Evolution of Sacoglossa (Opisthobranchia) with emphasis on their food (Ulvophyceae) and the ability to incorporate kleptoplasts

Evolution of Sacoglossa (Opisthobranchia) with emphasis on their food (Ulvophyceae) and the ability to incorporate kleptoplasts

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Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst habe, keine anderen Quellen und Hilfsmittel als die angegebenen verwendet habe und die Stellen der Arbeit, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen sind, als Entlehnung kenntlich gemacht habe.

Bonn, den 16.11.2010

Preface

Everyone reading this PhD thesis may be astonished by the fact that one and the same target of this study has been carried out in several types, e.g. amplification reactions or different primers for the same aim. The reason for this is the ongoing search for financial support and lab facilities during this project. So research was executed in three different laboratories in Bonn; Institut für Evolutionsbiologie und Ökologie, Institut für Zelluläre und Molekulare Botanik (IZMB) and the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK).

Eventually, financial support of the DFG (German Science Foundation) (Wa 618/8-3) and the opportunity to get my "own" place in the lab of the ZFMK led to working options free from travelling through different institutes with their different possibilities for the last months of lab research. Different methods according to different places are explicated where they occur.

At this point I would like to thank Prof. Dr. Heike Wägele (ZFMK), my supervisor, for her sustained effort that enabled me to process this thesis and Prof. Dr. V. Knoop (IZMB) who employed me for some months in his lab. I thank both of them for their help in getting a PhD grant from the University Bonn.

I am aware that the taxon Opisthobranchia does most likely not refer to a monophyletic clade. Nevertheless, the term is used within this thesis. It comprises the taxon groups of "sea slugs" (coll.) that share the same habitat and developed similar morphological structures, e.g. cerata (dorsal appendices) or rhinophores (paired chemosensory organs, located in the head region of the animal).

Three publications are included as separate hardcopies. Händeler et al. 2009 and Händeler et al. 2010 are part of this thesis, which is explained in detail in the chapter "Results". Wägele et al. 2010 is included since all publications that contain data of this thesis has to be added to the thesis according to the Promotionsordnung.

Contents

1 Introduction

1.1 Sacoglossa

Sacoglossa is a small taxon within Opisthobranchia (Gastropoda). The term Sacoglossa was mentioned at first by von Ihering (1876). The often used synonym Ascoglossa was published in the very same publication since von Ihering had sent his manuscript to Bergh, who had discovered the same taxonomic group and called it Ascoglossa. Bergh published his work in 1877 and 1878. The morphological aspect that led both of them to the same finding is the so-called "sacus" or "ascus" (figures 1.1). The radula is currently growing/renewed by odontoblasts at the beginning of the radula band. In opisthobranch groups, except for Sacoglossa, the old teeth at the end are usually shed or can be found in the slug's digestive tract (pers. communication Heike Wägele). In contrast to many other opisthobranch taxa, the radula of Sacoglossa is uniseriate and only one tooth, the leading tooth, is used to feed. The radula is distinguished in the upper limb, consisting of new, unused teeth, the leading tooth, and the lower limb that is constituted by the used teeth (figure 1.1 a)). In sacoglossans these end up and are maintained throughout the slug's lifetime in an anatomical structure formed by an epithelium, the ascus or sacus. Up to now,

