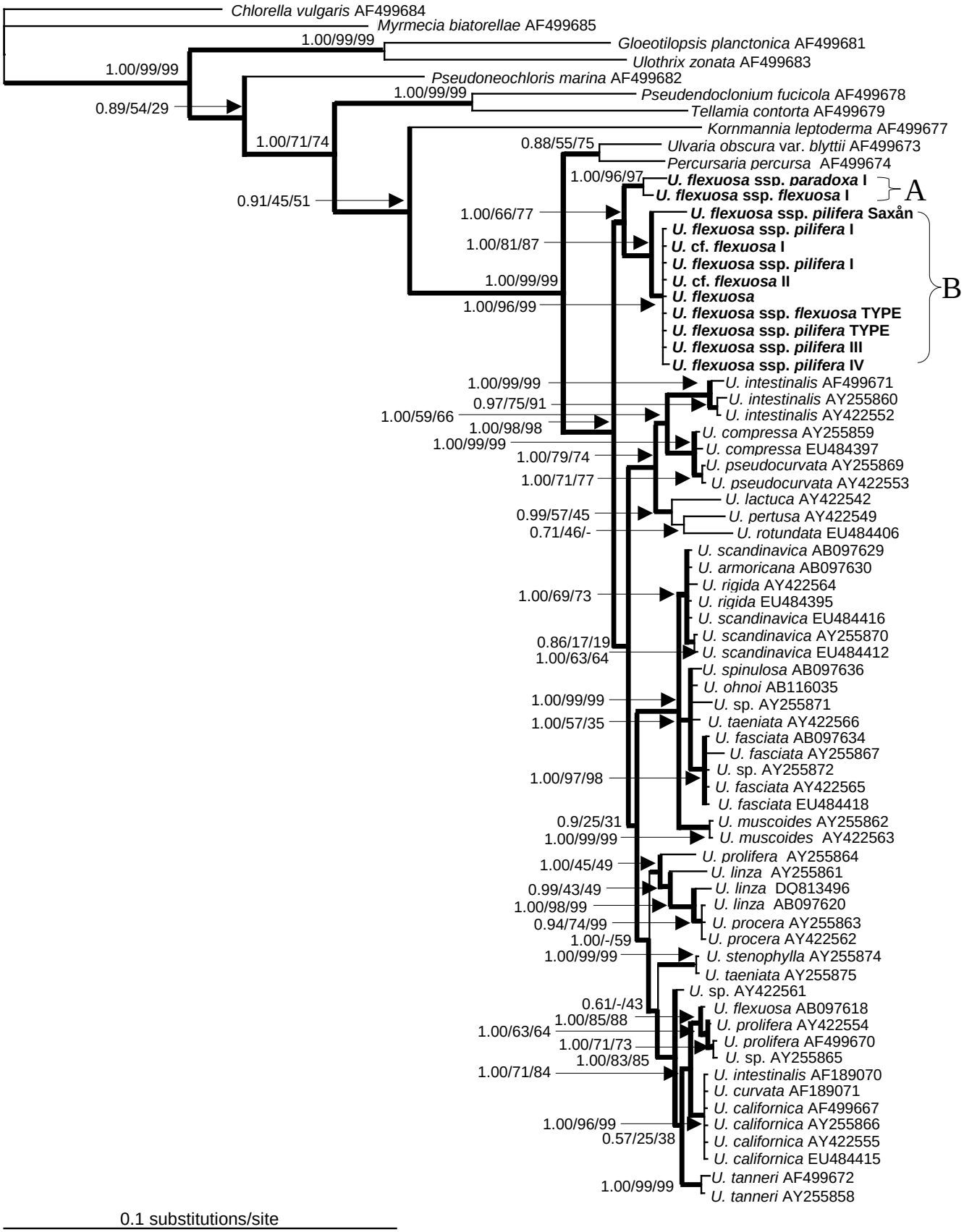

Figure 4. Bayesian tree based on *rbc*L sequences. Bayesian probabilities, MP and ML bootstrap values (1000 replications in all analyses) are given at the nodes in this shape: Bayes/MP/ML. Our original sequences are written in bold font. Branches present in Bayesian, MP and ML tree are drawn boldly (except final single-species branches). Sequences are labelled with taxon name and GenBank accession number (if available). The tree is rooted by *Chlorella vulgaris*, and branch lengths are proportional to sequence change. Clade A includes *Ulva flexuosa* subsp. *paradoxa* I and *U. flexuosa* subsp. *flexuosa.* All *U. flexuosa* subsp. *pilifera* samples are clustered in clade B together with further *U.* (cf.) *flexuosa* sequences.

The tree is rooted with *Chlorella vulgaris*. Furtherly, *M. biatorellae* (Trebouxiophyceae) and species of Ulvophycean genera other than *Ulva* (*Gloeotilopsis planctonica, Ulothrix zonata, Pseudoneochloris marina, Pseudendoclonium fucicola, Tellamia contorta* and *Kornmannia leptoderma*) are also distant from ingroup taxa. A clade formed by *Ulvaria obscura* var. *blyttii* and *Percursaria percursa* is divergent from the remaining outgroup taxa and in ML and Bayesian analysis, it is even sister to the *Ulva* clade (ML BP = 99 %, BI = 1.00).

All samples of *U. flexuosa* examined in our study consistently form a monophyletic clade (MP BP = 66 %, BI = 1.00, ML BP = 77 %). It is divided into two sister clades, one consists of all sequenced *U. flexuosa* subsp. *pilifera* specimens and several *U.* (cf.) *flexuosa* samples (MP BP = 81 %, BI = 1.00, ML BP = 87 %), and the second one includes *Ulva flexuosa* subsp. *flexuosa* from Sweden and *U. flexuosa* subsp. *paradoxa* I (Soos, Czech Republic) – MP BP = 96 %, BI = 1.00, and ML BP = 97 %.

Importantly, both analysed holotypes – *U. flexuosa* subsp. *pilifera* and *U. flexuosa* subsp. *flexuosa* – fell into the same clade, among isolates of *U. flexuosa* subsp. *pilifera*. In fact, *rbc*L sequences of the type specimens were totally identical.

Remaining sequences of *Ulva* are grouped in a single clade sister to *U. flexuosa* in this tree. This clade involves a lot of different species, including a cluster of three *U. intestinalis* specimens, and also a single sequence labelled *´Ulva flexuosa´* from a Japanese sample.

A matrix of sequence distances was calculated from the alignment. Pairwise distances of individual *rbc*L sequences from the *U. flexuosa* clade are shown in Tab. 7.

Table 7. Sequence distances among *rbc*L sequences of *Ulva* (cf.) *flexuosa*.

		2	3	4	5	6	7	8	9	10	11	12
1	***Ulva flexuosa* subsp. *paradoxa* I**	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.024	0.018	0.007
2	***Ulva flexuosa* subsp. *pilifera* I**		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.001	0.014
3	***Ulva* cf. *flexuosa* I**			0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.001	0.014
4	***Ulva flexuosa* subsp. *pilifera* II**				0.000	0.000	0.000	0.000	0.000	0.011	0.001	0.014
5	***Ulva* cf. *flexuosa* II**					0.000	0.000	0.000	0.000	0.011	0.001	0.014
6	***Ulva flexuosa***						0.000	0.000	0.000	0.011	0.001	0.014
7	***Ulva flexuosa* subsp. *flexuosa* TYPE**							0.000	0.000	0.011	0.001	0.014
8	***Ulva flexuosa* subsp. *pilifera* TYPE**								0.000	0.011	0.001	0.014
9	***Ulva flexuosa* subsp. *pilifera* III**									0.011	0.001	0.014
10	***Ulva flexuosa* subsp. *pilifera* Krusenberg**										0.012	0.019
11	***Ulva flexuosa* subsp. *pilifera* IV**											0.014
12	***Ulva flexuosa* subsp. *flexuosa***											

3.3.4. Combined data analysis

Acquired alignment was 1790 bp long and contained 223 parsimony-informative sites and 1459 conserved positions. The coding regions did not include any indels.

MP analysis of the alignment data produced 14 most parsimonous trees, that were collapsed into a 50 % majority rule consensus tree (l = 781, CI = 0.542, RI = 0.714, RCI = 0.387). A single tree was found by ML algorithm (-lnL = 6771.641), and a 50 % majority rule consensus tree was created from the two resulting trees from Bayesian analysis.

As the topology of final trees from all three types of analyses was very similar, the Bayesian tree, with consensual branches from MP and ML cladograms mapped onto it, is shown in Fig. 5.

The tree is rooted with *Percursaria percursa*, and two additional outgroup taxa, *Umbraulva olivascens* and *Ulvaria obscura* var. *blyttii*, are included.

Our samples of *Ulva* (cf.) *flexuosa* cluster together in a separate clade (MP BP = 47 %, BI = 0.99, ML BP = 64 %). This cluster is divided into two subclades exactly the same way as in previous analysis of *rbc*L data (part 3.3.3.) with consistently strong relative branch support from all analyses (MP BP ≥ 98 %, BI = 1.00, ML BP = 99,9 %).

A sister clade to *U. flexuosa* contains several species such as *U. linza*, *U. prolifera*, *U. californica*, etc. Another group of taxa, sister to the clade that includes all previously mentioned taxa, involves *U. fenestrata*, *U. lobata*, *U. compressa*, and also *U. intestinalis*.

Phylogenetic trees (not shown here) had been also computed from the separate ITS and *rbc*L data matrices to check their congruence, before they were merged. Their topologies were usually quite similar to the resulting tree of the combined analysis, but they had lower relative support values of some branches. Some of the higher branches had a tendency to swap in different types of analyses. The main dissimilarity was that in analyses of the partial ITS matrix, the clade of *U. flexuosa* subsp. *flexuosa* and *U. flexuosa* subsp. *paradoxa* I was sometimes in a clade sister to the remaining *U. flexuosa* sequences (together with other species), but the support of such topology was quite low (ML BP = 42%, MP BP = 75%). This pattern was similar as previously in the separate analysis of ITS data described in subchapter 3.3.2. Therefore, the topology of higher branches of the combined tree may not be taken as absolutely accurate.

Combined sequence distances were calculated from the alignment. Pairwise distances of individual *rbc*L+ITS sequences from the *U. flexuosa* clade are shown in Tab. 8.

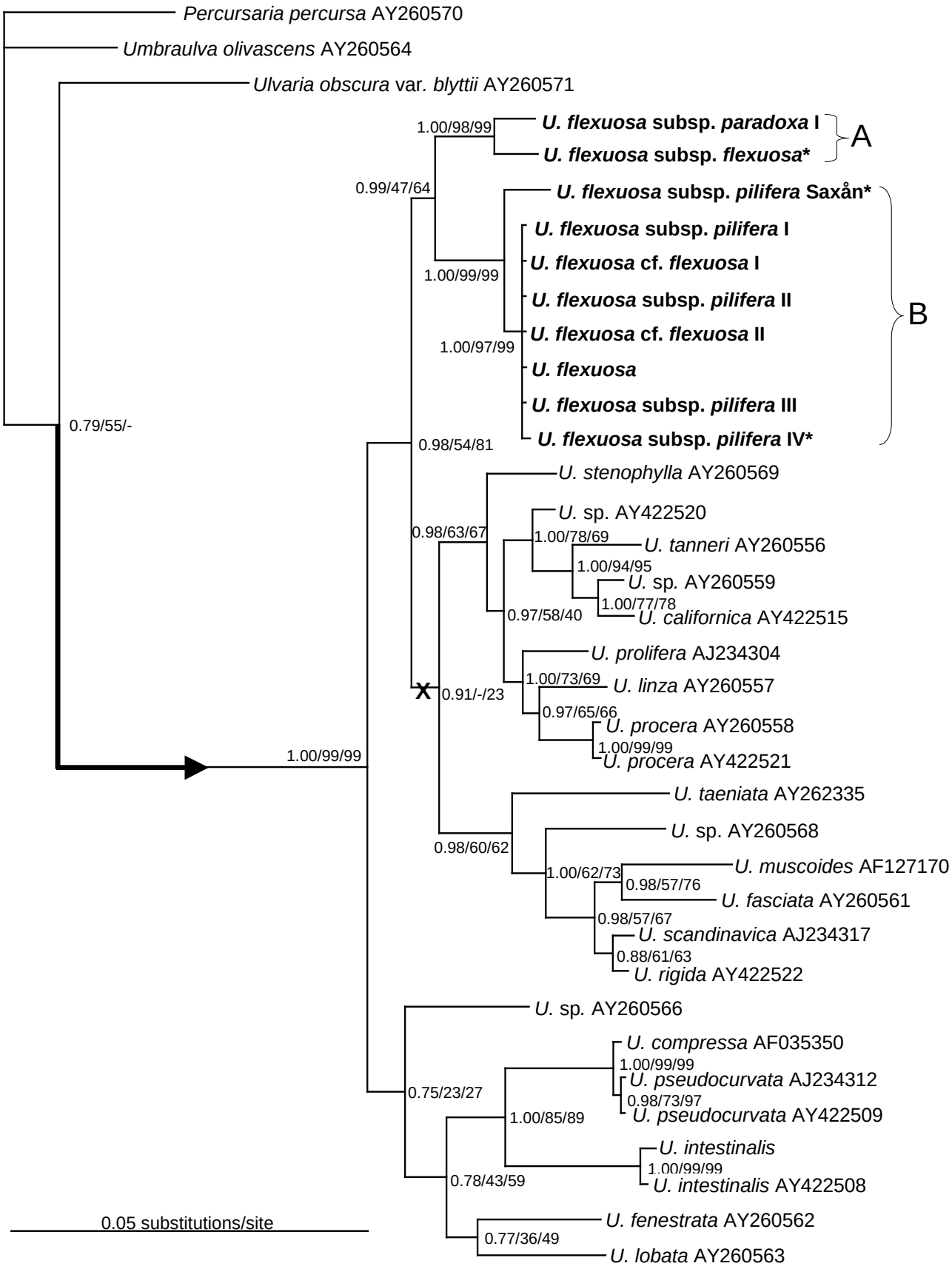

Figure 5. Bayesian tree based on combined ITS and *rbc*L sequences. Bayesian probabilities, MP and ML bootstrap values (1000 replications in all analyses) are given at the nodes in this shape: Bayes/MP/ML. Our original sequences are written in bold font. Sequences combined from our original *rbc*L part and previously known ITS part are marked with asterisk. All branches starting from the bold arrow were present in all trees except the branch marked with ´X´, which was undifferentiated in MP tree. Sequences are labelled with taxon name and GenBank accession number of ITS sequence. The tree is rooted by *Percursaria percursa*. Branch lengths are proportional to sequence change. Clade A includes *Ulva flexuosa* subsp. *paradoxa* I and *U. flexuosa* subsp. *flexuosa.* All *U. flexuosa* subsp. *pilifera* samples are clustered in clade B.

