TECHNISCHE UNIVERSITÄT MÜNCHEN

Forschungsdepartment Ökologie und Ökosystemmanagement
Fachgebiet Limnologie

Speciation in charophytes – a multidimensional approach

Michael Bögle

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. J. P. Geist
Prüfer der Dissertation:	1. Univ.-Prof. Dr. A. Melzer
	2. Univ.-Prof. Dr. W. Oßwald

Die Dissertation wurde am 07.03.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 04.06.2012 angenommen.

Speciation in charophytes – a multidimensional approach

Michael Bögle

CONTENTS

1. SUMMARY

Within the green algae genus *Chara,* several species are documented in which the morphology does not allow precise determination. Therefore it is necessary to include, in addition to morphologic traits, genetic and physiologic information as well as crossing experiment results for taxonomic determination. Beyond taxonomic interest, this information is also important for conservation of biodiversity. This study focuses on three species complexes within the genus *Chara* in which difficulties in traditional species determination based on morphology, ecology and mode of reproduction exist:

A) Specima which are difficult to separate from each other based on morphological characters were analysed. Due to the lack of clear morphological differentiation characteristics, *C. baltica* and *C. intermedia* are usually separated from each other by habitat salinity. In addition, the typical brackish water species *C. baltica* often occurs along with *C. horrida* and *C. liljebladii*. Among these three species, morphologically intermediate individuals are commonly found and their classification to one species or another is unclear.

B) *C. aspera* occurs both in brackish and fresh water but in contrast to the *C. intermedia – C. baltica* species complex, it is not separated into two species based on habitat salinity.

C) *C. canescens* is the only known *Chara* taxon where both sexual and parthenogenetic populations are known.

The following methods were used to investigate these three species complexes: a) analysis of morphological traits; b) genetic analyses using amplified fragment length polymorphism (AFLP); c) physiologic analyses with respect to photosynthesis parameters in order to detect possible differences in adaptation potential between individuals of putative species; and d) crossing experiments.

The objectives of this study were:

i) Evaluation of the morphological spectrum within genetically uniform sampling material and determination of morphological differences between genetically independent groups.

ii) Determination of the ecophysiological differences between the species and their adaptation abilities to salinity conditions.

iii) Determination of the genetic similarity between sexually interacting populations, as well as genetic differences between sexual separated populations.

iv) Investigation of the abilities for sexual reproduction within and between identified taxa based on morphological characteristics.

In our analyses (paper 1), none of the morphological characteristics traditionally used in the literature was able to distinguish between *C. baltica* and *C. intermedia*. In contrast, the physiologic and the genetic results clearly showed differences between *C. intermedia* and *C. baltica.* The physiologic adaptations possibly enabled individuals' better growth opportunities in their respective habitats and seem to be an adaptation to the different habitat conditions. Both the genetic and the physiological analyses separated populations from the Baltic Sea and from fresh water habitats. However, populations from the Salziger See and southern France exhibit intermediate properties, indicating a continuum. The existence of an intermediate taxon can be explained by phylogenetic evolution, where two differently specialised species have one common ancestor. A second possible explanation for an intermediate population is hybridization between *C. baltica* from the Baltic Sea and *C. intermedia* from fresh water habitats. We concluded that the *C. baltica – C. intermedia* complex either consists of three species or must be regarded as variations within one species. The present differentiation in two species is not supported by our data.

C. horrida and *C. liljebladii* occur in brackish water and are sometimes difficult to separate from *C. baltica*. In our analyses, *C. horrida* and *C. baltica* could be separated from each other based on morphological traits, whereas no morphologic separation characteristics for *C. liljebladii* were found. The three taxa showed no marked differences in physiological characteristics. Within the genetic analyses, *C. horrida* and *C. liljebladii* clustered together with *C. baltica* and formed one genetic group. In the crossing experiments, fertile oospores between *C. baltica* and *C. horrida* were found. We concluded that *C. horrida*, *C. liljebladii* and *C. baltica* are closely related to each other, irrespective of the morphological differences existing between *C. horrida* and *C. baltica*. Possible explanations are:

i) Frequent crossing events might occur between the three taxa.

ii) The morphologic variability with concurrent genetic similarity could be caused by DNA methylation, an undetected mutation or chromosome duplication. In view of these results, it will be necessary to re-evaluate species conservation programs and Red Lists. Assessment of

the significance of differences between morphological and genetic diversity will be one of many challenges in future discussions about biodiversity.

Morphologic variability is described in the *C. aspera* complex and *C. aspera* is found both in fresh and brackish water. This is according to physiologic analyses; differences between fresh and brackish water populations were detected. Genetic analyses however, showed no differentiation by habitat. Two explanations for the apparent discrepancy between the *C. baltica-intermedia* complex (different physiology, different genetics) and *C. aspera* (different physiology, no different genetics) are possible:

a) The separation between *C. baltica* and *C. intermedia* into two species could date back earlier than the separation of the *C. aspera* populations and therefore no genetic differences are yet established.

b) *C. aspera* is dioecious while *C. baltica* and *C. intermedia* are monoecious. Differences in the effectiveness of sexual reproduction between monoecious versus dioecious species can lead to a higher genetic uniformity in dioecious plants. In the crossing experiments, a higher amount of crossing events occurred within the dioecious *C. aspera* than in monoecious species. This could explain the genetic separation within the *C. baltica-intermedia*-complex and its absence in *C. aspera*.

C. canescens is dioecious and both parthenogenetic and sexual populations are found. No morphological trait is known which could separate the sexual from the parthenogenetic populations. However, in our study, genetic analyses show that differentiation into two groups is possible. Due to the fact that we found genetically distinguishable individuals within the same habitat, we assume that a clear separation is possible and might indicate the beginning of a speciation process based on different reproduction modes.

Our results show that the phenetic species concept does not explain all speciation processes in charophytes. Physiological results clearly show that charophytes adapt to habitat, and differences are found among species (*C. intermedia, C. baltica*) and within a population of one species (*C. aspera*, *C. canescens*). Genetic similarities on the DNA level follow the coefficient of sexual exchange. The crossing experiments do not support a separation of the *C. baltica-intermedia* or *C. aspera* complexes.

To understand the evolution of charophytes, morphological, physiological, and genetic analyses are necessary in addition to crossing experiments. Each analysis adds an element of understanding. These analyses suggest that the division of species in smaller units, as done by

the European authors, often is less supported than summing them up into larger units as done by Wood (1965).

2. INTRODUCTION

Charophytes are globally distributed and can be found in fresh, brackish, and in-land (athalassic) saline waters (Krause 1997). They possess nodes, internodes and leaf-like structures. The internodes consist of long, multinuclear haploid cells and the nodes consist of several cells, from where a set of branches originates. The sexual reproduction organs (oogonia and antheridia) are botanically unique and differ markedly from angiosperm flowers. In addition to sexual reproduction generating diploid oospores, reproduction via vegetative and parthenogenetic pathways has been observed. The sexual reproductive organs can be ephemeral; therefore finding charophytes with antheridia and oospores is often unpredictable. Vegetative reproduction occurs with special organs called bulbills, located in the root system, or from broken shoots. Within the charophytes are annual species and perennial species with irregular occurrence, both of which can grow in fresh water and brackish water habitats (Krause 1997). However, within the green algae genus *Chara*, several species are documented where the morphology does not allow precise determination (Mannschreck 2003). This may be due to several reasons:

First, in contrast to angiosperms, there are few clear characteristics, and typical flowers do not exist. Therefore the determination of charophytes is based on a small number of morphological characters. It is, however, unclear how morphology varies under different ecological conditions. Second, different authors disagree which morphological traits are considered important for species, variation, or subgroup differentiation (Wood 1965, Corillion 1972, Hollerbach 1983, Pankow 1990, Krause 1997, Blindow 2003a). For example, Wood (1965) discriminates 81 macrospecies globally, each including many varieties and forms. This is in contrast to Krause (1997) who differentiates 45 microspecies in Europe, each having less morphologic variability than the macrospecies of Wood (1965) (see table 1 for an example of denomination within the *Chara baltica-intermedia* group). This disharmony in taxonomy is not limited to Europe, species status for taxa in Australia are also under dispute (Casanova 2005). Third, within the charophytes intermediate forms and transitions are often observed. In order to separate species from each other in taxonomic analyses it is therefore necessary to include, in addition to the phenetic species concepts, other species concepts like biological and ecological species concepts.