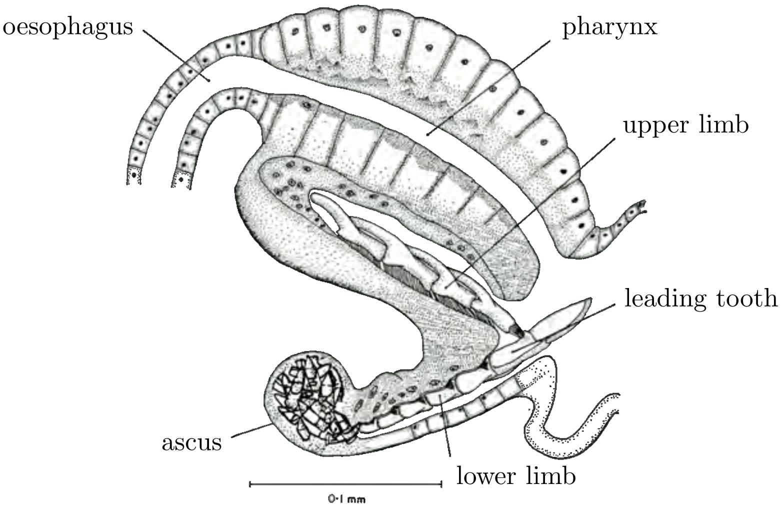


Figure 1.1 Sagital section of buccal region of the sacoglossan *Limapontia depressa*, modified after Gascoigne & Sartory 1974. The uniseriate radula is separated in the upper limb (new teeth), the leading tooth, the only effectively used tooth, and the lower limb, composed of the used teeth. These are maintained throughout the slug's lifetime in an epithelium-lined structure, the ascus or sacus. This unique structure combines the Sacoglossa.

there is no explanation for the evolution of such a structure especially since the teeth seem to be unspoiled in that epithelial sac and are not remineralized. Therefore Bergh's comment prevails until today: "ganz besonders merkwürdig ist ferner die Persistenz der ausgenutzten [...] einreihigen Zahnplatten, welche sich in einem besonderen Sacke an der Unterseite des Schlundkopfes, entweder spiralig aufgerollt oder in einen Haufen gesammelt, bis auf die erst gebildete, erhalten vorfinden." (Bergh, 1878, p.102) ["especially curious is furthermore the persistence of the used [...] single-rowed tooth-plates, which remain in a special bag situated under the pharynx, curled up or accumulated unregularly"].

In addition to the recently used synonyms Sacoglossa and Ascoglossa, one may find the term "Monostichoglossata" or "Stichoglossata" in older literature set up by Pagenstecher in 1874, but does not include all groups of Sacoglossa according to Bergh (1878).

Most sacoglossans are small animals of a size of less than one to about two cm. Very few reach four or five cm. Lately, a record of a 12cm long *Elysia ornata* has been reported (Jensen 2009), *Elysia crispata* can reach 15cm (Clark 1994). Sacoglossans are benthic organisms that can be found in the intertidal flat to a depth of about several 10m. They are not only small but can also be very cryptic on their food algae or the sediment, they crawl on (figure 1.2, see also figue 1.5b) and e), p. 6 or figure 3.19c), p. 72). Up to now, about 300 species have been described. Many undescribed species are documented in several online forums or even journals and books, e.g. Gosliner and coauthors presented *Elysia* sp. 1 to 25 in 2008.

Sacoglossa mainly feed on siphonous or siphonocladous green algae belonging to the taxon Ulvophyceae in the sense of Floyd and O'Kelly 1990. They pierce the algal cells with their leading tooth and suck the cell contents out. Some sacoglossans are able to incorporate the plastids of their food algae intracellularly in their digestive gland and keep these chloroplasts functional (Händeler et al. 2009). *Elysia chlorotica* is not only able to outlast, but also to reproduce on the photosynthetic products of their enslaved plastids alone (Rumpho et al. 2000).

In addition to kleptoplasts, many sacoglossans are reported to incorporate secondary metabolites from their food and use these as a chemical defence. Some sacoglossans modify this substances or produce their own feeding deterrents (Cimino et al. 1990; Cimino & Ghiselin 1998; Cimino et al. 1999; Cimino & Gavagnin 2006; Gavagnin et al. 1994a, b, c; Gavagnin & Fontana 2000; Marín & Ros 2004) (see figure 1.4d), p. 5 and 3.14c), p. 64).