Table 8. Sequence distances among *rbc*L+ITS sequences of *Ulva* (cf.) *flexuosa*.

		2	3	4	5	6	7	8	9	10
1	***Ulva flexuosa* subsp. *paradoxa* I**	0.023	0.023	0.023	0.023	0.023	0.023	0.027	0.024	0.010
2	***Ulva flexuosa* subsp. *pilifera* I**		0.000	0.000	0.000	0.000	0.000	0.008	0.001	0.022
3	***Ulva* cf. *flexuosa* I**			0.000	0.000	0.000	0.000	0.008	0.001	0.022
4	***Ulva flexuosa* subsp. *pilifera* II**				0.000	0.000	0.000	0.008	0.001	0.022
5	***Ulva* cf. *flexuosa* II**					0.000	0.000	0.008	0.001	0.022
6	***Ulva flexuosa***						0.000	0.008	0.001	0.022
7	***Ulva flexuosa* subsp. *pilifera* III**							0.008	0.001	0.022
8	***Ulva flexuosa* subsp. *pilifera* Krusenberg**								0.009	0.026
9	***Ulva flexuosa* subsp. *pilifera* IV**									0.022
10	***Ulva flexuosa* subsp. *flexuosa***									

4. Discussion

During our work on this study, we successfully employed both morphological and molecular assessment as two different but complementary tools for taxonomic research. Our main aim was to determine the identity of populations of a green-algal genus *Ulva* in the Czech Republic, which has been enigmatic for a long time.

Both methodical approaches have consistently shown that all collected samples (including several specimens from similar localities throughout Europe) should be assigned to the species *Ulva flexuosa* Wulfen.

According to a respected monography of Ulvales by Bliding (1963), *U. flexuosa* can be distinguished from other taxa the by presence of more than one (but not many) pyrenoids per cell, size and arrangement of cells (in typical rows at least in some parts of thallus), and occurrence of ramification or proliferation.

All these features were usually found in our specimens. In desiccated material, the number of pyrenoids could not be confirmed, since they were damaged or lost by drying. However, the overall morphology and anatomy of the thalli was quite similar to other samples, so that they could be identified at least as *U.* cf. *flexuosa*. Similarly, a couple of specimens did not show any branching (Pls. 4, 5). Despite that, they were similar to the remaining material of *U. flexuosa* by other previously mentioned characters. As the material did not include plants of every age, they perhaps represented only a developmental stage or an environmentally induced form of thallus.

Importantly, all sequenced specimens were clustered together (and with other available sequences of different *U. flexuosa* subspecies) in respective phylogenetic analyses (Figs. 3-5). This validates the classical morphological concept of *U. flexuosa sensu lato* (Bliding 1963), and confirms our determination.

The only two freely available sequences of *´Ulva flexuosa´* that were not included in this clade, belong to samples from Japan, which probably represent taxa different to European *U. flexuosa sensu* Bliding (1963).

In history, part of the contemporary *U. flexuosa* used to be included in another taxon *U. intestinalis* Linnaeus. Wærn (1952) notes that most probably *Enteromorpha pilifera* Kützing (an older synonym of *U. flexuosa* subsp. *pilifera* (Kütz) M. J. Wynne) was included even in the collections from Uppsala by Linnaeus himself as a freshwater form of *U. intestinalis*. The historical confusion between these two taxa is discussed in detail, and the author provides several different examples of mixing up *U. intestinalis* freshwater populations with *E. pilifera*.

In addition, another taxon, *Enteromorpha intestinalis* var. *tubulosa* Kützing, was also synonymized with *U. flexuosa* by Bliding (1963).

In today´s view, all these types certainly belong to *U. flexuosa*. Most importantly, they possess more than one pyrenoid per cell, while *U. intestinalis* has only one, but they are also typical with numerous branches and arrangement of cells as it was described above.

Unfortunately, these taxonomical discussions very probably have contributed to misidentifications of species also in the Czech Republic.

In accordance with the taxonomical view of his time, Hansgirg (1892) included only *U. intestinalis* with two varieties (var. *crispa* and var. *tubulosa*) in his compendium. Later, some authors stuck to this approach. For example, Wolgemuth et al. (1984) described several populations of *U. intestinalis* from the area of Třebíč. In their discussion of the species identity, they admitted to have doubts about their determination, but they were not able to assign it to description of any other known taxon.

We had an opportunity to study two different historical herbarium specimens collected in the Czech Republic that were originally identified as *U. intestinalis*. Both of them were found at localities in southern Moravia close to two of our sampling localities (Hlohovec, Lednice).

First of them, collected by Krist in 1934 from a fishpond in Vranovice, showed many characteristic features of *U. flexuosa*, including microscopic branching and cell organization.

The same can be stated about the second herbarium specimen from Lednice (Zimmermann, 19??), originally determined as *Enteromorpha intestinalis* var. *cylindracea* J. Agardh.

Since the material from Lednice was successfully sequenced (ITS) and fell among *U. flexuosa* subsp. *pilifera* in phylogenetic analysis (Fig. 3), it is most probable that these collections belong to *U. flexuosa*.

Phylogenetic distance of our samples from *U. intestinalis* is further proven by the position of sequences of this species in a separate lineage in the obtained cladograms (Figs. 3-5).

There is a similar situation concerning another herbarium specimen from a stream in Budapest, Hungary (leg. F. Filárszky, 19??) identified as *E. intestinalis* var. *crispa*. Our observations of anatomy and sequencing of the ITS region again clearly assign it to *U. flexuosa*.

Not all researchers did stick to the outdated determination concept in all cases. *U. flexuosa* was prevously referred to be present in the Czech Republic e.g. by Skácelová (2004) or Marvan et al. (1997). Nevertheless, these authors also describe *U. flexuosa* to be rather an additional taxon to the traditionally known *U. intestinalis*.

Marvan et al. (1997) even propose *U.* cf. *flexuosa* to be a non-indigenous and potentially invasive species, and they attribute its spreading to human-caused enrichment of certain habitats by salts.

Such concept is not in accordance with our results. We agree that the species is nowadays often found at secondary human-impacted localities with high concentration of chloride and sulphate ions (Tab. 3). Normal concentrations of these ions in surface freshwaters are usually lower (ones to tens of $mg.L^{-1}$ of chlorides and tens or low hundreds of $mg.L^{-1}$ of sulphates – Pitter 1999). However, given the identification of historical and presently collected specimens, we rather suggest that *U. flexuosa* is an expanding but indigenous species.

Still, this particular species has been reported to be able to form massive agglomerations of biomass (Lougheed and Stevenson 2004), and it should be monitored as a potential fouling species in human-influenced waters.

Interestingly, Lederer et al. (1998) reported *U. flexuosa* to occur at one of our sampling localities at the Soos salt marsh near Františkovy Lázně. These authors used a modern determination concept congruent with ours. Their samples were similar to our *U. flexuosa* subsp. *paradoxa* I from the same locality. This subspecies is typical with very slender (sometimes filiform) and intensively ramified thalli, rectangular cells ordered in quite long rows, and proliferations ended by a narrowed apical cell.

Older authors often identified specimens from this area as *U. intestinalis* (Hansgirg 1892, Brabez 1941, and others), which may have been due to older taxonomic view as it was discussed before.

Further occurence of similar types in inland habitats has been documented in our study. Herbarium specimens of *´E. intestinalis* var. *tubulosa´* collected by Teodorescu (19??) in stagnant water near Larga, Romania, and by Filárzsky (19??) near Budapest, Hungary (together with another type that will be described later), were examined. Both of them showed anatomical features very similar to the material from Soos (Pls. 11 and 13), and also to a specimen of *U. flexuosa* subsp. *paradoxa* from Baltic Sea (Pl. 16) that was included for comparison. Their morphological identity was confirmed by the position in a separate monophyletic clade in the constructed phylogenetic trees (Fig. 3).

Although Bliding (1963) describes the subspecies *paradoxa* as a marine taxon in Europe, he refers to its occurrence in almost freshwater conditions in the northern Baltic Sea. Our results indicate that it may also grow in inland habitats with increased concentration of salts.

Lougheed and Stevenson also reported this taxon as an exotic invader in Lake Michigan, USA. In our opinion, this should be furtherly proven by molecular analysis and comparison with indigenous taxa in America.

Recently, a suggestion has appeared (Kaštovský et al. 2006) that expanding populations of *Ulva* in the Czech Republic may belong to a non-indigenous species *U.* cf. *linza*.

This is not in agreement with any of our findings. *U. linza* according to Bliding (1963) possesses almost exclusively only one pyrenoid per cell, and it usually forms flat and silky, spirally wound, crisp, and mostly unbranched thalli at exposed marine coast. Moreover, all available sequences of *U. linza* were distant from the sequences of our specimens in all phylogenetic analyses (Fig. 3-5).

As the authors themselves doubt their identification, we consider it to be a mistake, perhaps caused by the flat and curly macroscopic morphology of some *U. flexuosa* thalli (also in our samples – Pls. 4, 5).

Finally, there are historical reports of *U. prolifera* (Fischer 1920, Zapletálek 1932, Brabez 1941) from the Czech Republic. These data were neither confirmed nor disproven by our results.

Although it cannot be excluded that other taxa may occur inside borders of the Czech Republic, our results lead us to suggest *U. flexuosa* to be the most common and apparently indigenous species in the area.

One of our clearest results is the close genetic relationship among all collected fresh specimens, with exception of the previously described sample from Soos. As for the chosen molecular markers, these samples are identical (Figs. 3-5, Tabs. 6-8.).

Two of the respective specimens (collected in Hradec Králové and Oleksovice, in small streams) were determined with confidence up to the subspecies level as *U. flexuosa* subsp. *pilifera*. According to Bliding (1963) and Koeman and van den Hoek (1984), this subspecies is typical with ramified, tube-shaped thalli, irregularities in cell arrangement, rounded-polygonal shape of cells, and mostly also with abundant uniseriate proliferations terminated by a globose apical cell (Pls. 1, 2, 7, 8, 15).

Interestingly, further two foreign samples examined by us (collected by Małgorzata Sitkowska from a fishpond in Lodz, Poland, and E. Leskinen from a lake, Mälaren, Sweden), and further samples collected and determined by E. Leskinen (from lake in Mälaren and river Saxån, Sweden) were also identified as *U. flexuosa* subsp. *pilifera* and fell into the same clade (Fig. 3).

Moreover, this group included also sequences of *Ulva flexuosa* from a fishpond in Lednice (coll. by author, 2007), *U.* cf. *flexuosa* I from a fishpond in Hlohovec (coll. by author, 2008), *U.* cf. *flexuosa* II from a water tank in Nemilkov (coll. by author, 2008), *U.* cf. *flexuosa* IV from a stream in Budapest, Hungary (leg. F. Filárszky 19??), and *U.* cf. *flexuosa* V from a dike near Lednice (leg. H. Zimmermann, 19??). All these samples were examined for morphology and anatomy, and they showed shape and arrangement of cells typical of *U. flexuosa* subsp. *pilifera*. Microscopic branching was observed only in the material from Budapest.

A sample obtained from the Žitava alluvium, Slovakia (leg. A. Palaticka 2008) was morphologically almost identical to the previous *U. flexuosa* subsp. *pilifera* specimens, but it could not be sequenced due to fixation by formaldehyde.