Table 1: Nomenclature of selected *Chara* species as given in different taxonomic works

Wood (1965)	Krause (1997)	Schubert (2003)	This study
***"Chara baltica* group"**			
Chara hispida var. *baltica f. baltica*	*C. baltica*	*C. baltica* var. *baltica*	*C. baltica*
C. hispida var. *baltica f. liljebladii*	*C. baltica f. elongata*	*C. baltica* var. *liljebladii*	*C. liljebladii*
C. hispida var. *baltica f. fastigiata*	*C. horrida*	*C. horrida*	*C. horrida*
C. hispida var. *major f. intermedia*	*C. intermedia*	*C. intermedia*	*C. intermedia*

Solving taxonomic conflicts within the genus *Chara* is only one reason for taxonomic research on charophytes. Beyond taxonomic interest, this information is also necessary for conservation of biodiversity and habitat characteristics:

First, single species are recognised as worthy for protection based on their uniqueness and ecological function. For example, charophytes play an important role as primary producers (Blindow 2003b, Noges 2003, Küster 2004) and provide unique habitats which serve as refuges for a variety of invertebrates (Wolfram 1999, Kotta 2004).

Second, in the discussion for assigning Red List species status and how to best manage their conservation, it is important to be able to differentiate species, to recognise which morphological forms are variations of one species, and to know how stable these variations are through a range of habitat types and communities (e.g. AG charophytes, for Germany, Blindow (2000) for Sweden, Blazencic (2006) for the Balkans, Casanova (2005) for Australia).

Third, if species are different not only in their morphology but also in their specific ecological requirements, then these species can be used for characterising habitats. Melzer (1988, 1999) established an index with macrophytes for the trophic characterisation of lakes. As indicator organisms, charophytes have been used for evaluating and monitoring lake and river systems (Stelzer 2005, Meilinger 2005). Within the EU Water Framework Directive, water bodies within the EU are to be assessed using different chemical and biological methods, and macrophytes – among them charophytes – are important indicators in this context. However, to use species as indicators, knowledge of their ecology is mandatory.

Fourth, more than 500 mya ago green algae belonging to the class charophyceae emerged from their aquatic habitat to colonize the land, and gave rise to more than 3.000 land plant

species currently found on our planet. In contrast to the large diversity of the land plants, only a few thousand charophycean species are living today. This group exhibits great variability in cellular organisation and reproduction. Six monophyletic groups exist, given here in the order of increasing complexity: The charales consist of one extant family (characeae) with six genera: *Chara, Nitella, Lamprothamnium, Tolypella, Lychnothamnus* and *Nitellopsis*. (McCourt 1996, Meiers 1997, McCourt 1999). The charales are suggested to be the extant sister group to all land plants (Karol 2001, McCourt 2004). This has been confirmed with morphological and ultra-structural analyses, as well as with genetic methods (McCourt 2004). However, new results show that charales are not the closest living relatives to embryophtes (Wodniok 2011). Recent studies identified a number of homologous genes involved in both the gametophyte-dominated life cycle of charophytes and the developmental process of flowering plants, which makes charophytes model organisms for studying developmental processes (Tanabe 2005, Braun 2007, Nishiyama 2007).

Therefore it is important to clarify the taxonomic status of charophyceae species. The focus of this study was on three species complexes within the genus *Chara:* the *Chara baltica – intermedia* group, the *Chara aspera* group, and parthenogenetic as well as dioecious *Chara canescens* (table 2). The algae were sampled along a north-south gradient throughout Europe (papers 1-5).

The species *C. baltica* and *C. intermedia* are difficult to distinguish by morphology (see paper 1), therefore the main separation criterion is habitat salinity. *C. intermedia* mainly occurs in fresh water whereas *C. baltica* is found in brackish water. However, a conflict exists when using this separation criterion for species determination - the first description of *C. intermedia* was based on individuals sampled from a brackish inland lake.

C. baltica often occurs along with *C. horrida* and *C. liljebladii*. In contrast to *C. baltica* and *C. intermedia*, morphological separation is possible using various determination keys (Krause 1997, Blindow 2003a). However, morphologically-intermediate individuals are found and their determination is unclear.

Table 2: Overview of the species of this study. * Morphological separable means a morphologic separation criterion exists that separate this species from other species within the complex. **C. hispida has been added to the genetic comparison and because of a close morphological relationship. It is not part of the complex

Species name	Habitat (water)	Morphological separable*	Sexual reproduction form	Species status
Species of the *C. baltica- intermedia* complex				
C. baltica	brackish	no	monoecious	
C. liljebladii	brackish	not sure	monoecious	unclear
C. horrida	brackish	yes	monoecious	
C. intermedia	fresh	no	monoecious	
C. intermedia Salziger See	brackish	no	monoecious	first description of *C. intermedia*
C. baltica Mediterranean Sea	brackish	no	monoecious	
*C. hispida***	brackish & fresh	yes	monoecious	
Species of the *C. aspera* complex				
C. aspera var. *curta*	brackish & fresh	yes	dioecious	unclear
C. aspera var. *aspera*	brackish & fresh	yes	dioecious	unclear
C. aspera var. *subinermis*	brackish & fresh	yes	dioecious	unclear
Species of the *C. canescens* complex				
C. canescens	brackish	no	parthenogenetic	unclear
C. canescens	brackish	only female individuals	dioecious	unclear

In contrast, *C. aspera* occurs both in brackish and freshwater (Krause 1997) and three different morphotypes have been described in literature (*C. aspera* var. *aspera*, *C. aspera* var. *subinermis,* and *C. aspera* var. *curta*, Wood 1965, Krause 1997). However, they are regarded as variations and are not considered to be individual species by these authors.

C. canescens is the only known *Chara* taxon where sexual and parthenogenetic reproduction occurs (Wood 1965, Krause 1997, Blindow 2003a).

This exemplifies the ambiguity in the determination and separation of charophyte species.

Methods

Various methods can be used to determine differences between species. As described above, there are several difficulties in using the phenetic species concept for *Chara*. In addition, the most recent analyses demonstrate that morphology is not a habitat independent characteristic. Different environmental parameters, for example light or salinity, can impact the morphology (Schneider 2006, Blindow 2006).

Based on new methods, the morphological taxonomy is supported by genetic or ecological data. Accurate determination of charophyte genera and species can be based on genetic analyses (Karol 2001). With genetic methods, the similarity between individuals, populations and species can be analysed independent of habitat. Sakayama (2004) separated different *Nitella* species using multiple markers of nuclear DNA (internal transcribed spacers ITS and 5.8S ribosomal RNA). In contrast, Donner (2006) analysed the large subunit of rubisco (*rbcL*) and showed that within the genus *Chara,* few differences between species occurred. However, the small number of these analyses does not allow us to conclude that the method can be used for species determination. Many investigations show that the amplified fragment length polymorphism (AFLP) technique is applicable for the differentiation of species (Says-Lesage, 2002), populations (Hongtrakul 1997, Roldan-Ruiz 2000) and the degree of relatedness (Kardolus 1998). Mannschreck (2002, 2003) demonstrated that this method could be adapted to charophytes, leaving open the possibility to make statements of similarity between charophyte species. However, genetic information cannot be used to determine the degree of adaptation of a specific species to conditions within a habitat. For example, it is not possible to interpret whether a species is poorly or advantageously adapted to its environment. In order to obtain information about the species' physiological capabilities and limitations, physiological analyses are necessary.

In this context it is important to direct attention to the ecological requirement of species. Abiotic and biotic conditions were compared with physiological measurements to detect differences in the adaptation potential between individuals of different species. Küster (2004) shows that differences can be found in charophytes; *C. baltica, C. canescens,* and *Lamprothamnium papulosum* growing at the same sampling site showed specific adaptations to light and salinity (Küster 2004). Comparable to genetic differences between species,

physiological adaptations are representative of species or populations. However, characteristics that are affected by climate or habitat also must be compared by analyses under laboratory conditions. In the field, Küster (2000) found apparent differences between *Lamprothamnium papulosum* populations that could not be reproduced under laboratory conditions.