The distribution of Sacoglossa is bound to the distribution of their food algae; high diversity can be found in the warm waters of the Indo-Pacific and the Caribbean, but some species can also be found in cold waters. Figure 1.3 gives an overview of sacoglossan records around the world according to Jensen 2007. The northernmost species are *Placida dendritica*, *Alderia modesta*, *Limapontia capitata* and *Limapontia senestra*; they occur in the northernmost part of Norway. The latter three and *Limapontia depressa* were recorded in the White Sea and Russian part of the Barents Sea (Northwest of Russia) (Jensen 2007 and references therein). The probably southernmost species were found in Patagonia: *Elysia* cf. *hedgpethi*, *Ercolania evelinae*, *Aplysiopsis brattströmii* and *Limapon-*

tia sp. (Schrödl 1996). Actually, sacoglossans are reported from any middle European coast; Mediterranean, Atlantic, North Sea, Baltic Sea, but some species have not been listed lately. E.g. *Alderia modesta* has not been found for about the last fifteen years along the German or Danish coasts (personal communication K. Jensen and own "non-observation" on field trips).

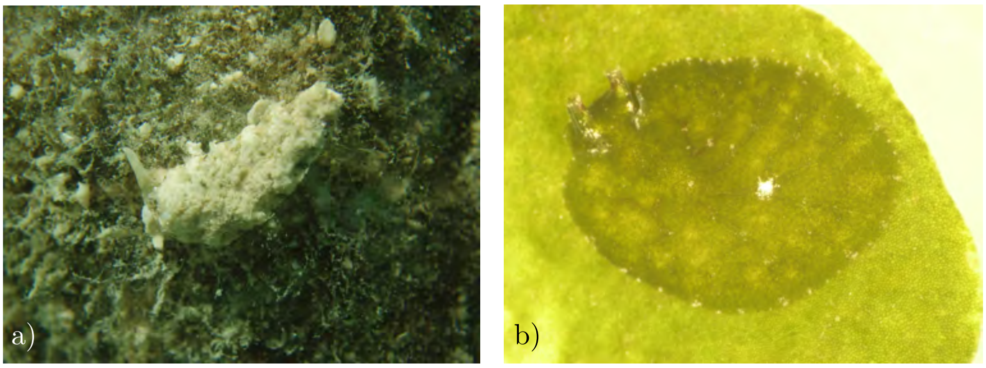


Figure 1.2 Cryptic sacoglossan species. a) *Plakobranchus ocellatus* (Guam, USA 2009) is almost invisible on the ground. Additionally to its colouration it is covered with sediment. b) *Bosellia mimetica* in its rest position on its food alga *Halimeda tuna* (Banyuls-sur-Mer, France 2006).

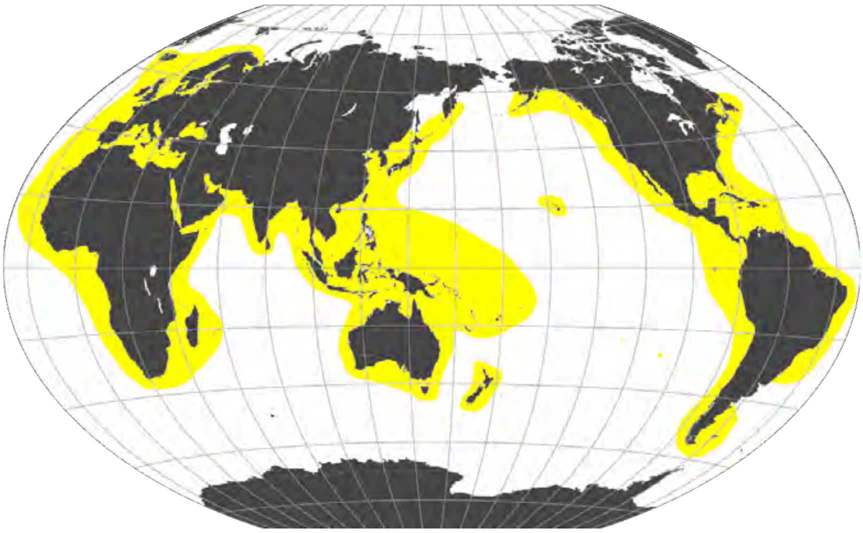


Figure 1.3 Distribution of Sacoglossa around the world according to Jensen (2007). Of course, data are biased by collection activity. Notice the information gap at the south-eastern coast of South America. Sacoglossa could also be found on the Easter Island (Schrödl 1996), and three of the Pitcairn Islands (Preece 1995); marks are too small. Map: http://www.mygeo.info/weltkarten.html.