Finally, a herbarium sample from a fishpond in Vranovice (leg. Krist 1934) resisted DNA sequencing. However, it was morphologically similar to the previous *U.* cf. *flexuosa* specimens, and possessed some microscopic branches.

It should be emphasized, that although the specimens determined as *U.* cf. *flexuosa* I-V showed typical anatomical features of *U. flexuosa* subsp. *pilifera*, some of them consisted of unbranched, inflated, saccate, or even flat and leaf-like thalli. Such morphology has not been referred to this subspecies before.

Nevertheless, given the obvious similarity of molecular data, anatomical features, and evident origin of some of the leaf-like thalli by unfolding from a tube, we think that all

these specimens should be regarded as *U. flexuosa* subsp. *pilifera*. In addition, all the samples were found in freshwaters, and *U. flexuosa* subsp. *pilifera* is known as a typical, and almost only species of *Ulva* that is able to survive entirely freshwater conditions (Wærn 1952, Bliding 1963, Koeman and van den Hoek 1984).

In such scenario, the originally tubular and ramified thalli could gain leaf-shaped form and lose branching with age, or in dependence on environmental factors. The occurence of attached tubular thalli in streams (Hradec Králové, Oleksovice, Budapest), and unfolding, free-floating plants in stagnant water with highest concentration of salts (Nemilkov, Hlohovec – Tab. 3) would support these suggestions.

Moreover, it was previously shown by other scientists that some species of *Ulva* drastically change their macroscopic morphology due to shift in environmental conditions.

For instance, Reed and Russell (1978) reported formation of a ´bottle-brush´ (proliferous) morhology in *U. intestinalis* at extreme salinity, and Blomster et al. (2002) observed occurrence of sheet-like morphology of the same species when it grew in ´green tides´. In dependence of variables like salinity, irradiance, temperature, or nutrient levels, *U. intestinalis* is also affected as for growth rate and productivity (Martins et al. 1999, Taylor et al. 2001, McAvoy and Klug 2005), or even size of cells (an osmotic response – Young et al. 1987).

Another species, *U. muscoides* includes so different ecotypes that they used to be described as separate taxa, and they were only recently joint together using molecular analysis (Blomster et al. 1999).

Collected data on *U. flexuosa* subsp. *pilifera* also improve our knowledge about the geographical distribution of this taxon in Europe. Previously, it has been reported from Sweden, the Netherlands, France, England and Croatia (summarized by Bliding 1963, Koeman and van den Hoek 1984), Germany (Kützing 1849) and Poland (Sitkowska 1999) as far as it is known to us. Our findings further include the Czech Republic, Slovakia, and Hungary. It is most probable that this species occurs at similar freshwater localities at least throughout the whole Europe, but possibly also in other parts of the world.

Holotypes of *U. flexuosa* subsp. *pilifera* and *U. flexuosa* subsp. *flexuosa* were included in our study after we obtained first results, in attempt at getting a better background for taxonomical conclusions.

The type specimen of *U. flexuosa* subsp. *pilifera* somewhat differs from our samples by its rather filiform macroscopic morphology (Pl. 19). Regarding previous discussion of variable morphology of the thalli, this feature need not be necessarily so important.

In terms of anatomy, the same specimen was previously shown (Bliding 1963 – p. 98, Fig. 56.) to possess typical features (cell shape and arrangement, microscopic branches) that are also present in our samples of this taxon.

In addition, the *rbc*L gene of the holotype was repeatedly sequenced with identical results, and it fell into the same clade with our samples of *U. flexuosa* subsp. *pilifera* in phylogenetic trees (Fig. 4), thus validating our proposals.

On the other hand, the results of analysis of the second type specimen, *U. flexuosa* subsp. *flexuosa* are problematic and ambiguous.

Both macro- and micromorphologically, the holotype is separated from most samples. Its thallus is thin, but only scarcely branched in the basal region, the rectangular or quadrungular cells are organized in rather long rows and possess only 1-2 pyrenoids per cell (Bliding 1963 – p. 77, Fig. 40). As for classical taxonomy, the specimen is also similar to the sample of *U. flexuosa* subsp. *flexuosa* collected in Skagerrak by E. Leskinen (1995).

However, a single obtained *rbc*L sequence of the type was totally identical to that of the previous holotype of *U. flexuosa* subsp. *pilifera*, and consequently it was included in the same clade (Fig. 4, Tab. 7).

This contradictory results may have two different causes. First, it has to be taken into consideration that the two specimens´ DNA was extracted and analysed at the same time, and PCR amplification of the subsp. *flexuosa* type was extremely difficult (it was successfully completed only once from many attempts). Therefore, it cannot be excluded, that some of the subsamples or PCR reactions was contaminated with the other type´s DNA.

To validate or reject the present results, it is absolutely necessary to carry out a new DNA extraction and amplification of the type specimen of *U. flexuosa* subsp. *flexuosa* in a separate and extremely careful analysis. If the result was eventually validated, it would probably lead to unification of the holotypes under a common name (probably *U. flexuosa* subsp. *flexuosa* Wulfen as an older name), and the designated morphological differences between these two taxa would lose their validity.

Importantly, the morphologically similar specimen of *U. flexuosa* subsp. *flexuosa* was placed outside this clade together with the only *rbc*L-sequenced of the previously commented samples of *U. flexuosa* subsp. *paradoxa* (Figs. 4 and 5). In analysis of ITS data, with more sequences available for comparison, it formed a completely separate clade sister to *U. flexuosa* subsp. *pilifera* together with *U.* cf. *flexuosa* from the Ythan estuary in Scotland.

If the two holotypes should be unified in the end, these unfamiliar specimens would probably need a new characterization and typification.

Regardless to these considerations and their possible implications, it is interesting to observe how ecologically and morphologically divergent the individual specimens and subspecies can be.

Speaking in more exact terms of ITS nucleotide sequence distance (Tab. 6), the members of the subsp. *paradoxa* clade did not differ at all among themselves, but had a 1.1-1.6 % distance from the subsp. *pilifera* clade and 1.3-2.1 % distance from subsp. *flexuosa*. Moreover, the sequences inside the subsp. *pilifera* clade were also identical, but they differed by 1.1-2.1 % form *U. flexuosa* subsp. *flexuosa*. The *U.* cf. *flexuosa* VI and *U. flexuosa* subsp. *flexuosa* sequences had a 1.1 % distance.

Similarly, most of the members of the subsp. *pilifera* clade were identical in *rbc*L sequence (Tab. 7), except *U. flexuosa* subsp. *pilifera* from Krusenberg that differed by 1.1 % from the others. Their distance from subsp. *paradoxa* ranged between 1.8-2.4 %, and the difference from *U. flexuosa* subsp. *flexuosa* from Skagerrak was 1.4-1.9 %. Subspecies *paradoxa* and *flexuosa* were more similar, with a 0.7 % distance.

Compared to previously published studies, these values stand at the boundary of species and infraspecific ranks of taxa. Blomster et al. (1998, 1999, 2002), Hayden et al. (2003), Hayden and Waaland (2004), and Loughnane et al. (2008) report ranges of sequence distance among conspecific taxa of *Ulva* to lie between 0-2.2 % for ITS, and 0-0.56 % for *rbc*L, and more than 7.5 % (ITS) or 0.67 % (*rbc*L) between different species.

Considering these results, together with present anatomical and ecological disparities between the subspecies, we hypothesize that after a more comprehensive investigation of the type material, the individual subspecies may eventually be given a status of separate species.

Whether they are classified as species or not, it is certain that they form a phylogenetically consistent group of taxa with a common evolutionary history, yet diversified in their phenotypes and life histories.

5. Conclusions

1. All collected specimens of *Ulva* in the Czech Republic clearly belong to the species *Ulva flexuosa*.
2. Previous findings identified as *U. intestinalis* or *U. linza* very probably should be assigned to *U. flexuosa*, too.
3. Most of the Czech specimens fall into *U. flexuosa* subsp. *pilifera*.
4. Analysis of *U. flexuosa* subsp. *pilifera* samples from our field collections and European herbaria contributed to our knowledge of its distribution and morphological variability. We also confirmed its definition as a taxon typical for freshwater habitats.
5. The specimen collected in the Soos National Nature Reserve salt marsh was identified as *U. flexuosa* subsp. *paradoxa*. Examination of the sample and herbarium material from Hungary and Romania extended the known occurrence of this prevailably marine taxon to certain inland waters.
6. Preliminary results of the analysis of type specimens suggest possible identity of holotypes of *U. flexuosa* subsp. *pilifera* and *U. flexuosa* subsp. *flexuosa*. However, these results are doubtful, and further investigation of the type material is necessary.
7. The subspecies of *U. flexuosa* form a phylogenetically consistent unit. Nevertheless, partial differences between the individual subspecies might lead to their division into separate species in future.

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Appendix

Photographic documentation of 19 specimens of *Ulva*

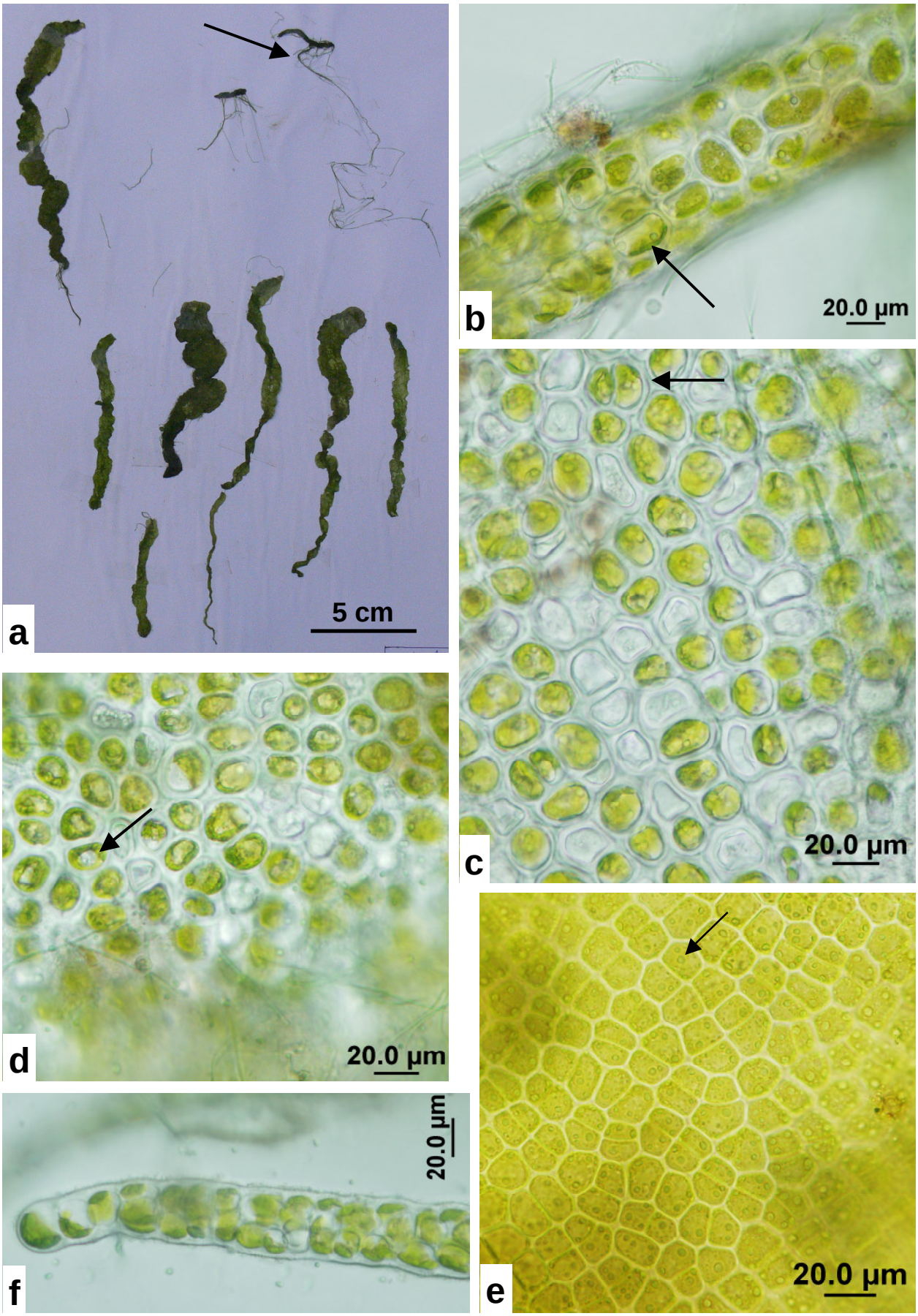

Plate 1. *Ulva flexuosa* subsp. *pilifera* I: **a** – macroscopic view, intensive branching in basal parts (arrow); **b** – rectangular cells in a narrow branch (arrow); **c** – some cells remain in common cell wall after division (arrow); **d** – chloroplasts form a hollow cylinder (transverse section – arrow); **e** – irregularly arranged cells with 2-3 pyrenoids (arrow); **f** – a branchlet with a globose apical cell.