Different species concepts consider the ability for sexual reproduction between individuals, populations or species as a main characteristic for differentiation (Wägele 2000). Crossing is primarily used within zoological species differentiation rather than in botanical taxonomy. This is reflected by the often unclear situation in plants with hybrids and intermediate forms. In charophytes, little information about fertility and crossing abilities exists. A successful crossing event within charophytes could be interpreted as a possible relatedness between individuals.

Therefore the three *Chara* species complexes where analysed with the following methods:

A) Morphological analyses (see method description in paper 1)
B) Ecophysiological analyses (see method description in papers 2 and 3)
C) Genetic analyses using AFLP and sequence analyses (see method description in papers 1-5)
D) Crossing experiments (see method description in paper 4 and Blindow 2010).

Objectives

Within this study, the following objectives were investigated and compared for *C. aspera, C. baltica, C. intermedia, C. horrida, C. liljebladii,* and *C. canescens:*

i) Evaluation of the morphological spectrum within genetically uniform sampling material and determination of morphological differences between genetically different groups.
ii) Determination of the ecophysiological differences between the species and their adaptation abilities to salinity conditions.
iii) Determination of the genetic similarity between sexual interacting populations, as well as genetic differences between sexual separated populations. Comparison of the genetic similarity within and between different species complexes (*C. baltica, C. intermedia*; *C. aspera*; *C. canescens*) as well as determination of genetic

differences between morphological varieties of these complexes (*C. aspera* var. *aspera*, *C. aspera* var. *subinermis*, *C. aspera* var. *curta*, *C. horrida*, *C. liljebladii*).

iv) Investigation of the abilities for sexual reproduction within and between taxa identified solely upon morphological characteristics.

v) Recording the usability of different methods for differentiation based on ecophysiological parameters, morphological acclimation reactions, genetic differences, and the ability for sexual reproduction.

3. RESULTS & DISCUSSION

Differentiation between *C. baltica* and *C. intermedia*

One goal of the study presented here was to test the morphological separation criteria of Wood (1965), Corillion (1972), Hollerbach (1983), Pankow (1990), Krause (1997), and Blindow (2003a) using modern methods. This is especially important for *C. baltica* and *C. intermedia* because in the literature, both species are separated based on different determination criteria. In our analyses, none of the morphological characteristics used in the literature could separate *C. baltica* and *C. intermedia* into two species (paper 1). In addition, the morphological variability within populations of these species does not allow distinction of individual populations (paper 1).

In contrast to the morphological similarities, different adaptations to the climate and habitat conditions in the Baltic Sea, Salziger See, Lauterbach, and southern France sample sites could be found. These adaptations were stable under laboratory conditions independent of climate and abiotic and biotic influences (paper 2). *C. baltica* from the Baltic Sea (brackish water) and *C. intermedia* from the Lauterbach (fresh water) show major differences in their physiological reactions. These differences are constant over a range of salinities from fresh to brackish water. Therefore these differences cannot be regarded as variable, short term reactions to different site conditions, but as long term, constant adaptations. These adaptations allowed individuals to grow better in their respective habitats. A probable explanation for this improvement is the adaptation to the different habitat conditions. Separation of *C. baltica* from the Baltic Sea and *C. intermedia* from fresh water as two species based on different habitats, as per the literature (e.g. Krause 1997), is supported by our physiological results.

Within the field experiments, the individuals sampled from the Salziger See and southern France demonstrate different physiological responses to habitat conditions. However, these are obviously short term adaptations to the different climate conditions because under controlled laboratory conditions, these differences vanished and the populations reacted similarly (paper 2). The climate and chemical conditions between these sites differ considerably. The salinity of the Mediterranean water bodies fluctuates seasonably as a

consequence of evaporation in the summer months. The Salziger See is also brackish; however the salinity is based on the local geology with abundant potassium ions and varies little during the year. Despite the differences in habitat specific conditions, the results suggest that the algae have comparable physiological adaptation mechanisms. However, no studies exist which compare the reaction to brackish water with the reaction to potassium ion concentrations. It is important to note that the physiological reactions of the individuals from the Salziger See and southern France are different from the individuals of the Baltic Sea and the Lauterbach (fresh water). Some parameters, e.g. E_k or P_{max}, of the individuals sampled from the Salziger See and southern France were more similar to individuals from the Baltic Sea, whereas other parameters, e.g. Chl a/Car ratio, were more similar to individuals from the Lauterbach. Further research must determine whether these varying reactions could be explained by different physiological requirements (paper 2).

The genetic analyses show a separation between *C. baltica* and *C. intermedia*, while the specima from the Salziger See and southern France are intermediate (paper 2). The genetic results therefore support the physiologic results, but are in contrast to morphology, where no differences could be found. Species that are morphologically undeterminable but genetically separable are called 'cryptic species' (e.g. Adams 2005). Our results show that *C. baltica* and *C. intermedia* are cryptic species.

Within the genetic analyses, individuals from the Mediterranean and the Salziger See sampling sites are grouped in between *C. baltica* and *C. intermedia* individuals (paper 2). This suggests the possible necessity of similar adaptations to the habitat (Winter 1991). The importance of habitat salinity selecting those genotypes adapted to the environmental condition of a water body was also stressed by Triest (2009) for the aquatic macrophyte *Ruppia*.

The results of Blindow (2010) show, that under laboratory conditions crossing events can be found within the different species of the C. *baltica* - *C. intermedia* complex (table 3). However, only a small number of successful crossing events were observed: a quantitative analysis of crossing events is impossible. In the analyses, the number of fertile oospores was counted as crossing success, the highest number (six) was found in between samples from the Salziger See and southern France. This confirms the results of the physiological and genetic analyses.

Table 3: (Blindow 2010): Number of oospores in male-sterilised plants (bold) and number of replicates (in brackets) for cross-fertilisation experiments during 2004 and 2005. The shaded cells represent autogamy (self-fertilisation). Dashes represent crosses that were not attempted. * These two oogonia were fertilised (darkened), but did not develop into ripe oospores.

	Male-fertile plants					
Male sterilised plants	"French *baltica*"	*C. baltica*	*C. horrida*	*C. liljebladii*	"*intermedia* S"	*C. intermedia*
"French *baltica*"	**12** (9)	**3** (10)	-	-	**6** (4)	-
C. baltica	**0** (10)	**0** (10)	**0** (5)	**0** (5)	**0** (5)	**0** (5)
C. horrida		**4** (5)	**1** (7)	**0** (7)	-	**1** (7)
C. liljebladii		**0** (7)	**0** (7)	**1** (7)	-	**0** (7)
"*intermedia* S"	**2*** (5)	**0** (5)	-	-	**8** (4)	-
C. intermedia		**0** (7)	**0** (7)	**0** (7)	-	**0** (7)

Two evolutionary processes could possibly explain this result. First, the genetic results could be based on phylogenetic evolution, where two different specialised species have one common ancestor. *C. intermedia* individuals are better adapted to fresh water habitats and *C. baltica* individuals grow better in brackish water. The adaptations necessary to survive and reproduce in the new habitat resulted in a selection process, leading to genetic separation. Within the charophytes, a common mechanism for salinity adaptation is found (Winter 1996) therefore it is probable that salinity adaptation is a primary adaptation. We can assume that the common ancestor has a broad physiological tolerance to salinity and was not specialised to one particular habitat. The presumed ancestor is most likely comparable to the Mediterranean population. In this region, great annual climatic variations are common. Due to high precipitation in winter the salinity of water is low while in summer, due to high evaporation, salinity is high. Therefore in charophytes, a broad tolerance is necessary. In contrast to the other habitats examined in this study, the Mediterranean region was not covered with ice during the glacial period. From this refuge, other habitats could be colonised during glacial retreat. The present Mediterranean populations could be considered as survivors of the common ancestor of *C. baltica* and *C. intermedia*. However, this is no explanation for the similarity between the Mediterranean and the Salziger See samples. The Salziger See differs in comparison to the other brackish water habitats with respect to ion composition. Comparable studies on genetic diversity show that the highest degree of genetic diversity is found within the oldest populations (Wägele 2000). If the hypothesis that the brackish water *C. baltica* and the freshwater *C. intermedia* descended from a common

ancestor similar to the Mediterranean/Salziger See populations is true, further investigations are necessary. It has yet to be shown whether there is a higher degree of genetic diversity in charophytes of the Mediterranean region compared to the *C. baltica* and *C. intermedia* group. Differentiation of the submerged macrophyte *Zannichellia* into, among others, haplotypes from western European freshwaters has been shown, with an especially close evolutionary relationship occurring between those from the Baltic Sea and southern France (Triest 2007).