Table 1.1 gives a survey of the classification of the genera of Sacoglossa following Jensen 2007, completed or changed according to own data.

The shelled Sacoglossa, the Oxynoacea (figure 1.4), present only about one fifth of the group. Most sacoglossan species belong to the group Plakobranchacea, the shell-less Sacoglossa. Two main groups are distinguished: the Lima–pontioidea and the Plakobranchoidea. Limapontioidean slugs bear dorsal appendices (figure 1.5), the so-called cerata. Plakobranchoidean slugs are characterised by lateral parapodia (figure 1.6).

Table 1.1 Classification of Sacoglossa. Number of described species is given in brackets (Jensen 2007, changed or completed according to own data).

Oxynoacea		Cylindrobullidae	*Cylindrobulla* (5)
		Volavatellidae	*Volvatella* (16)
			Ascobulla (7)
		Juliidae	*Julia* (6)
			Tamanovalva (3)
			Berthelinia (10)
		Oxynoidae	*Lobiger* (5)
			Lophopleurella (1)
			Oxynoe (8)
			Roburnella (1)
Plakobranchacea	PLAKOBRANCHOIDEA	Boselliidae	*Bosellia* (5)
		Plakobranchidae	*Elysia* (80)
			Elysiobranchus (2)
			Pattyclaya (2)
			Plakobranchus (1)
			Thuridilla (24)
		Platyhedylidae	*Gascoignella* (3)
			Platyhedyle (1)
	LIMAPONTIOIDEA	Polybranchiidae	*Caliphylla* (1)
			Cyerce (11)
			Mourgona (3)
			Polybranchia (8)
			Sohgenia (1)
		Costasiellidae	*Costasiella* (13)
		Limapontiidae	*Alderella* (1)
			Alderia (3)
			Alderiopsis (2)
			Calliopea (2)
			Ercolania (22)
			Limapontia (4)
			Olea (1)
			Placida (11)
			Stiliger (9)
		Hermaeidae	*Aplysiopsis* (9)
			Hermaea (13)