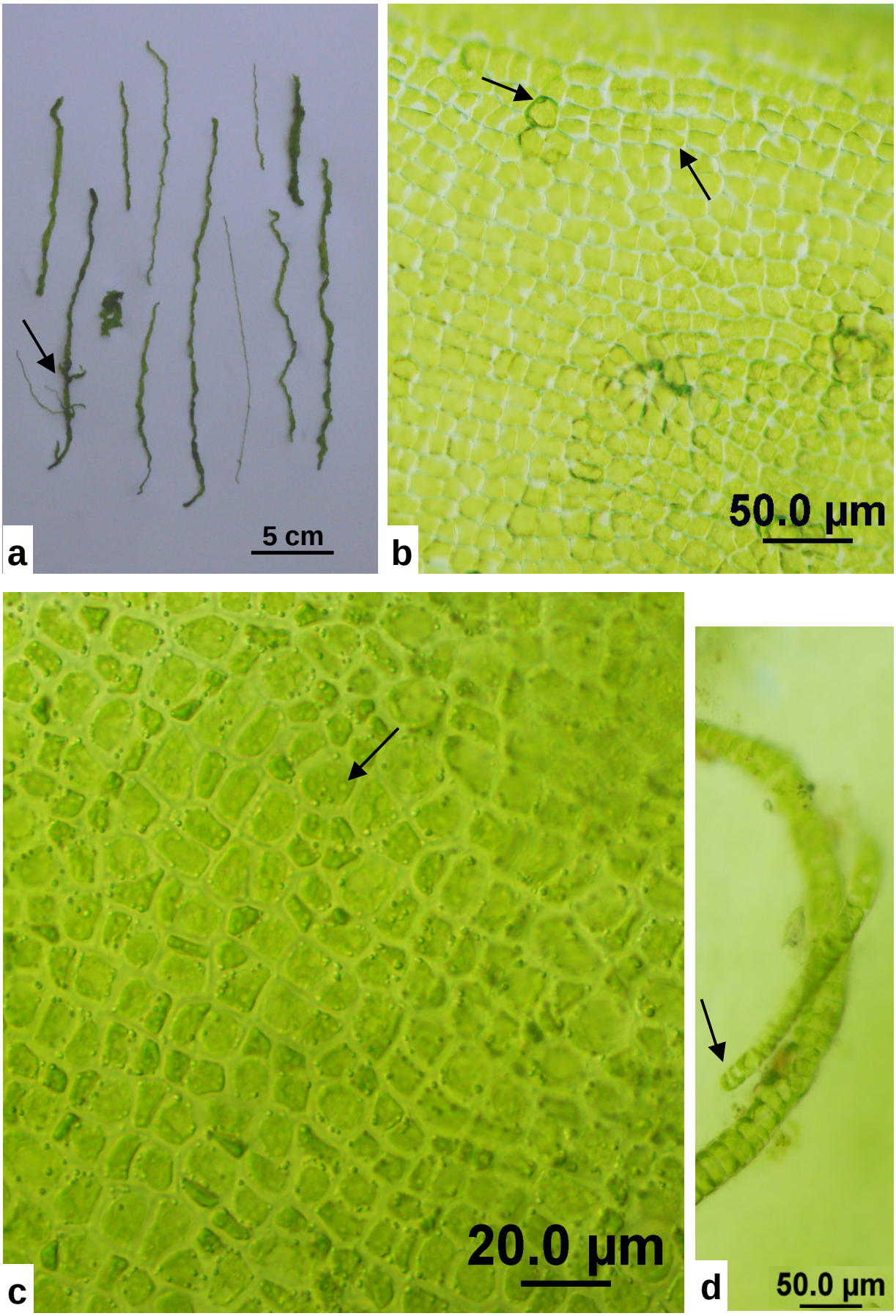

Plate 2. *Ulva flexuosa* subsp. *pilifera* II: **a** – macroscopic view, branching in basal parts (arrow); **b** – rectangular cells (rows) in marginal part of the thallus (right arrow), chloroplasts form hollow cylinders (transverse section – left arrow); **c** – irregularly arranged cells with 2-3 pyrenoids (arrow); **d** – branchlet with a globose apical cell (arrow).

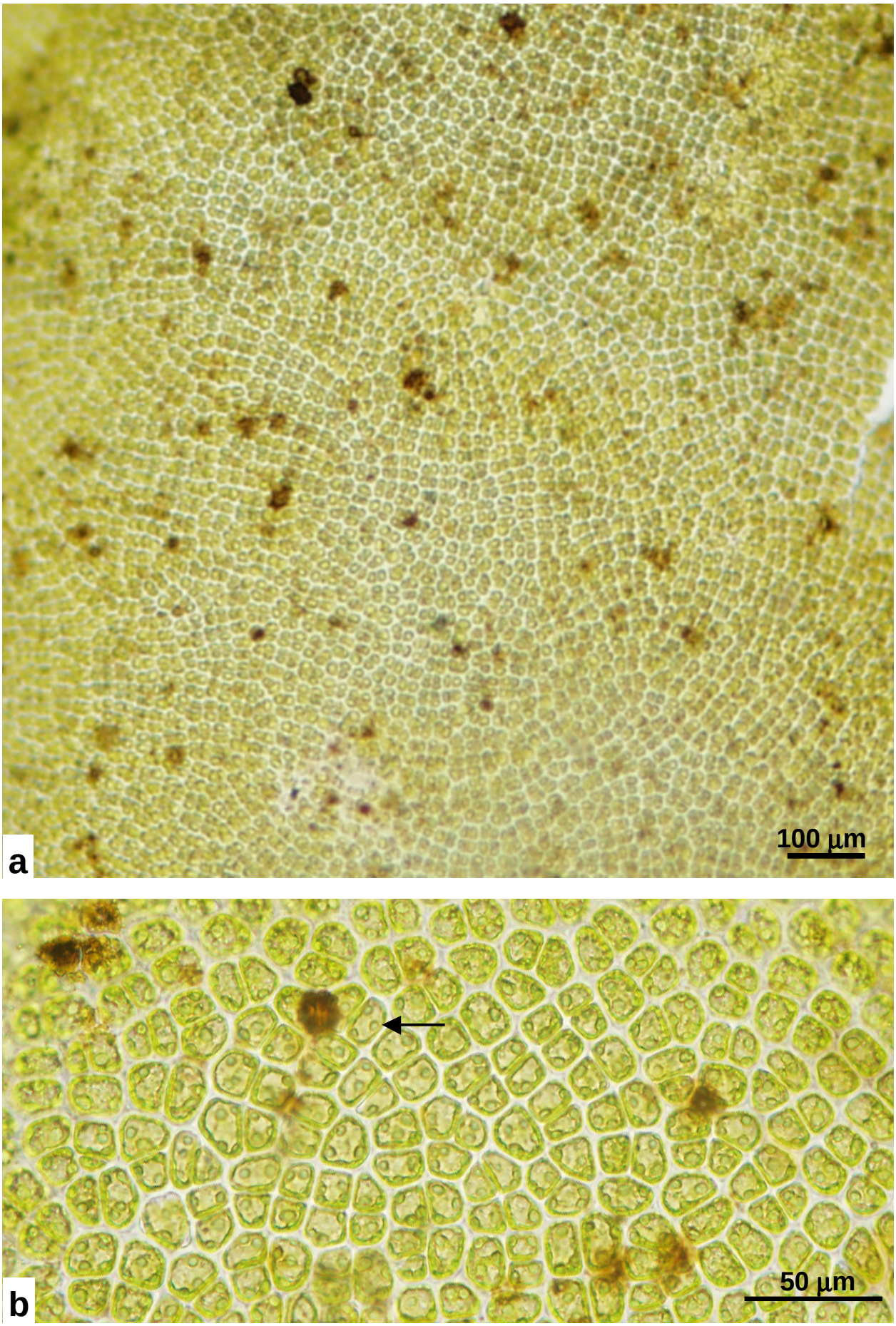

Plate 3. *Ulva flexuosa*: **a** – round cells arranged in curved rows or irregularly; **b** – rounded-polygonal cells with parietal chloroplast and 2-3 pyrenoids.

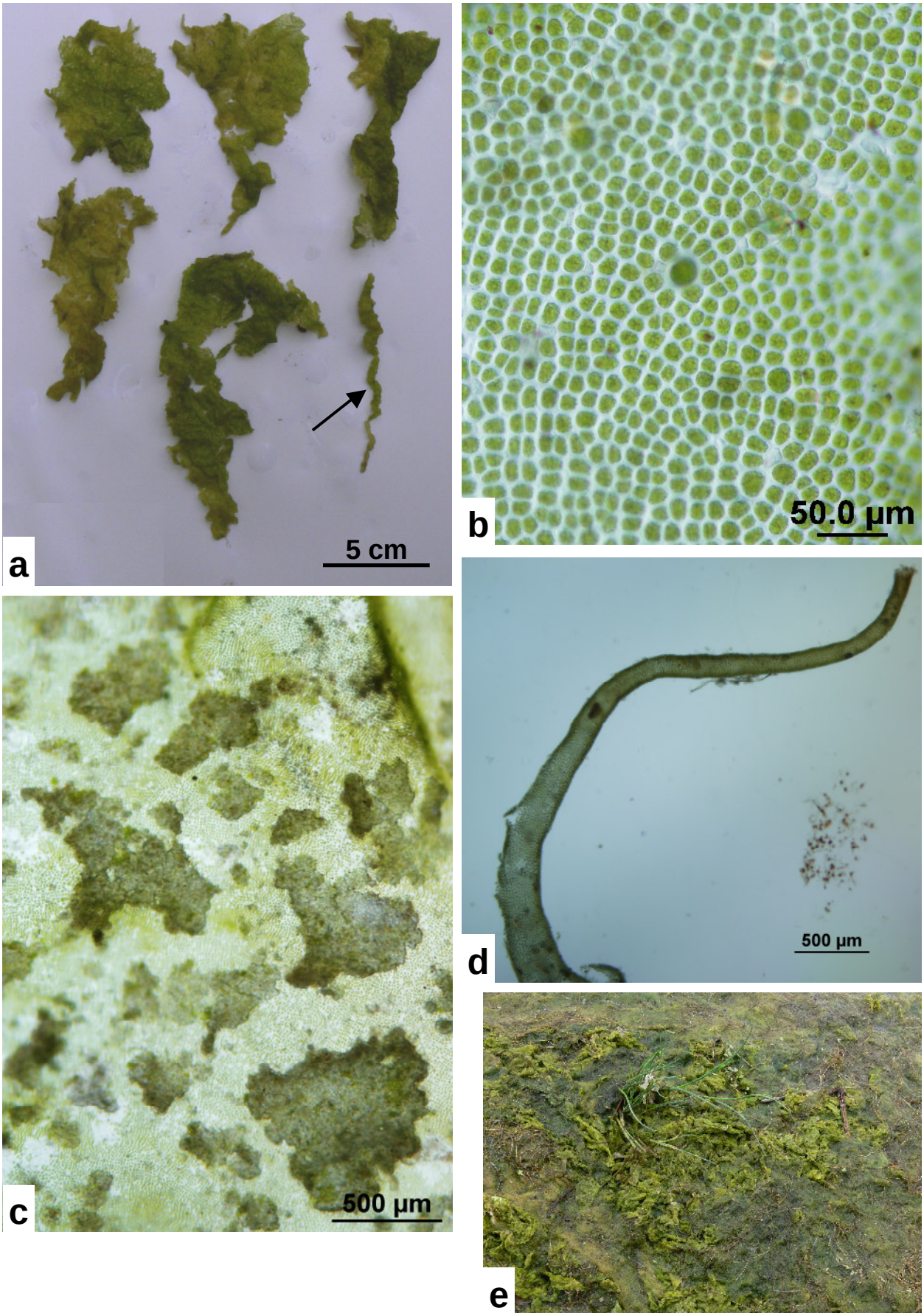

Plate 4. *Ulva* cf. *flexuosa* I: **a** – macroscopic view, young thalli are tubular (arrow); **b** – irregularly arranged cells; **c** – intensive mineral incrustation; **d** – basal part of a tubular thallus; **e** – densely agglomerated biomass at the boundary of mud and water.