A second explanation for an intermediate population is hybridization between *C. baltica* from the Baltic Sea and *C. intermedia* from fresh water habitats. Populations resulting from hybridization between species would also exhibit intermediate ecophysiological characteristics, as was observed in our experiments. The hybrid could have been distributed by waterfowl all over Europe and survived in special habitats with respect to salinity regime. Distribution of charophytes by waterfowl was described by Charalambidou (2005). However, the results of Blindow (2010) showed no crossing events between *C. intermedia* and *C. baltica*. Therefore, the hypothesis that the populations of the Salziger See and southern France are established by crossing of *C. baltica* and *C. intermedia* individuals seems to be improbable. Nevertheless, it is unclear how frequently crossing events happen within monoecious populations.

Both the genetic and the physiological analyses separated populations from the Baltic Sea and from fresh water habitats, but populations from the Salziger See and southern France appeared intermediate, indicating that a continuum exists. Morphological analysis however, revealed no distinct groups. This result is consistent with the assumption that the variation in the *C. baltica – C. intermedia* cluster can be seen as habitat-specific forms of one species - similar to the brackish water and freshwater forms of *C. aspera*. Alternatively, it is also possible that the *C. baltica – C. intermedia* cluster is real. It can be assumed that there exist either cryptic genotypes with no morphological differences or that other characters not assessed in our work such as gametangia size (cf. Corillion 1972) differentiate the groups. Blume (2009) found differences in oospore morphology between *C. baltica* populations from the Baltic Sea and from France. We therefore conclude that the *C. baltica – C. intermedia* complex either consists of at least three species: one corresponding to the samples from the Baltic Sea, one to the samples collected from freshwater and one to the samples collected from the Salziger See and the Mediterranean, or the mentioned complex must be regarded as

variation within one species. The present differentiation in two species (Corillion 1972, Hollerbach 1983, Pankow 1990, Krause 1997, Blindow 2003a) is not supported by our data.

Differentiation between *C. horrida* and *C. liljebladii*

C. horrida and *C. liljebladii* grow in brackish water in the Baltic Sea, i.e. in the same habitat as *C. baltica*. In our analyses, *C. horrida* and *C. baltica* were distinguished morphologically by two rows of stipulodes for *C. baltica* and by more than two rows in *C. horrida* (Donner 2006, paper 3). In contrast, *C. liljebladii* is known as a huge *C. baltica* and is difficult distinguish (Wood 1965, Blümel 2003). Therefore the species status of *C. liljebladii* is discussed controversially within literature (Blümel 2003). In our analyses, no morphologic separation characteristics were found (Donner 2006, paper 3). Within other species e.g. *C. contraria*, enlarged forms were also observed with slightly varying morphology in contrast to the classical species description (Krause 1976). In *C. contraria*, this growth form is found in deep water sites (15 - 20 meters depth). However, this pattern does not differentiate the two growth forms as different species. The *C. liljebladii* in this study was sampled in a water depth of 2 - 4 meters, which is much shallower than in the case of *C. contraria*. Therefore, the morphological variation of *C. liljebladii* could not be explained by water depth.

In our ecophysiological analyses of *C. horrida*, *C. liljebladii*, *C. baltica*, and *C. intermedia* individuals, no clear separation was found (Peters 2006). These results are in contrast to those of *C. baltica* and *C. intermedia*, which were distinguished as different species. A reasonable explanation could be the differences in the experimental design; in the above mentioned study *C. baltica* and *C. intermedia* were grown under continuous light conditions, which was likely a stress factor. In the other study, *C. horrida*, *C. liljebladii*, *C. baltica*, and *C. intermedia* were exposed to 16 hours daylight followed by an eight hour night phase (Peters 2006). The differences between the *C. baltica* and *C. intermedia* populations were only apparent under additional stress conditions (continuous light conditions). It was not possible to separate *C. horrida* and *C. liljebladii* from neither each other nor *C. baltica* and *C. intermedia* from *C. horrida* and *C. liljebladii*. In comparable experiments, Blindow (2003b) and Küster (2004) independently showed that physiological differences can be found between *Chara* populations and species. However in both studies, *C. aspera* (Blindow 2003b) and *C. baltica*, *C. canescens* (Küster 2004) were exposed to higher photon conditions than were used in our

experiments. This suggests that methodology plays an important role in determining species that are closely related.

In the genetic analyses, *C. horrida* and *C. liljebladii* clustered together with *C. baltica* in one group (paper 3). These genetic results are in contrast to the morphological analyses, where *C. horrida* could be separated from the others.

Within the crossing experiments, four fertile oospores of the *C. baltica* populations and the *C. horrida* individuals could be found (table 3). These are probably based on genetic similarities between these species. The harmonisation of the gene pool inhibits separation into genetic groups; therefore the morphological differences must be interpreted as variability of one group rather than independent species. Based on the small number of crossing events within the *C. horrida, C. liljebladii, C. baltica* and *C. intermedia* populations, the significance of the method can still be doubted. The reasons for the small number of successful crossing experiments could be many: first, there are crossing barriers between the different species and therefore only a small number of crossing events could be observed. However, a higher number of crosses within one population would be expected. Second, an overall low fertilisation success in male-sterilised plants can be explained by lower success of allogamous compared to autogamous fertilisation (Blindow 2010). Third, within laboratory experiments, the number of crossing events is lower than within natural habitats. This is based on the poor condition of the male-sterilised plants due to repeated removal of the upper whorls, combined with the fact that these plants were stunted and only possessed one to two intact whorls (Blindow 2010). Additionally, the formation of oospores does not allow the conclusion that the crossing partners belong to the same species. In many crossing experiments, oospores formed either did not germinate or the germinated plants were self-sterile (Proctor 1975). Only a few oospores were formed by male-sterilised plants, preventing any conclusions about incompatibility between the combined taxa. In the experiments, oospores were not even formed in all combinations within the same population. However, lower fertilisation success was generally observed in crosses among populations (all representing different taxa) than within populations (Blindow 2010). This could indicate the existence of reproductive barriers among single taxa of the '*Chara baltica* species complex' (Blindow 2010).

Four different explanations are possible for this apparent discrepancy between the methods:

First, in monitoring studies of Swedish charophytes, many forms were found that could not be defined unambiguously (Irmgard Blindow personal comments). It seems that a high

morphological variability exists within these species. All three species occur closely together in one habitat. Although there is little evidence in our experiments, crossing events can cause intermediate forms and morphological variability as well as the genetic similarity. Although it is assumed that within the monoecious species, vegetative or clonal reproduction is more frequent, sexual reproduction must occur within one habitat. Otherwise we would not observe one genetic group, but only broadly distributed clones. This would explain why *C. intermedia* can be genetically separated from *C. baltica*, *C. horrida*, and *C. liljebladii*. This conclusion only explains the observed genetic similarity but does not indicate why different morphotypes emerged within the Baltic Sea.

Second, the morphologic variability can be caused by adaptation to habitat conditions. Adaptation results in morphological differentiation which is not genetically fixed and can be due to various reasons:

i) Physiological adaptations: Our own physiological analysis only showed small differences in response to salinity and light variability within *C. baltica*, *C. horrida*, and *C. liljebladii* (Peters 2006). All three species grow close together under the same environmental conditions, which makes a physiological adaptation advantage for one species unlikely.

ii) Grazing pressure: Grazing of charophytes by fish (Ten Winkel 1984), snails (Sheldon 1987), crayfish (Nyström 1996) and amphipods (Proctor 1999) has been recorded. Proctor (1999) hypothesised that secondary structures like spine cells may protect against grazing. Grazing experiments can be used to test this hypothesis. However, preliminary results have not yet provided evidence that a higher grazing pressure leads to the formation of more spine cells or stipulodes (Schneider unpublished research, Senft 2003).

iii) Unidentified chemical or physical reactions: Idestam-Almquist (1995) found differences in the phenotype of *Potamogeton pectinatus* which were caused by different nutrient conditions of the sediment. Since small-scale differences in the nutrients of the sediment commonly occur (Brandl 1993), they could in theory explain differences in *Chara* morphology even if the specimens grow very close to each other in the same habitat. Growth experiments at different nutrient concentrations should be used to test whether nutrient concentrations cause

differences in plant length and number of spines and stipulodes within the *C. baltica* group.

iv) Inter- and intra-specific competition: These interactions may cause morphologic variation, however no studies exist for charophytes as yet.