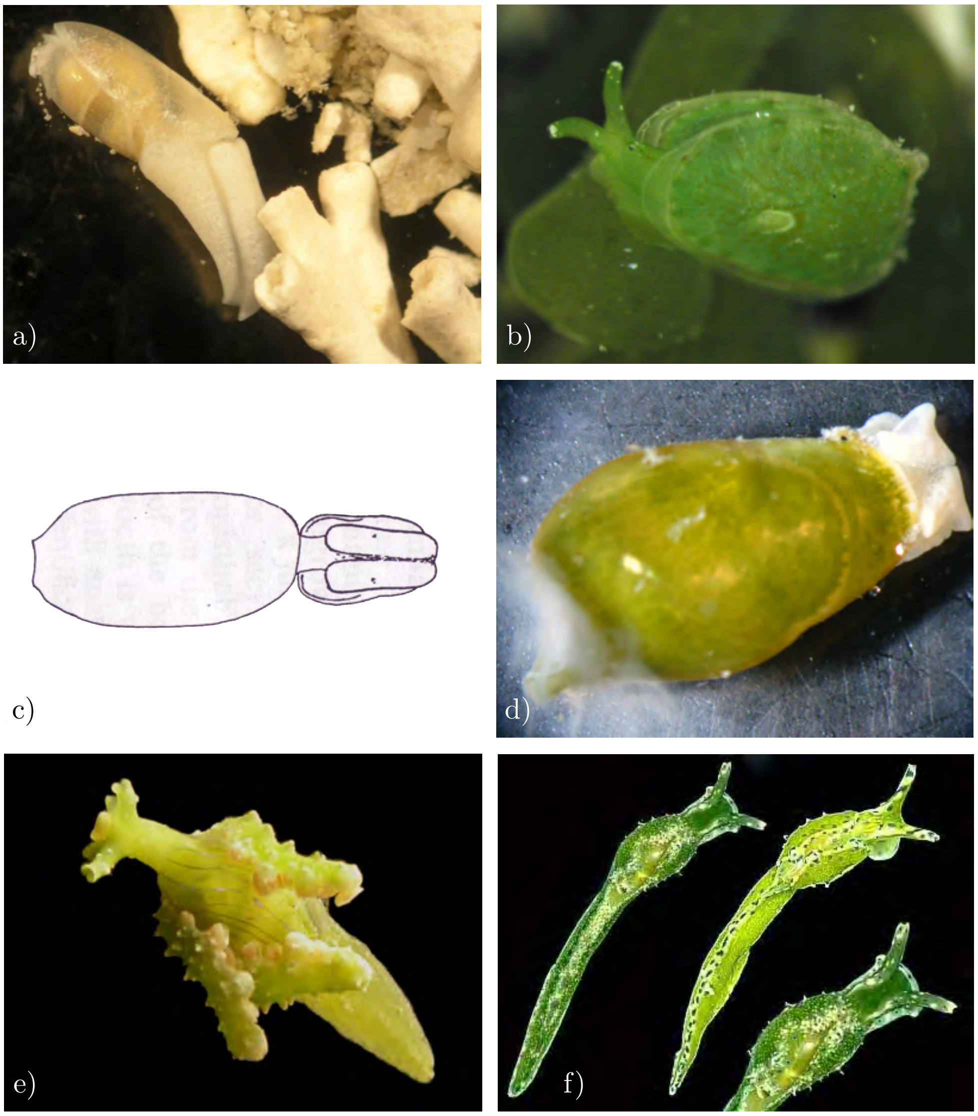


Figure 1.4 Oxynoacea, the shelled sacoglossans. Approximation of length is given. a) *Cylindrobulla* sp. (Cylindrobullidae). The cephalic shield is used to bury into the sediment. Guam, USA 2009, 1cm. b) *Tamanovalva limax* (Juliidae) with a bivalved shell. Photo: Heike Wägele. c) *Ascobulla fischeri* (Volvatellidae). Figure: Burn 1972. d) *Volvatella* cf. *ventricosa* (Volvatellidae), Lizard Island, Australia 2008, 1.5cm. The shell of *Volvatella* narrows in its posterior end into a spout. White mucus is excreted when the animal is disturbed. Although they did not develop typical rhinophores, they have head tentacles and oral lobes. e) Members of the Lobigeridae have a tail and four lateral parapodia. *Lobiger viridis* has a translucent shell, that may be decorated with bluish stripes, Lizard Island, Australia 2008, 1.5cm. f) *Oxynoe viridis* (Lobigeridae). The thin shell is covered by their parapodia. Photo: Bill Rudman, available from seaslugforum.net/factsheet/oxynviri.

Figure 1.5 Plakobranchacea: Limpantioidea, shell-less sacoglossans that possess dorsal appendices, the cerata. Approximation of length is given. a) *Placida cremoniana* (Limapontiidae), Guam, Australia 2009, 1cm. Species of the family Limapontiidae have digitiform cerata. b) *Ercolania "annelyleorum"* (Limapontiidae) in its food alga *Boodlea*, Lizard Island, Australia 2008, 0.2cm. c) *Cyerce nigra* (Polybranchiidae) Guam, USA 2009, 2cm. Species of the family Polybranchiidae have broad, flat cerata and bifurcate rhinophores. One oral tentacle can be seen here. d) *Polybranchia orientalis* (Polybranchiidae), Lizard Island 2008, 2cm. e) *Costasiella* cf. *kuroshimae* (Costasiellidae) on its food alga *Avrainvillea erecta*. Lizard Island, Australia 2008. Characteristic for this group is the close position of their eyes. f) *Hermaea bifida* (Hermaeidae) feeds on red algae, e.g. *Griffithsia*. Photo: Bjørnar Nygård, Norway, available from http://www.seaslugforum.net/find/23416.