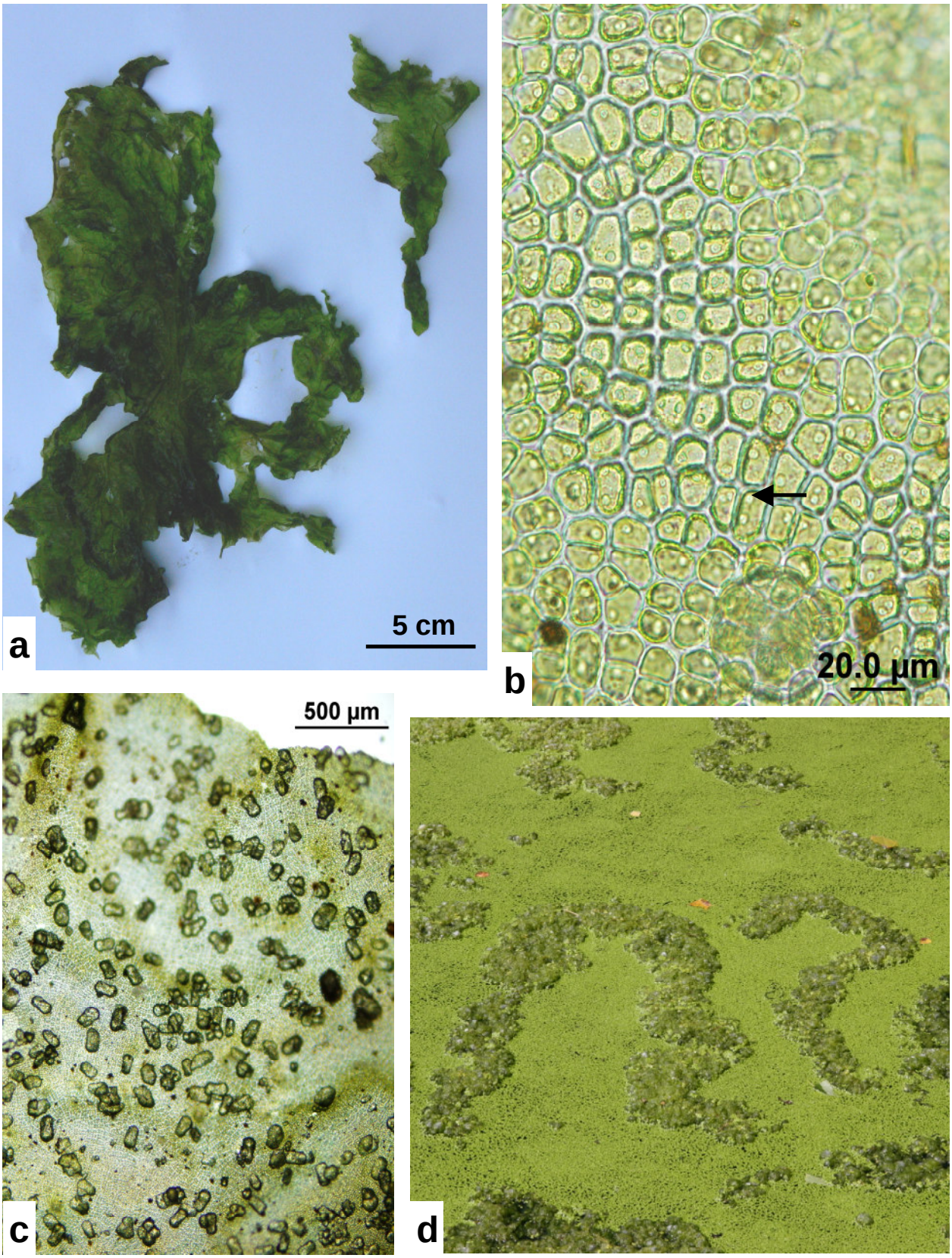

Plate 5. *Ulva* cf. *flexuosa* II: **a** – macroscopic view; **b** – cells arranged irregularly or in short rows, parietal chloroplasts possess 2 pyrenoids in average (arrow); **c** – intensive mineral incrustation; **d** – floating thalli surrounded by a film of *Lemna minor*.

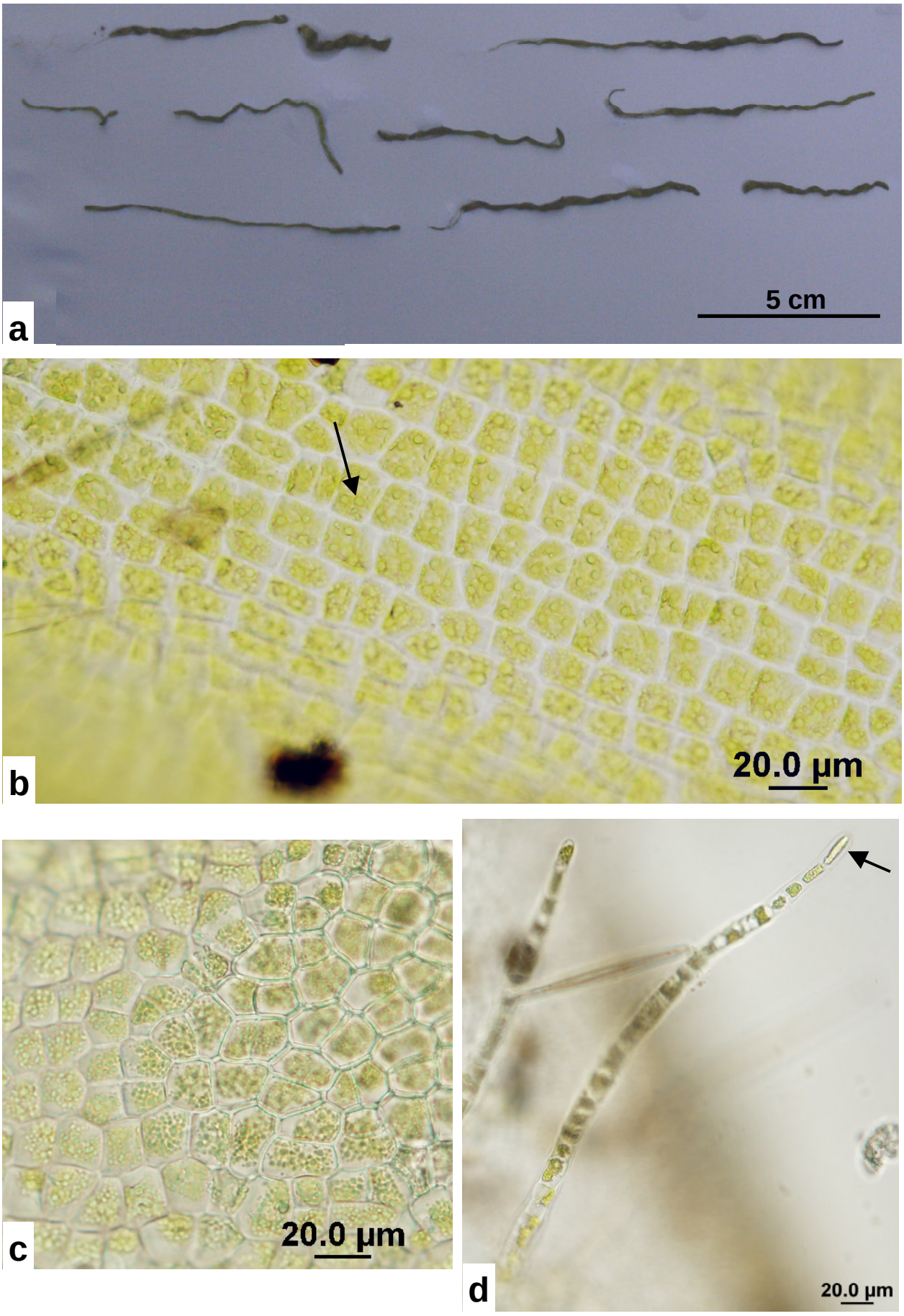

Plate 6. *Ulva flexuosa* subsp. *paradoxa* I: **a** – macroscopic view; **b** – rectangular cells in long rows in marginal part of the thallus, chloroplasts with 2-4 pyrenoids (arrow); **c** – irregularly arranged cells in central parts of broad thalli; **d** – branchlet with an elongate and narrowed apical cell (arrow).

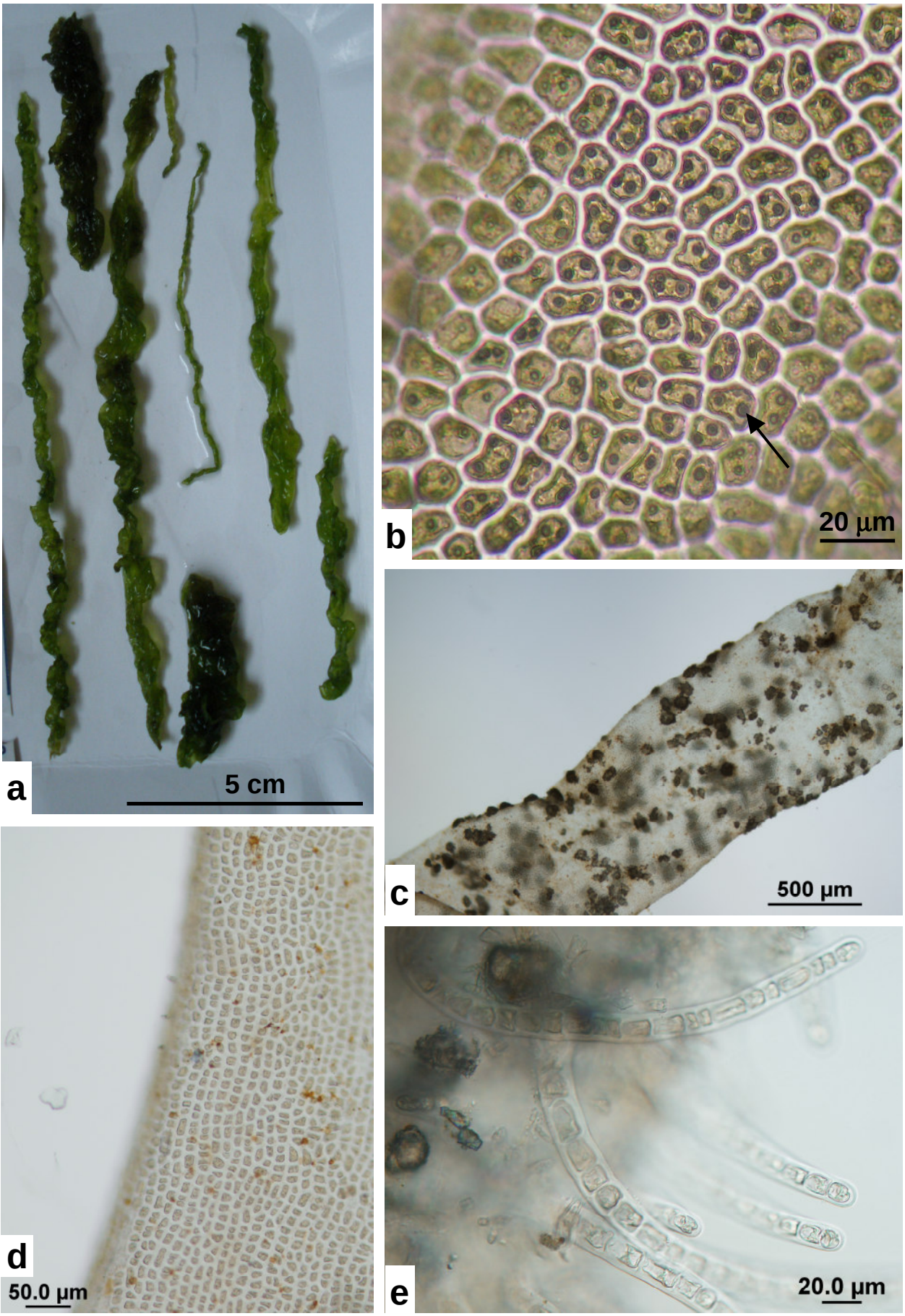

Plate 7. *Ulva flexuosa* subsp. *pilifera* III: **a** – macroscopic view; **b** – irregularly arranged cells in central parts of broad thalli, chloroplasts with 2-3 pyrenoids (arrow); **c** – cells in long rows in marginal part of the thallus; **d** – branchlets with widely rounded apical cells.
Figures **a** and **b** - photo M. Sitkowska