Third, a mutation that causes the morphologic differences is not detected by the AFLP analyses we used due to nature of the mutation or location on the genome. However, AFLP technique is usually regarded as being a rather high resolution method which can be used to detect even small genetic differences (Schaeffer 2002, Murphy 2003, Erting 2004). Nevertheless, very small genetic differences between *C. baltica*, *C. horrida,* and *C. liljebladii* cannot be completely excluded. Furthermore, the results show that the morphological traits of plant size, arrangement and length of spine cells and number and length of stipulodes are of minor importance for differentiation among these *Chara* species. Our data therefore support the conclusions of Wood (1965), who described *C. baltica*, *C. liljebladii* and *C. horrida* as different forms of one species.

Fourth, a possible explanation for morphological differences in the '*C. baltica* group', in the absence of genetic differences detectable by AFLP technique, is chromosome duplication. Autopolyploidy can lead to morphological differences (Liu 2007) and duplication of chromosomes cannot be detected by AFLP method. This hypothesis is supported by the observation that *C. aspera* can have either 12 or 14 chromosomes (Guerlesquin 1996) only one study reported the number of 21 chromosomes (Ravanko 1988 in Guerlesquin 1996). It can be assumed that different chromosome numbers of *C. aspera* would correspond with different morphological variations. However, no studies exist to prove this. *C. baltica* has 28 chromosomes (Guerlesquin 1996), but to our knowledge the chromosome number of *C. horrida* is unknown. Here, further investigation in needed.

In view of these genetic studies, it will be necessary to re-evaluate species conservation programs and Red Lists. For example *C. horrida* is regarded as 'vulnerable' in Sweden and action plans to protect this 'species' have started, whereas *C. baltica* is rather common and thus not Red-Listed (http://www.artdata.slu.se/rodlista/). Assessment of the significance of differences between morphological and genetic diversity is going to be one of many challenges in future discussions about biodiversity.

Differentiation between *C. aspera* in contrast to *the C. baltica- intermedia*-complex

Morphologic variability in form of different growth types is also described within the *C. aspera* complex. Three different variations can be found in literature: *C. aspera* var. *subinermis*, *C. aspera* var. *aspera*, and *C. aspera* var. *curta* (Krause 1997, Blindow 2003a). Similar to *C. baltica*, *C. horrida*, and *C. liljebladii*, these morphological variations can occur in the same habitat. In contrast to *C. baltica*, *C. horrida*, and *C. liljebladii*, Krause (1997) and Blindow (2003a) do not regard the *C. aspera* varieties as different species.

Comparable to the physiological experiments performed on *C. baltica* and *C. intermedia* in this study, Blindow (2003b) analysed *C. aspera* individuals sampled from different salinities, ranging from brackish to fresh water. Different responses were found to occur with respect to origin. For individuals originating from fresh water habitats, the maximum values for maximum photosynthesis capacity, light saturation, and Ek-values occur under fresh water conditions. In contrast, individuals originating from brackish water habitats show optimal adaptation responses within salinities ranging between 5 and 10 PSU (Blindow 2003b).

These results are comparable to our experimental results performed with *C. baltica* and *C. intermedia*. The fresh and brackish water individuals differed in their maxima in laboratory experiments. Using physiology as a determination criterion, it could be argued that just as *C. baltica* and *C. intermedia* individuals were classified into different groups, it is also possible for *C. aspera* to be distinguished into two distinct species.

Mannschreck (2003) demonstrated that *C. aspera* individuals were not genetically separable between brackish and freshwater populations; these results are confirmed by the results presented here, as no differentiation by habitat was observed (paper 4). This result is in direct opposition to the separation of *C. baltica* and *C. intermedia* by different habitat conditions. The difference between the three species cannot be explained by variations in methods or experimental design, as the same protocol was used in both analyses. Habitat could also not explain these differences between the species. *C. aspera* grows in brackish and fresh water and the populations are therefore forced by the same adaptation mechanisms. However, within our physiological experiments, adaptation reactions to the different habitats could be observed within all species. Within *C. baltica* and *C. intermedia*, adaptations to different

salinity caused a genetic separation whereas this separation was not observed by genetic analysis in *C. aspera*.

However, neither Mannschreck (2003) nor O'Reilly (2007) could genetically separate *C. aspera* var. *subinermis*, *C. aspera* var. *aspera*, and *C. aspera* var. *curta* in distinguishable groups. Genetically separable groups could be found only in populations from different locations. The morphological differences between the *C. aspera* forms could be due to the same reasons as stated above for *C. horrida, C. liljebladii* and *C. baltica*.

Two explanations for the apparent discrepancy between the *C. baltica-intermedia* complex and *C. aspera* are possible. First, the separation of *C. baltica* and *C. intermedia* into two species could date back earlier than the separation of the *C. aspera* populations. If the *C. aspera* brackish water population and the freshwater individuals were not isolated long enough, random mutations could not generate sufficient identifiable genetic differences, therefore no separation can be found currently. Alternatively, phylogenetic background and habitat differences are not the only reasons for the separation of species, as the distribution abilities (i) and the reproduction mechanisms (ii) must also be considered:

(i) The main distribution vector for charophytes is transport by waterfowl. This could be by external transport with shoot pieces trapped within the coats of birds (Charalambidou 2005), or internally, as oospores are still viable after intestinal passage within birds (Charalambidou 2005). Transport by waterfowl is one reason why charophytes are considered as pioneer species (Krause 1997) and are therefore able to successfully colonise new environments. Species-specific differences in transport frequency, grazing incidence, and settlement success could explain the differences between the *C. aspera*, *C. baltica*, and *C. intermedia* species.

(ii) Within charophytes, asexual and sexual reproduction is very common. Asexual reproduction occurs from shoot pieces, special organs called bulbills, or through parthenogenetic reproduction. However, parthenogenetic reproduction was described only for *C. canescens* (Braun 1857, Ernst 1918, 1921, Krause 1997) and it is unknown to which extent it occurs in other species. The different reproduction mechanisms may significantly alter the evolutionary process. With a higher sexual reproduction rate, higher genetic similarity exists, whereas with asexual reproduction, genetic differences could be established faster. Sexual reproduction can be found in monoecious and dioecious species. *C. aspera* is dioecious in contrast to the monoecious species *C. baltica* and *C. intermedia*. Differences in the success rate of sexual reproduction between monoecious versus dioecious species can be assumed.

Within laboratory crossing experiments, a higher amount of crossing events occurred within the dioecious *C. aspera* species (paper 4 and Blindow 2010), in contrast to the monoecious *C. baltica* and *C. intermedia* species. If it is assumed that this is not based on laboratory conditions, the differences in sexual reproduction rates could be one explanation of the genetic differences. With higher amounts of sexual reproduction smaller genetic differences can be found for *C. aspera* than for the *C. baltica-intermedia complex*. Beside the reproduction mode, the differences between the *C. baltica-intermedia* complex and *C. aspera* could also be based on success rate in clonal reproduction. Higher amounts of clonal reproduction should than be found within the *C. baltica-intermedia* complex. However, it is unknown how often clonal reproduction happened within the *C. baltica-intermedia* complex in contrast to *C. aspera* or other charophyte species. Assuming that differences in sexual reproduction are more important than differences in the migration process, this could explain best the genetic separation within the *C. baltica-intermedia*-complex and its absence in *C. aspera*.

Differentiation within *C. canescens*

No morphologic separation exists within the *C. canescens* populations. In ecophysiological experiments, Küster (2004) found only small differences in *C. canescens* populations.
C. canescens is well suited example to compare genetic differentiation within a single species. Within *C. canescens*, both parthenogenetic and sexual reproduction occurs. In many water bodies only female plants exist, but these plants produce fertile oospores. No morphological analysis is known to separate the sexual from the parthenogenetic reproducing female plants. However, in our study, genetic analyses show that differentiation into two groups is possible (paper 5). One group represents populations with male and female and therefore sexual reproductive plants. The other group represents populations with only female and thus parthenogenetically reproductive plants. Even within the mixed populations, some female plants clustered within the parthenogenetically reproducing plants. Due to the fact that genetically distinguishable individuals were found within the same habitat, we assume that a clear separation is possible and might indicate the beginning of a speciation process with different reproduction modes. To determine whether this has already resulted in crossing barriers, further studies are necessary.