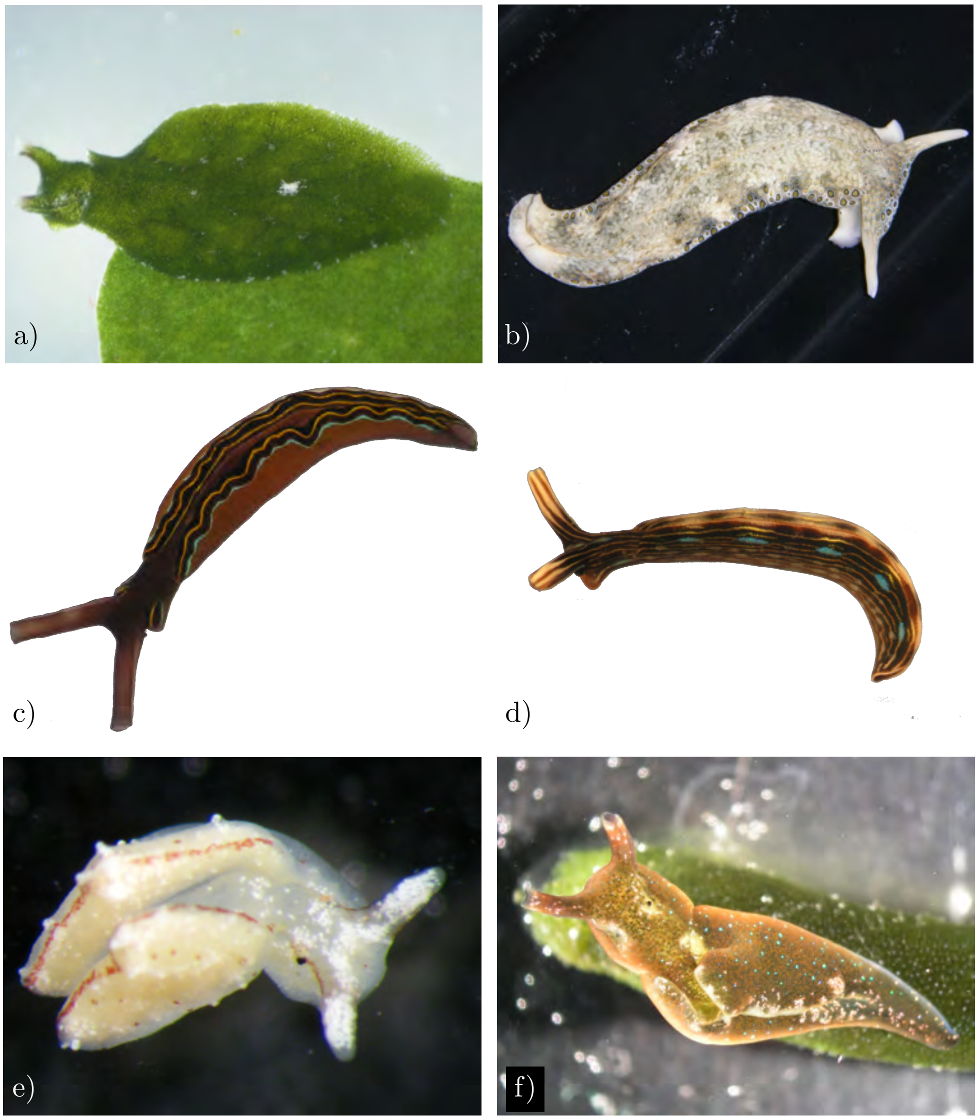


Figure 1.6 Plakobranchacea: Plakobranchoidea, shell-less sacoglossans that possess lateral flaps, the parapodia. Approximation of length is given. a) *Bosellia mimetica* (Boselliidae) on its food alga *Halimeda tuna*, Banyuls-sur-Mer, France 2006, 1cm. *Bosellia mimetica* does not have parapodia, its morphology is highly derived and difficult to compare to other Plakobranchoidea. b) *Plakobranchus ocellatus* (Plakobranchidae), Lizard Island, Australia 2008, 4cm. c) *Thuridilla livida* (Plakobranchidae), Guam, USA 2009, 1.5cm. d) *Thuridilla gracilis* (Plakobranchidae), Guam, USA 2009, 1.5cm. e) Juvenile *Elysia bennettae* (Plakobranchidae), Lizard Island, Australia 2008, 3mm. f) *Elysia viridis* (Plakobranchidae) on *Codium*, Ferrol, Spain 2009, 1.5cm.

The fossil record of Sacoglossa traces back to the early Eocene (Ypresian: 55.8-48.6Ma) (Le Renard et al. 1996; Tracey et al. 1993). The known fossils had been restricted to members of the shelled Juliidae for a long time but *Volvatella faviae* were described in 2000 (Valdés & Lozouet) from the Aquitaine Basin (Early Miocene: Aquitanian: 23-20.4Ma). Until now, there have been no findings of shells belonging to the genera *Cylindrobulla*, *Ascobulla*, *Oxynoe* or *Lobiger*.

The first species of the taxon Juliidae was described as *Berthelinia elegans* Crosse, 1875 - as a bivalve. The bivalved shell of Juliidae reminds one at first sight of the shells of Bivalvia (figure 1.4b)). The recent species *Berthelinia schlumbergeri* was described in 1895, but only based on the shell material (Dautzenberg, 1895). Dautzenberg was not sure whether to consider *Berthelinia* a bivalve or a gastropod. Eventually, Kawaguti and Baba (1959) found a living animal, *Tamanovalva limax*, and the shell's origin could be re-evaluated.

By now fossil shells of sacoglossans have been described all around the world from Britain, the Caribbean to several spots in the Indo-Pacific/Pacific, i.e. Borneo, Japan and Australia (see review in Schneider et al. 2008). Schneider and colleagues propose the origin of Juliidae as being in middle Europe since the oldest fossils (Eocene) have been found in the Paris Basin.