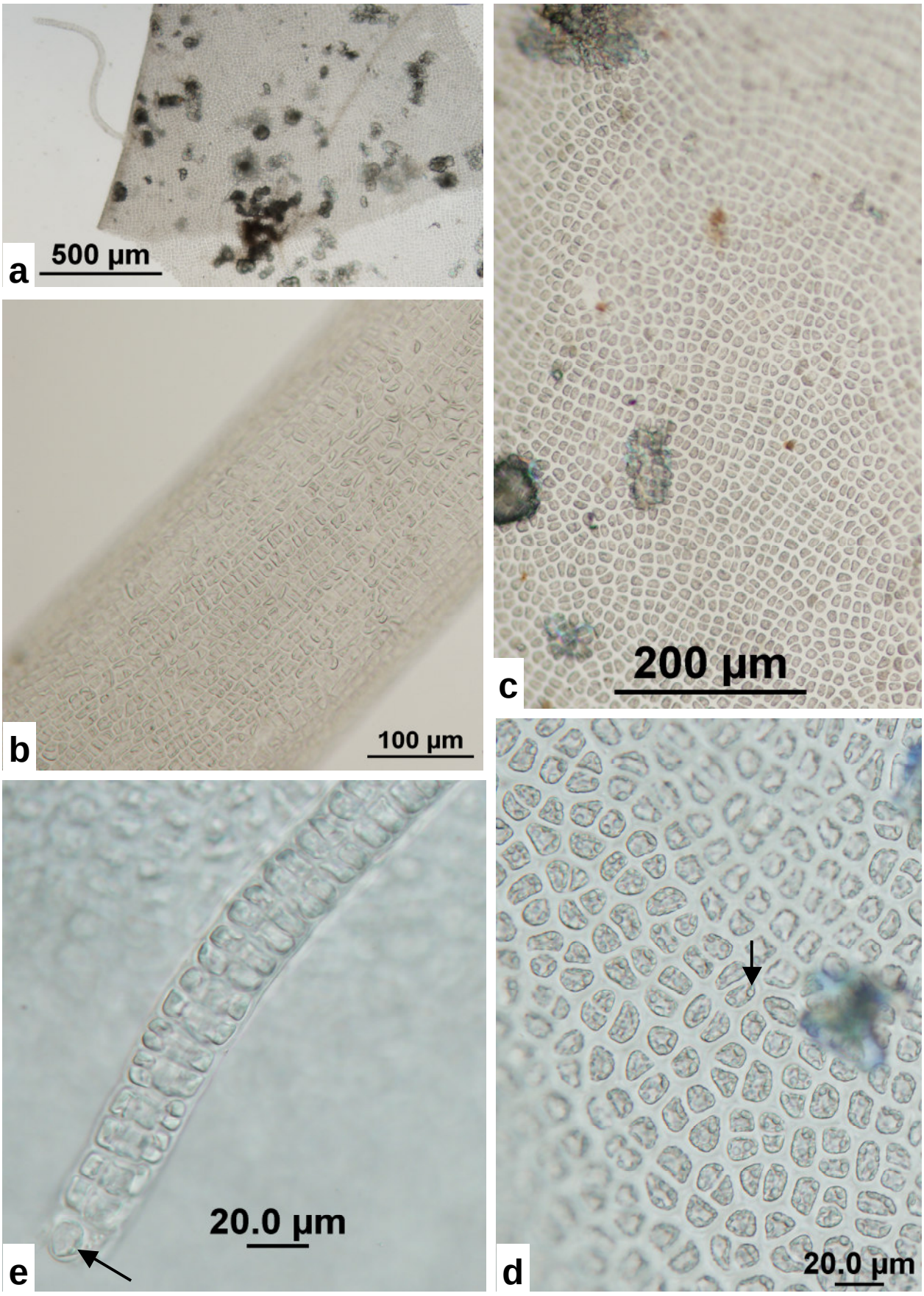

Plate 8. *Ulva flexuosa* subsp. *pilifera* IV: **a** – thallus with mineral incrustations; **b** – rectangular cells (rows) in a narrow thallus; **c** – cells arranged in short rows or disordered in central parts of the thallus; **d** – cells with parietal chloroplasts that contain 2-4 pyrenoids (arrow); **e** – a polyseriate branchlet with a globose apical cell (arrow).

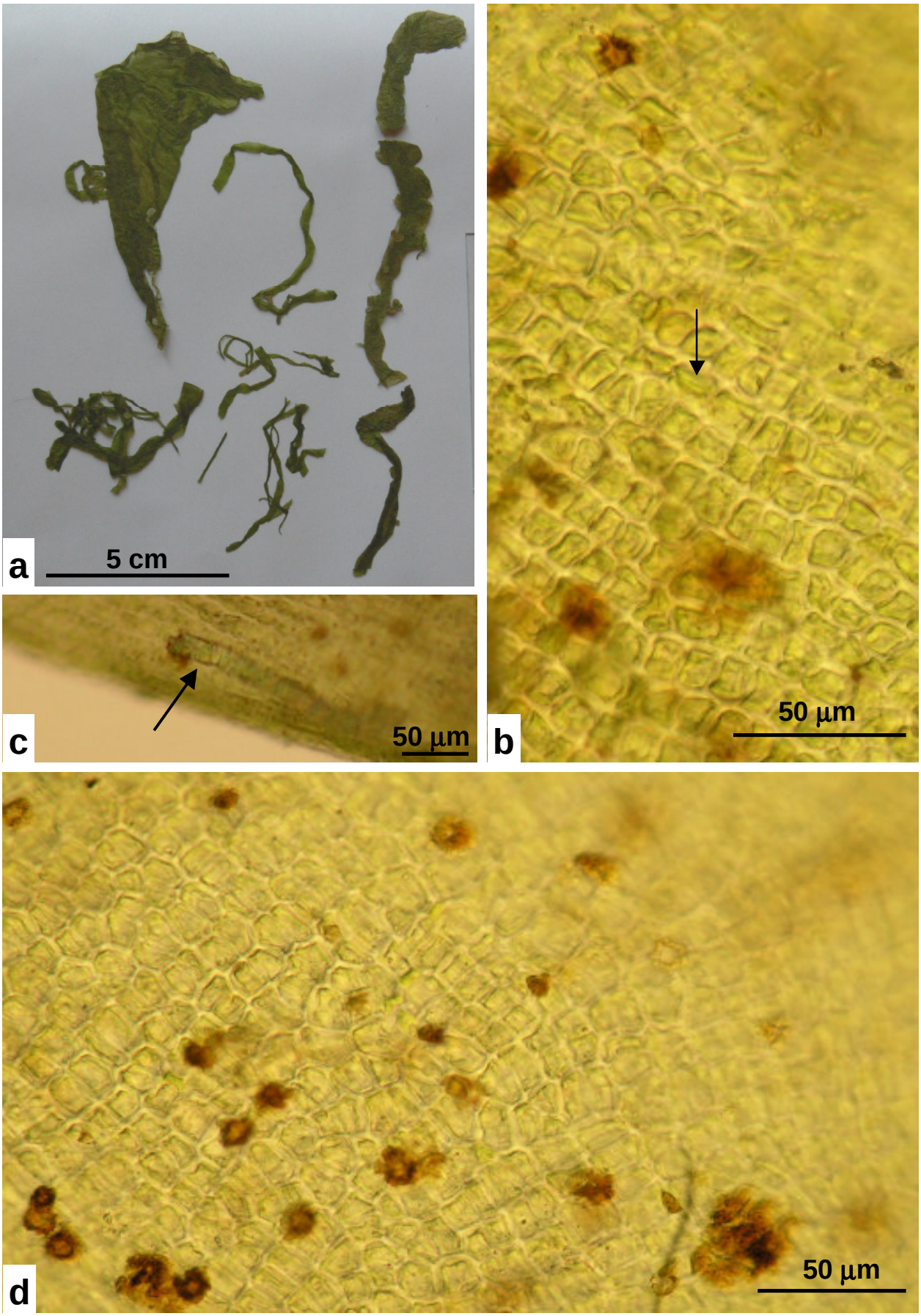

Plate 9. *Ulva* cf. *flexuosa* III: **a** – macroscopic view of tubular and unwound thalli; **b** – rectangular cells (rows) in marginal parts of the thallus, parietal chloroplasts sometimes form almost complete hollow cylinders (arrows); **c** – a small microscopic branch (arrow); **d** – cells arranged in shorter rows or disordered in central parts of the thallus.

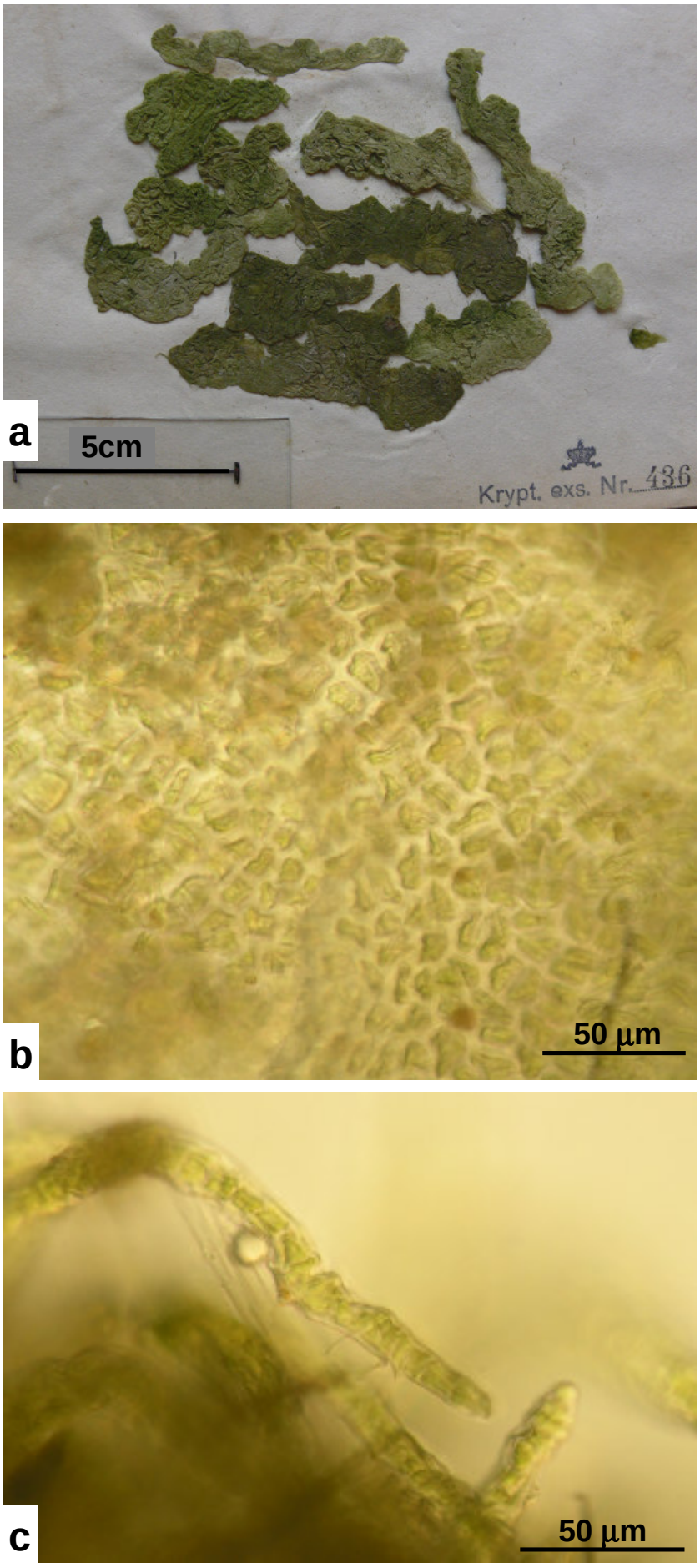

Plate 10. *Ulva* cf. *flexuosa* IV: **a** – macroscopic view (arrow); **b** – irregularly arranged cells; **c** – microscopic branchlets.

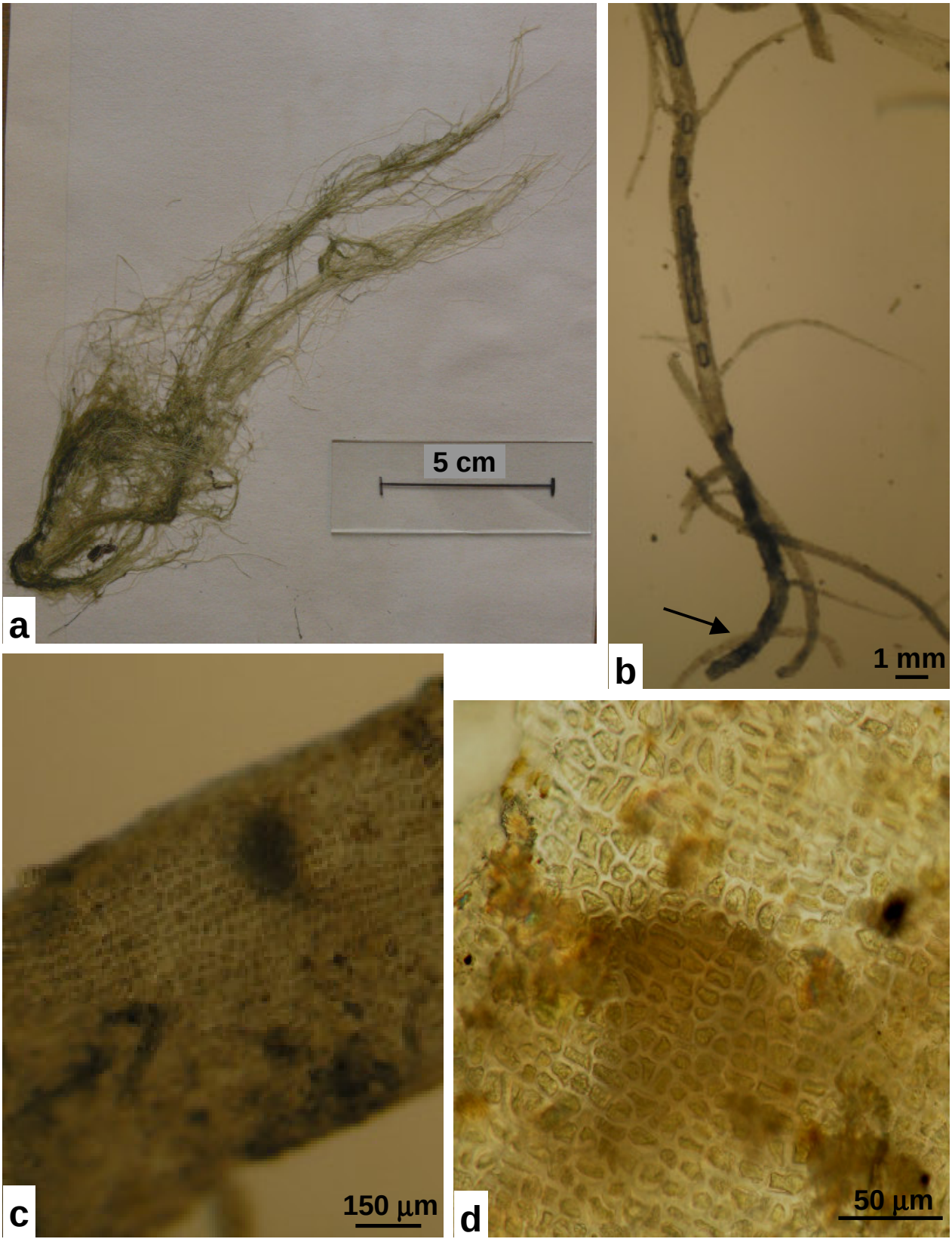

Plate 11. *Ulva flexuosa* subsp. *paradoxa* II: **a** – macroscopic view; **b** – microscopic view of the thallus, differentiated basis (arrow); **c** – cells organized in paralell rows; **d** – somewhat irregularly arranged cells in central parts of broad thalli.