If different reproduction modes foster a separation within one species, it could be assumed that comparable mechanisms also work in other species. It would then be expected that within dioecious species, a higher amount of sexual exchange exists than within monoecious species. This can explain why genetic separation occurs within the monoecious *C. baltica - intermedia* cluster but not in *C. aspera*, where no separation can be found. Therefore, the two different reproduction modes could be separated genetically: on one side the *C. baltica - intermedia* cluster and the parthenogenetically reproducing *C. canescens* populations and on the other side the *C. aspera* and the sexual reproducing *C. canescens* populations. To test this hypothesis, the genetic variability within the different populations should be analysed; for the sexual and dioecious reproduction modes, higher genetic similarity must be found than within the parthenogenetic or monoecious reproducing plants. For these analyses, nearly equal numbers of populations are necessary to compare the genetic variability within these populations. However, no other sexually reproducing *C. canescens* populations are known than those analysed here.

Species concepts in charophytes

Our results show that the phenetic species concept does not explain all speciation processes in charophytes (table 4). In the ecological species concept, the adaptation factors to similar and different habitats are integrated. Physiological results clearly show that charophytes adapt to their habitat. These responses were found at both the phenetic species (*C. intermedia*, *C. baltica*) and population (*C. aspera*, *C. canescens*) levels. In our study, the analyses support the distinction of the *C. baltica* individuals from the Baltic Sea and fresh water *C. intermedia* as two groups. However, the individuals of the Salziger See and the Mediterranean region connect the two groups genetically and physiologically. A clear separation into two species does not exist.

Elucidating speciation processes in charophytes by means of ecophysiological analysis is difficult because of differences in experimental design. Therefore it is not possible to postulate separation into two species for *C. aspera* even though differences are found in our ecophysiological analyses. Thus only limited information about ecophysiological diversity between or within species or populations can be derived.

To understand speciation processes in charophytes, knowledge of additional factors is important. This is true for morphological separation between *C. baltica, C. horrida* and *C. liljebladii* as well as between the *C. aspera* varieties. Our results indicate that morphologic differences in charophytes exist which do not correspond to ecophysiologic or genetic differences. At the moment, charophytes are used as indicator organisms for the trophic status of inland waters (Melzer 1988). However, if more information about the ecological reasons for the occurrence of the different morphotype exists, the different varieties of the species may explain other ecological conditions.

It is not possible to use the ecological species concept within charophytes because only few data are available for this purpose. These data show large differences to the previously used nomenclature, that is to say, the habitat specific separation of *C. aspera* origin from fresh or brackish water. Differences in their adaptation potential, for example, to different salinity conditions as described in Blindow (2000), or eutrophication (Melzer 1988, Blindow 2000), should be integrated in the species delineation descriptions. However, the reasons for the occurrence of e.g. the different *C. aspera* varieties, is still unclear or even if there is a speciation connection to habitat characteristics.

The phylogenetic species concept defined in Wägele (2000) states that each "species" forms a group of ancestors and their offspring irreversibly diverge in their genetic composition from other groups. Both groups or "species" do not include an irreversibly diverged sub-group. The advantage of genetic analyses is that various parameters are analysed independently of habitat adaptations or morphological variability. Similarities within DNA follow the coefficient of sexual exchange and sexual or asexual dispersion or the absence of these mechanisms.

The results of our genetic investigations show that *C. baltica* and *C. intermedia* are different even though they are morphologically similar. Even the individuals of the Salziger See and the Mediterranean Sea form their own group. Other groups, such as the varieties of *C. aspera* (var. *aspera,* var. *curta,* and var. *subinermis*) and *C. baltica* (*C. horrida* and *C. liljebladii*) cannot be distinguished genetically, although they are morphologically separable. Following the phylogenetic species concept, these are not taxonomically distinguishable and should not be denominated as species. Therefore, it is not possible to determine *C. liljebladii* and *C. horrida* as independent species by genetic analyses as claimed by Krause (1997) and Blindow (2003a). The reasons for the morphological differences could be similar for both groups; in the cases of *C. horrida, C. liljebladii* and *C. baltica* as well as the *C. aspera* variations, the

onset of speciation can be observed. Currently, different morphotypes exist but no genetic separation can be detected. For genetic differentiation to occur, an adaptation process must exist, e.g. different habitat conditions. Therefore *C. intermedia* from fresh water can be separated genetically from *C. liljebladii*, *C. horrida* and *C. baltica*. However, it is unclear if the morphological differences follow an adaptation or are only random. Alternatively, the morphologic differentiation could be based upon a process called gene silencing. In this case, different chromosome numbers cause variations in the expression of the morphological criteria. The morphological variation is then not part of an adaptation process but is determined solely by a random chromosome composition. As yet, no studies on the different chromosome numbers of the specific morphotypes exist.

As the limitations of the phylogenetic concept show, the differences in phenotype between species should be based on genetic investigations and interbreeding experiments. This is realised in the biological species concept. Therefore crossing experiments must be added to the analyses; these show a higher number of fertile oospores for *C. aspera* than for the *C. baltica* - *intermedia* complex. There are two possible explanations. First, dioecious *C. aspera* individuals may be easier to handle within the laboratory. In the experimental process, the antheridia (male reproduction organs) of the monoecious individuals must be removed in order to act as male sterilised plants. This can have a negative impact on the plant and therefore cause a lower reproduction success in the monoecious *C. baltica* - *intermedia* complex compared to dioecious *C. aspera*. Second, it is unclear how often sexual reproduction happens for charophytes. Blindow (2010) showed that more gametangia and greater fertilisation success resulted in a far higher number of oospores per plant from temporary water bodies compared to plants from permanent water bodies. This confirms the hypothesis and the suggestion by Casanova (1994), that the formation of oospores is more important for the persistence in temporary habitats. In permanent habitats however, it unclear how often sexual reproduction happens compared to asexual reproduction. For monoecious plants, the frequency of allogamy versus autogamy is unknown. For dioecious plants sexual reproduction is the only way to produce oospores and this always results in a homogenisation of the gene pool. This is in contrast to monoecious plants: if autogamy happens more often, genetic differences can be established easier than within dioecious species. However, all results show that a separation within the *C. baltica* - *intermedia* complex, as well as within the *C. aspera* complex, is not supported by the biological species concept.

Table 4: Overview of the results of this study (n.d. = no data) *C. hispida has been added for genetic comparison and because of a close morphological relationship. It is not part of the complex.

Species name	Morphologic separation possible	Ecophysiological separation possible	Genetic separation	Sexual separation
Taxa of the *C. baltica- intermedia* complex				
C. baltica Baltic Sea	no	no	yes, *intermedia* fresh water	unclear
C. liljebladii	not sure	no	yes, *intermedia* fresh water	unclear
C. horrida	yes	no	yes, *intermedia* fresh water	unclear
C. intermedia	no	no	yes, *baltica* Baltic Sea	unclear
C. intermedia Salziger See	no	no	no	unclear
C. baltica Mediterranean Sea	no	no	no	unclear
*C. hispida**	yes	n.d.	yes, all above	n.d.
Taxa of the *C. aspera* complex				
C. aspera var. *curta*	yes	n.d.	no	n.d.
C. aspera var. *aspera*	yes	n.d.	no	n.d.
C. aspera var. *subinermis*	yes	n.d.	no	n.d.
C. aspera brackish water	no	yes, from fresh water	no	no
C. aspera fresh water	no	yes, from brackish water	no	no
Species of the *C. canescens* complex				
C. canescens dioecious	no	no	yes, from dioecious	n.d.
C. canescens parthenogenetic	only female individuals	no	yes, from parthenogenetic	n.d.

Each of the discussed species concepts has its advantages and disadvantages (table 4). To understand the evolution of charophytes, morphological, physiological and genetic analyses are necessary as well as crossing experiments. Each analysis adds an element to the greater understanding of speciation. As a result of our studies it appears that the division of species in

smaller units, as done by the European authors (Corillion 1972, Hollerbach 1983, Pankow 1990, Krause 1997, Blindow 2003a), cannot be supported. Subsuming of larger units as done by Wood (1965) seems to be a more reasonable approach. However, only the combination of all information allows an understanding of the speciation process. Therefore, multidimensional analysis is also necessary in further studies.