Since the shells are quite small, fragile, easily-destroyed by the power of water movement they may be seldom bequeathed. If fossilised and found, fossil sacoglossan shells may be mistaken for bivalves (Juliidae), for other opisthobranch species or their stemline. E.g. the recent *Ascobulla fragilis* had originally been described as *Cylichna fragilis* based only on the shell by Jeffreys (1856) until he transferred it to the genus *Cylindrobulla* (1882).

All time data are given according to Gradstein and Ogg (2004).

The first phylogeny of Sacoglossa based on morphological data was presented by Jensen (1996) applying Hennigian principles (figure 1.7a)). Except for four species of the genus *Elysia*, her analysis was performed at genus level. She used a 'hypothetical cephalaspid ancestor' as outgroup, deriving character polarity from *Haminaea*, *Akera* and *Cylindrobulla*. She found the two major lineages Oxynoacea, shelled Sacoglossa, and Plakobranchacea, shell-less Sacoglossa, monophyletic. Plakobranchacea comprised the monophyletic Plakobranchoidea and the monophyletic Limapontioidea with Hermaeidae and Limapontiidae as only monophyletic families. Mikkelsen re-evaluated Jensen's data set (1998). The result differed mainly in the fact that *Cylindrobulla* was situated within the Oxynoacea.

A first analysis based on one molecular marker did not follow until 2007 (Händeler & Wägele). The result of this study (figure 1.7b)) was considered as preliminary since only one gene (16S rDNA) was used and taxon sampling was not depletive. Major differences between this analysis and the former lie in the paraphyly of the Oxynoacea and Limapontiidae.

Gosliner (1995) performed a morphologically based analysis on Plakobranchidae concentrating on the genus *Thuridilla*. Another molecular analysis based on 16S rDNA, *coxI* and H3 focussed on the family Plakobranchidae (Bass & Karl 2006).