Plate 12. *Ulva* cf. *flexuosa* V: **a** – macroscopic view, thalli divide into broad branches (arrow); **b** – cells arranged in rows or irregularly.

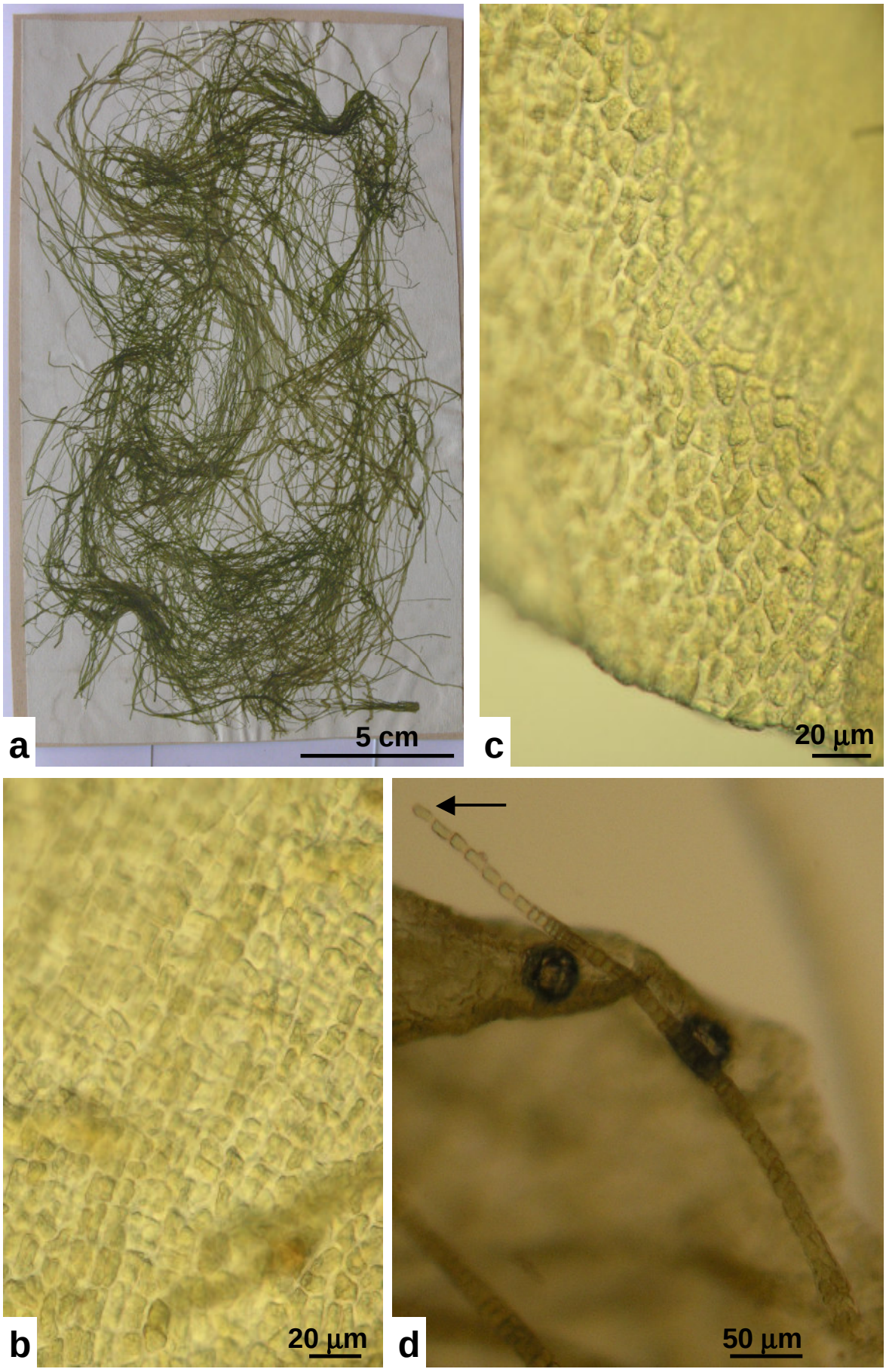

Plate 13. *Ulva flexuosa* subsp. *paradoxa* III: **a** – macroscopic view; **b** – cells organized in parallel rows; **c** – somewhat irregularly arranged cells in broader thalli; **d** – a branchlet with a narrowed and elongate apical cell (arrow).

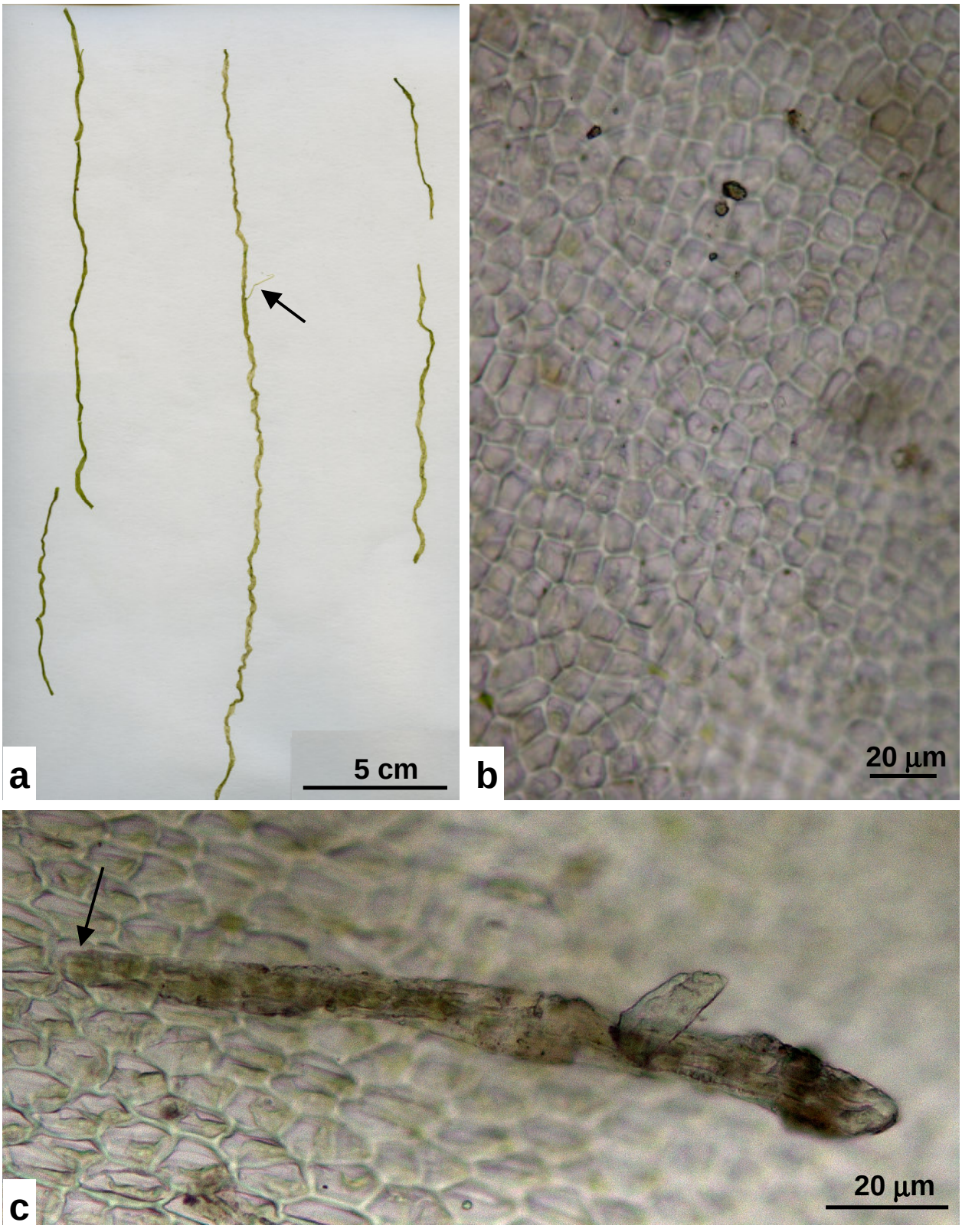

Plate 14. *Ulva* cf. *flexuosa* VI: **a** – macroscopic view, thalli with occasional branches (arrow); **b** – cells arranged in short rows or irregularly; **c** – a microscopic branchlet with a rounded apex (arrow).

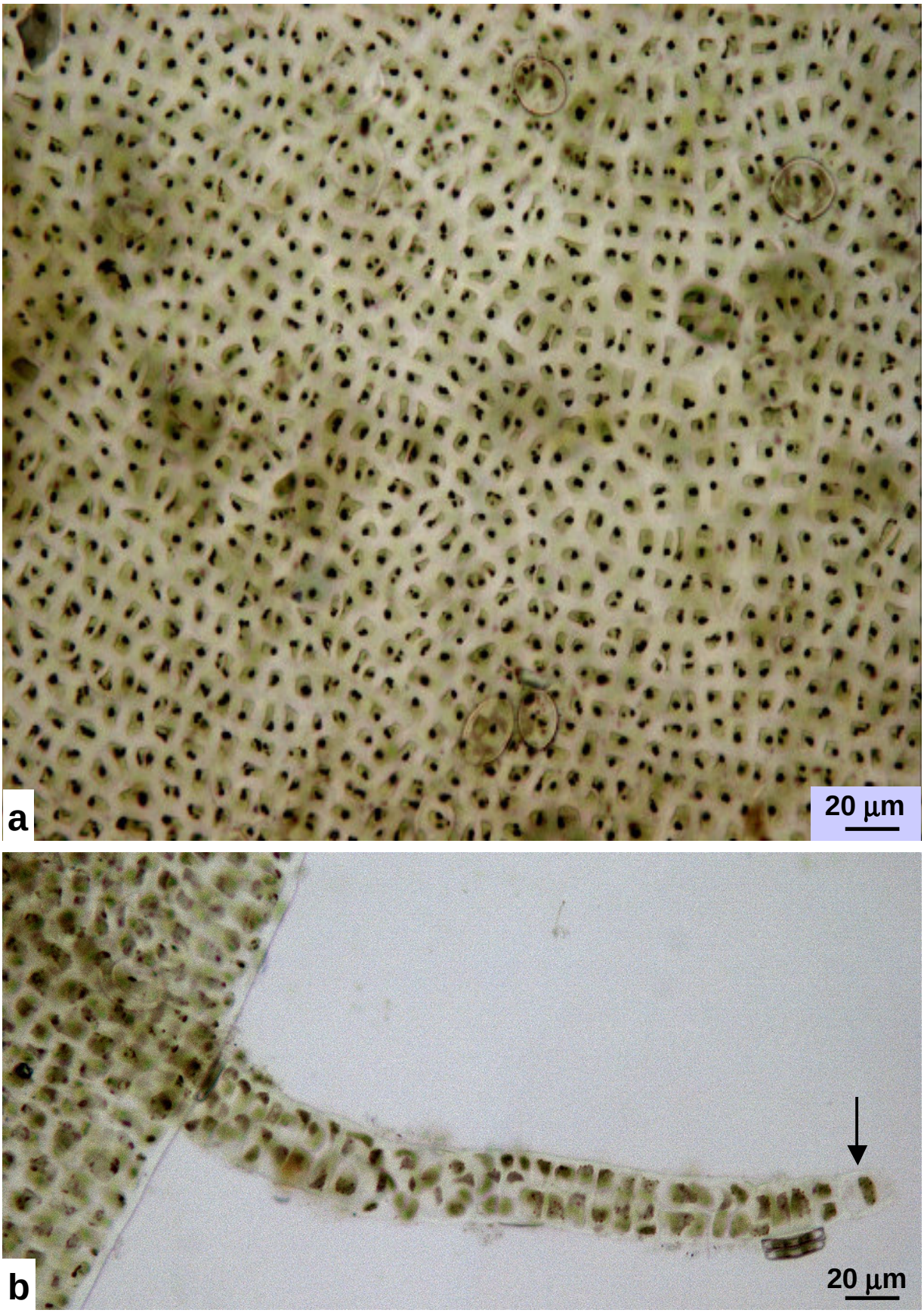

Plate 15. *Ulva flexuosa* subsp. *pilifera* IV: **a** – cells arranged in curved rows or irregularly; **b** – a microscopic branchlet with a round apical cell (arrow).