4. REFERENCES

Adams R.P., Morris J.A., Pandey R.N., Schwarzbach A.E. (2005) Cryptic speciation between *Juniperus deltoides* and *Juniperus oxycedrus* (Cupressaceae) in the Mediterranean. Biochemical Systematics and Ecology 33: 771–787.

Blazencic J., Stevanovic B., Blazencic Z., Stevanovic V. (2006) Red data list of charophytes in the Balkans. Biodiversity and Conservation 15: 3445-3457.

Blindow I. (2000) Distribution of Charophytes along the Swedish coast in relation to salinity and eutrophication. International Review of Hydrobiology 85: 707-717.

Blindow I., Koistinen M. (2003a) Determination key for charophytes in the Baltic Sea. In: Schubert H., Blindow I. (Eds.), Charophytes of the Baltic Sea. Gantner Verlag, Rugell, pp. 27–36.

Blindow I., Dietrich J., Mollmann N., Schubert H. (2003b) Growth, photosynthesis and fertility of *Chara aspera* under different light and salinity conditions. Aquatic Botany 76: 213–234.

Blindow I., Schütte M. (2006) Elongation and mat formation of *Chara aspera* under different light and salinity conditions. Hydrobiologia 584: 69-76.

Blindow I., Erichsen I. (2010) Cross fertilisation within the *Chara baltica* species complex. Charophytes 2: 59-65.

Blümel C. (2003) *Chara baltica* Bruzelius 1824. In: Schubert H., Blindow I. (Eds.), Charophytes of the Baltic Sea. Gantner Verlag, Rugell, pp. 53–63.

Blume M., Blindow I., Dahlke S., Vedder F. (2009) Oospore variation in closely related *Chara* taxa. Journal of Phycology 45: 995–1002.

Bhattacharya D., Medlin L. (1998) Algal phylogeny and the origin of land plants 1. Plant Physiology 116: 9-15.

Brandl H., Hanselmann K.W., Bachofen R., Piccard J. (1993) Small-scale patchiness in the chemistry and microbiology of sediments in lake Geneva, Switzerland. Journal of General Microbiology 139: 2271–2275.

Braun A. (1857) Über Parthenogenese bei Pflanzen. Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin 10: 311–376.

Braun M., Foissner I., Lühring H., Schubert H., Thiel G. (2007) Characean green algae: still a valid model system to examine fundamental principles in plants. Progress in Botany 68: 193–220.

Casanova M.T. (1994) Vegetative and reproductive responses of charophytes to water-level fluctuations in permanent and temporary wetlands in Australia. Australian Journal of Marine and Freshwater Research 45: 1409-1419.

Casanova M.T. (2005) An overview of *Chara* L. in Australia (Characeae, Charophyta). Australian Systematic Botany 18: 25-39.

Charalambidou I., Santamaria I. (2005) Field evidence for the potential of waterbirds as dispersers of aquatic organisms. Wetlands 25: 252-258.

Corillion R. (1972) Les Charophycees de France et d' Europe Occidentale. Otto Koeltz Verlag, Koenigstein-Taunus.

Donner A. (2006) Morphologische und genetische Untersuchungen an Characeen. Diplomarbeit, Universität Rostock, Rostock.

Ernst A. (1918) Bastardisierung als Ursache der Apogamie im Pflanzenreich. Gustav Fischer Verlag, Jena.

Ernst A. (1921) Die Nachkommenschaft aus amphimiktisch und apogam entstandenen Sporen von *Chara crinita*. Zeitschrift für induktive Abstammungs- und Vererbungslehre 25: 185-197.

Erting L., Daugbjerg N., Moller Pedersen P. (2004) Nucleotide diversity within and between four species of *Laminaria* (Phaeophyceae) analysed using partial LSU and ITS rDNA sequences and AFLP. European Journal of Phycology 39: 224–256.

Feist M., Feist R. (1997) Oldest record of a bisexual plant, Nature 385: 401.

Guerlesquin M. (1996) On the variability of the known chromosome numbers observed in the characeae. In: Chaudhary B.R., Agrawal S.B. (Eds.) Cytology, Genetics and Molecular Biology of Algae pp. 95-113. Academic Publishing, Amsterdam.

Hollerbach M.M., Krasavina L.K. (1983) Charavye Vodorosli – Charophyta. Oprodelitel Presnovodnych Vodoroslej SSSR. Izdatielstvo Nauka, Leningrad, pp. 190. [in Russian]

Hongtrakul V., Huestis G.M., Knapp S.J. (1997) Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm: genetic diversity among oilseed inbred lines. Theoretical and Applied Genetics 95: 400-407.

Idestam-Almquist J., Kautsky L. (1995) Plastic responses in morphology of *Potamogeton pectinatus* L. to sediment and above-sediment conditions at two sites in the northern Baltic proper. Aquatic Botany 52: 205–216.

Kotta J., Torn K., Martin G., Orav-Kotta H., Paalme T. (2004) Seasonal variation in invertebrate grazing on *Chara connivens* and *Chara tomentosa* in Koiguste Bay, NE Baltic Sea. Helgoland Marine Research 58: 71–76.

Kardolus J.P., Van Eck H.J., Van den Berg S.J. (1998) The potential of AFLPs in biosystematics: a first application in *Solanum* taxonomy (Solanaceae). Plant Systematic and Evolution 210: 87-103.

Karol K.G., McCourt R.M., Cimino M.T., Delwiche C.F. (2001) The closest living relatives of land plants. Science 294: 2351-2353.

Krause W. (1976) Characeae aus Bayern. Teil 1. Bestimmungsschlüssel und Abbildungen. Bericht der Bayerischen Botanischen Gesellschaft 47, 229-257.

Krause W. (1997) Charales (Charophyceae). In: Ettl H., Gärtner G., Heynig H., Mollenhauer D. (Eds.) Süßwasserflora von Mitteleuropa. Band 18, Gustav Fischer Verlag, Jena.

Küster A., Schaible R., Schubert H. (2000) Light acclimation of the charophyte *Lamprothamnium papulosum*. Aquatic Botany 68:205-216.

Küster A., Schaible R., Schubert H. (2004) Light acclimation of photosynthesis in three charophyte species. Aquatic Botany 79: 111–124.

Liu G.F., Li Z.N., Bao M.H. (2007) Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology. Euphytica 157: 145–154.

Mannschreck B., Fink T., Melzer A. (2002) Biosystematics of selected *Chara* species (Charophyta) using amplified fragment length polymorphism. Phycologia 41: 657-666.

Mannschreck B. (2003) Genetische und morphologische Differenzierung ausgewählter Arten der Gattung *Chara*. Doktorarbeit TU- München. Shaker Verlag, Aachen.

Marin B., Melkonian M. (1999) Mesostigmatophyceae, a new class of streptophyte green algae revealed by SSU rRNA sequence comparisons. Protist 150: 399-417.

McCourt R.M., Karol K.G., Guerlesquin M., Feist M. (1996) Phylogeny of extant genera in the Family Characeae (Division Charophyta) based on *rbcL* sequences and morphology. American Journal of Botany 83: 125-131.

McCourt R.M., Karol K.G., Casanova M.T., Feist M. (1999) Monophyly of genera and species of characeae based on *rbcL* sequences, with special reference to Australian and European *Lychnothamnus barbatus* (Characeae: Charophyceae). Australian Journal of Botany 47: 361–369.

McCourt R.M., Delwiche C.F., Karol K.G. (2004) Charophyte algae and land plant origins. Trends in Ecology & Evolution 19: 661-666.

Meiers S.T., Rootes W.L., Proctor V.W., Chapman R.L. (1997) Phylogeny of the Characeae (Charophyta) inferred from organismal and molecular characters. Archiv für Protistenkunde 148: 308-317.

Meilinger P., Schneider S., Melzer A. (2005) The Reference Index Method for the Macrophyte-Based Assessment of Rivers – a contribution to the implementation of the European Water Framework Directive in Germany. International Review of Hydrobiology 90: 322-342.