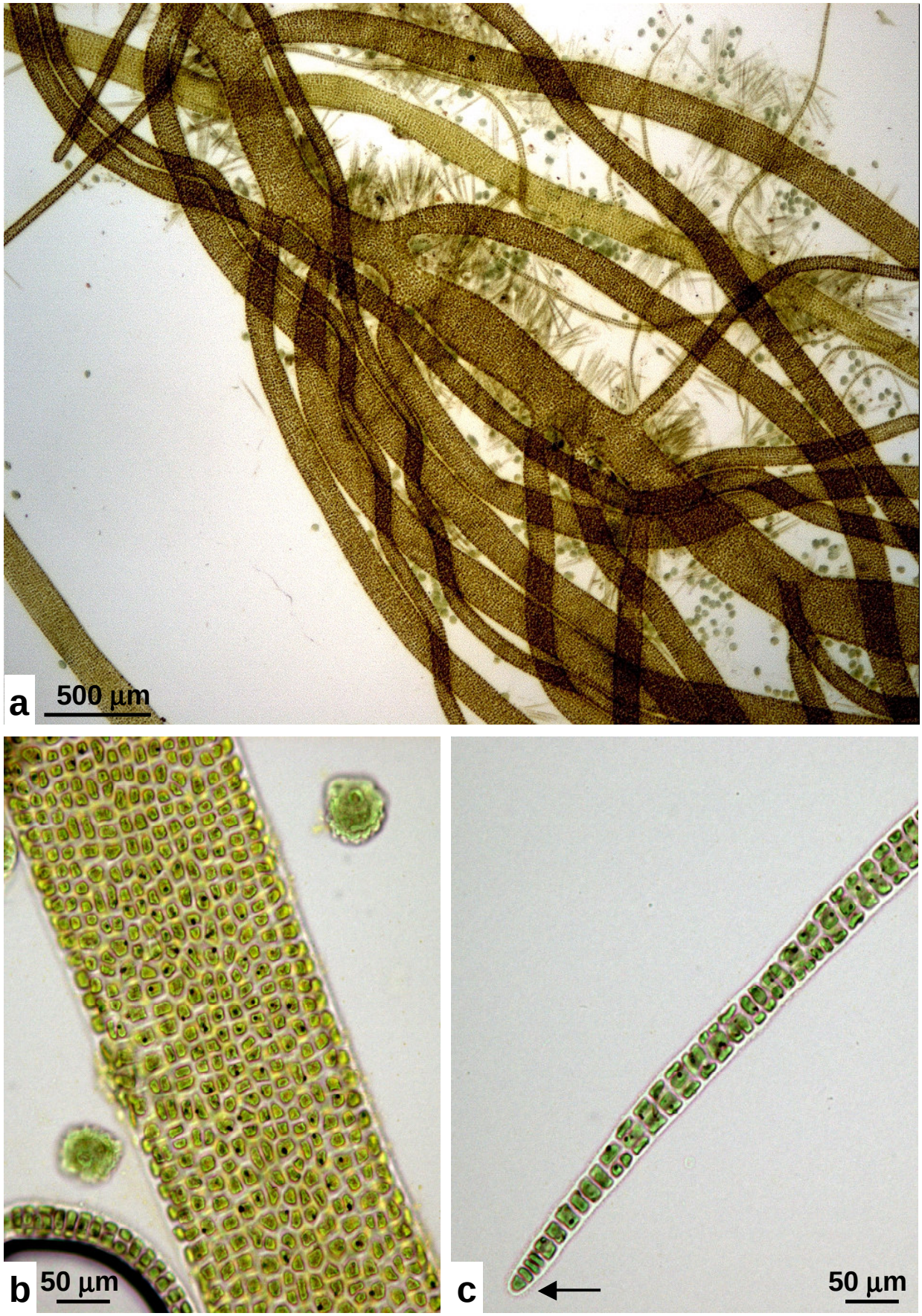

Plate 16. *Ulva flexuosa* subsp. *paradoxa* IV: **a** – intensive ramification of the thallus; **b** – cells organized in parallel rows at the margin, but somewhat disordered in the middle of the thallus; **c** – a branchlet with a rouded-conical apical cell (arrow).

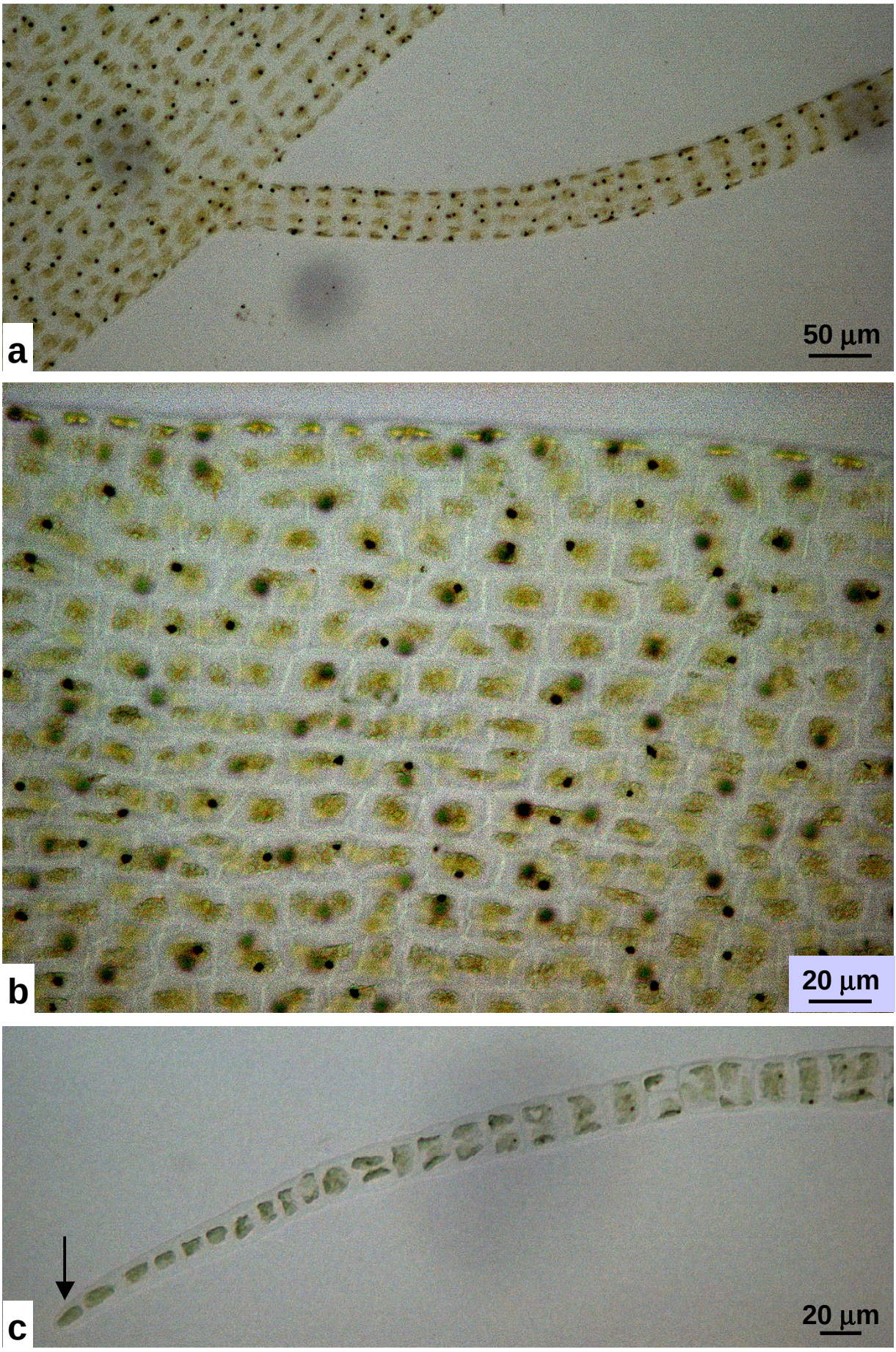

Plate 17. *Ulva flexuosa* subsp. *flexuosa*: **a** – a branching thallus; **b** – rectangular cells organized in parallel rows; **c** – a branchlet with a narrowed and elongate apical cell (arrow).

Plate 18. *Ulva flexuosa* subsp. *pilifera* – the type specimen from herbarium L.

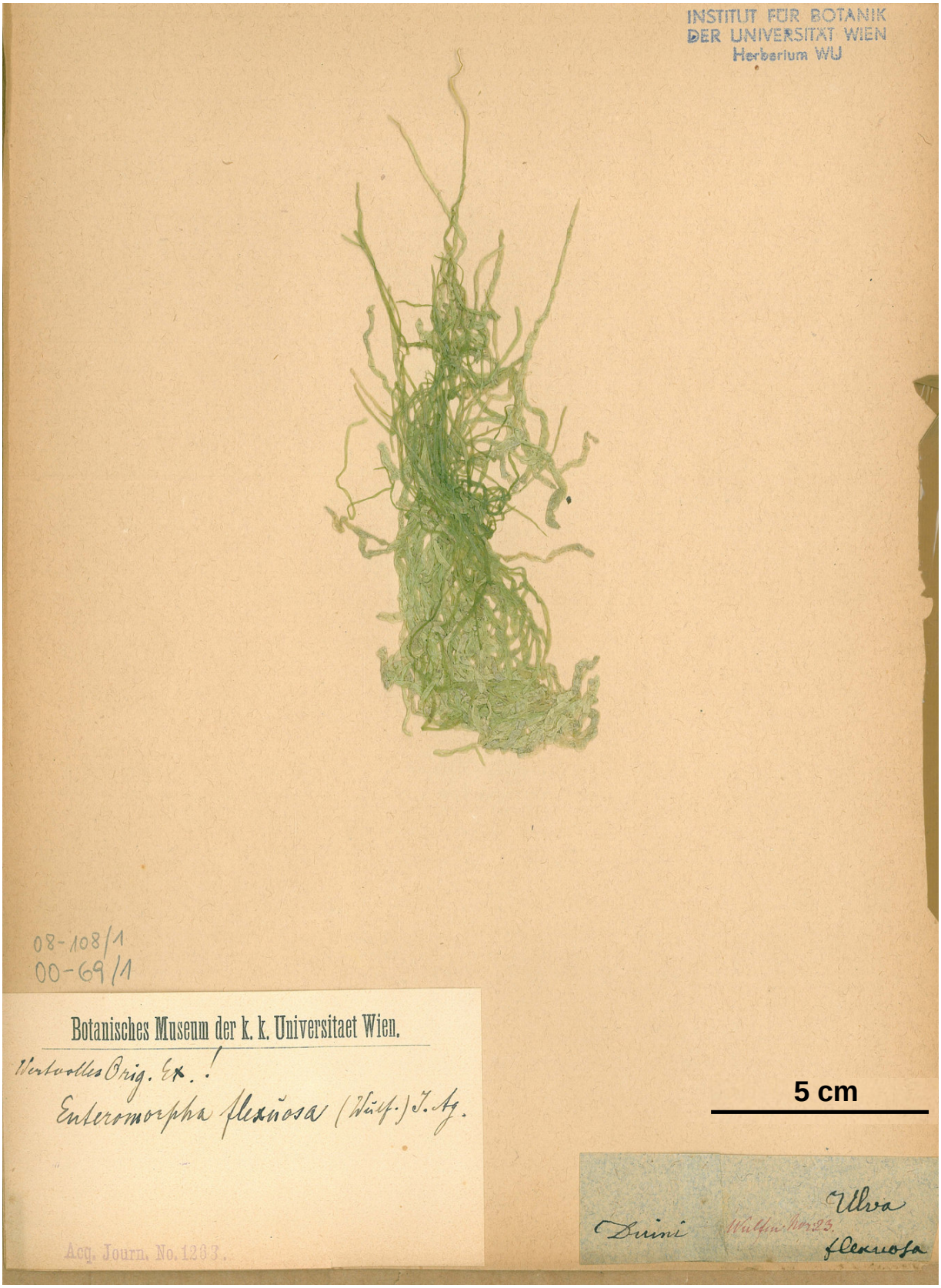

Plate 19. *Ulva flexuosa* subsp. *flexuosa* – the type specimen from herbarium WU.