Melzer A. (1988) Der Makrophytenindex – eine biologische Methode zur Beurteilung der Nährstoffbelastung von Seen. Habilitationsschrift TU München. 249 pp.

Melzer A. (1999) Aquatic macrophytes as tools for lake management. Hydrobiologia 395/396: 181–190.

Murphy N.E., Schaffelke B. (2003) Use of amplified fragment length polymorphism (AFLP) as a new tool to explore the invasive green alga *Caulerpa taxifolia* in Australia. Marine Ecology Progress Series 246: 307–310.

Noges P., Tuvikene L., Feldmann T., Tonno I., Kunnap H., Luup H., Salujoe J., Noges T. (2003) The role of charophytes in increasing water transparency: a case study of two shallow lakes in Estonia. Hydrobiologia 506: 567–573.

Nishiyama T. (2007) Evolutionary developmental biology of nonflowering land plants. International Journal of Plant Sciences 168: 37–47.

Nyström P., Strand J. (1996) Grazing by a native and an exotic crayfish on aquatic macrophytes. Freshwater Biology 36: 673–682.

O'Reilly C.L., Cowan R.S., Hawkins J.A. (2007) Amplified fragment length polymorphism genetic fingerprinting challenges the taxonomic status of the near endemic *Chara curta* Nolte ex. Kütz. (Characeae). Botanical Journal of the Linnean Society 155: 467–476.

Paper 1: Boegle M.G., Schneider S., Mannschreck B., Melzer A. (2007) Differentiation of *Chara intermedia* and *C. baltica* compared to *C. hispida* based on morphology and amplified fragment length polymorphism, Hydrobiologia 586: 155-166.

Paper 2: Boegle M.G., Schneider S.C., Schubert H., Melzer A. (2010) *Chara baltica* Bruzelius 1824 and *Chara intermedia* A. Braun 1859—Distinct species or habitat specific modifications, Aquatic Botany 93: 195-201.

Paper 3: Boegle M.G., Schneider SC, Melzer A., Schubert H. (2010) Distinguishing *Chara baltica*, *C. horrida* and *C. liljebladii* – conflicting results from analysis of morphology and genetics, Charophytes 2: 53-38.

Paper 4: Blindow I., Möllmann N., Boegle M.G., Schütte M. (2009) Reproductive isolation in *Chara aspera* populations, Aquatic Botany 91: 224-230.

Paper 5: Schaible R., Bergmann I., Bögle M., Schoor A., Schubert H. (2009) Genetic characterisation of sexually and parthenogenetically reproductive populations of *Chara canescens* (Charophyceae) using AFLP, *rbcL*, and SNP markers, Phycologia 48: 105-117.

Pankow H. (1990) Ostsee – Algenflora. Gustav Fischer Verlag, Jena.

Peters K. (2006) Anpassungen von Characeen an Salz- und Lichtstress. Diplomarbeit. Technische Universität München, München.

Proctor V.W. (1975) The nature of charophyte species. Phycologia 14: 97–113.

Proctor V.W. (1999) Charophytivory, Playas y Papalotes, a local paradigm of global relevance. Australian Journal of Botany 47: 399–406.

Roldan-Ruiz I., Denauw J., Van Bockstaele E., Depicker A., De Loose M. (2000) AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). Molecular Breeding 6: 125-134.

Sakayama H., Hara Y., Arai S., Sato H., Nozaki H. (2004) Phylogenetic analyses of *Nitella* subgenus Tieffallenia (Charales, Charophyceae) using nuclear ribosomal DNA internal transcribed spacer sequences, Phycologia 43: 672-681.

Schaeffer T.N., Smith G.J., Foster M.S. (2002) Genetic differences between two growthforms of *Lithophyllum margaritae* (Rhodophyta) in Baja California Sur, Mexico. Journal of Phycology 38: 1090–1098.

Schneider S., Ziegler C., Melzer A. (2006) Growth towards light as an adaptation to high light conditions in *Chara* branches. New Phytologist 172: 83–91.

Schubert H., Blindow I. (2003) Charophytes of the Baltic Sea. The Baltic Marine Biologists Publication No. 19. Gantner Verlag, Ruggell.

Senft M. (2003) Morphologische Veränderung an Characeen infolge von grazing durch Makroinvertebraten. Diplomarbeit, Fachhochschule Bingen.

Sheldon S.P. (1987) The effects of herbivorous snails on submerged macrophyte communities in Minnesota lakes. Ecology 68: 1920–1931.

Stelzer D., Schneider S., Melzer A. (2005) Macrophytebased assessment of lakes – a contribution to the implementation of the European Water Framework Directive in Germany. International Review of Hydrobiology 90: 223–237.

Tanabe Y., Hasebe M., Sekimoto H., Nishiyama T., Kitani M., Henschel K., Munster T., Theissen G., Nozaki H., Ito M. (2005) Characterization of MADS-box genes in charophycean green algae and its implication for the evolution of MADS-box genes. Proceedings of the National Academy of Sciences of the United States of America 102: 2436–2441.

Ten Winkel E.H., Meulemans J.T. (1984) Effects of fish upon submerged vegetation. Hydrobiological Bulletin 18: 157–158.

Triest L., Thi V.T., Sierens T. (2007) Chloroplast microsatellite markers reveal *Zannichellia* haplotypes across Europe using herbarium DNA. Belgian Journal of Botany 140: 109–120.

Triest L., Sierens T. (2009) High diversity of *Ruppia* meadows in saline ponds and lakes of the western Mediterranean. Hydrobiologia 634: 97–105.

Wägele J.W. (2000) Grundlagen der Phylogenetischen Systematik. Verlag Dr. Friedrich Pfeil, München.

Winter U., Soulié-Märsche I., Kirst G.O. (1996) Effects of salinity on turgor pressure and fertility in Tolypella (Characea). Plant, Cell and Environment 19: 869-879.

Winter U., Kirst G.O. (1990) Salinity response of a freshwater charophyte, *Chara vulgaris*. Plant, Cell and Environment 13: 123–134.

Winter U., Kirst G.O. (1991) Partial turgor pressure regulation in *Chara canescens* and its implications for a generalized hypothesis of salinity response in Charophytes. Botanica Acta 104: 37–46.

Wodniok S., Brinkmann H., Glöckner G., Heidel A.J., Philippe H., Melkonian M., Becker B. (2011) Origin of land plants: Do conjugating green algae hold the key?. BMC Evolutionary Biology 11: 104.

Wolfram G., Donabaum K., Schagerl M., Kowarc V.A. (1999) The zoobenthic community of shallow salt pans in Austria – preliminary results on phenology and the impact of salinity on benthic invertebrates. Hydrobiologia 409: 193–202.

Wood R.D. (1965) Monograph of the Characeae. In: J Cramer (Ed.) Wood R.D., Imahori K. A Revision of the Characeae. Weinheim.

Declaration of contributions as co-author

The first three papers are predominantly based on my own work. However, my work was supported by contributions of several co-authors:

Paper 1: Susanne Schneider and Arnulf Melzer participated in the conceptual design, the interpretation of the results and the writing of the manuscript.

Paper 2 and 3: Susanne Schneider participated in the conceptual design, the interpretation of the results and the writing of the manuscript. Hendrik Schubert and Karla Peters supported the physiological analyses and the interpretation, and together with Arnulf Melzer the discussion and writing of the manuscript.

Paper 4: resulted from a joint effort of Nils Möllmann, Irmgard Blindow, Manuela Schütte and myself. We discussed the topic in detail and outlined the manuscript together. Cross-fertilization and Oospore and bulbil germination were organised by Nils Möllmann and Manuela Schütte under supervision of Irmgard Blindow, the genetic results were analysed by myself. Irmgard Blindow led the writing of the manuscript and Nils Möllmann and I revised all drafts until completion.

Paper 5: resulted from a joint effort of Ralf Schaible, Ingo Bergmann, Arne Schoor, Hendrik Schubert and myself. We discussed the topic in detail and outlined the manuscript together. My part was the AFLP analyses and there implementation in the manuscript. Ralf Schaible led the writing of the manuscript and Hendrik Schubert, Arne Schoor and I revised all drafts until completion.

The diploma thesis of Karla Peters and Antje Donner were written within the project; Karla Peters supervised by myself Susanne Schneider and Antje Donner by Ralf Schaible and myself.