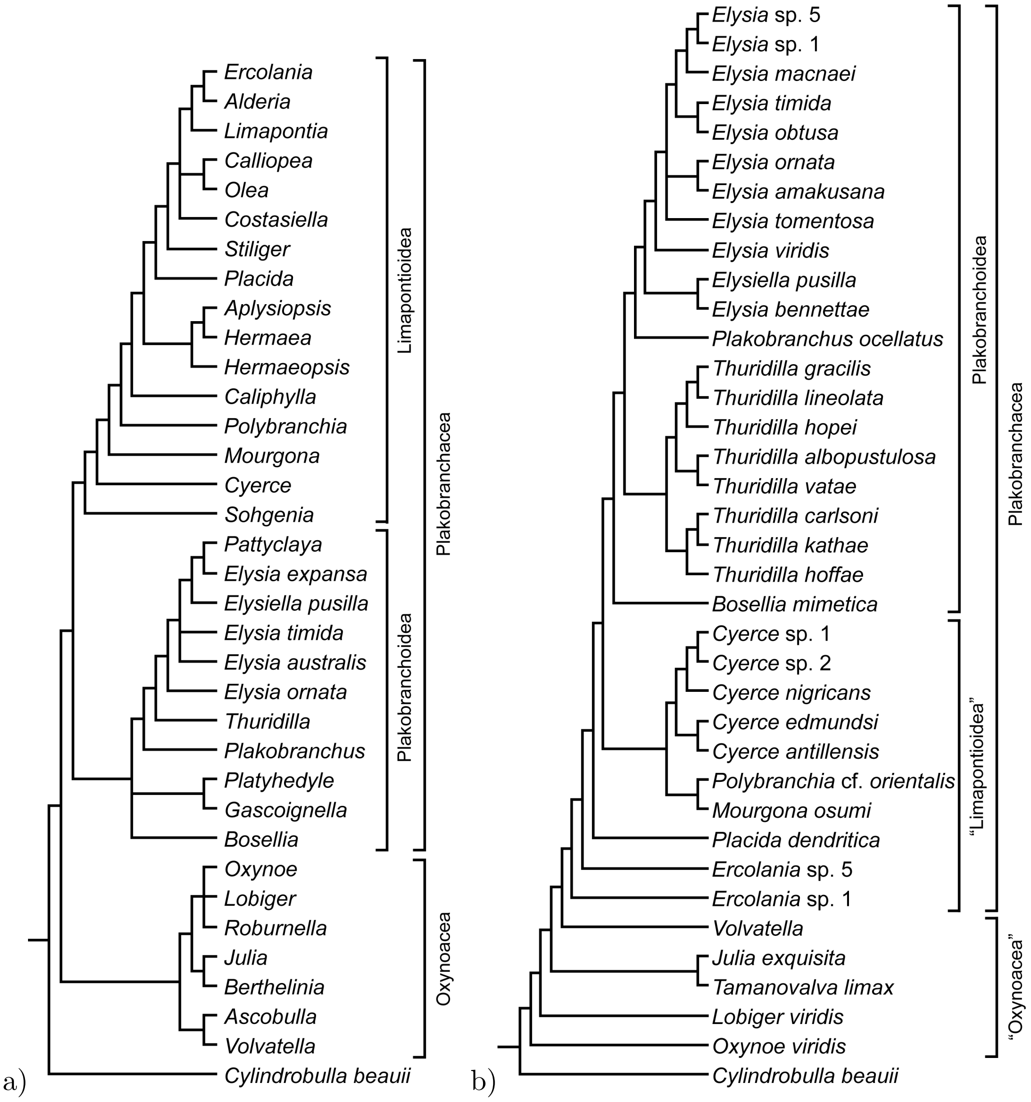


Figure 1.7 Phylogenies of Sacoglossa. a) Based on morphological data (Jensen 1996). The genus *Hermaeopsis* has been merged into *Hermaea* (Jensen 2007). b) By means of partial 16S rDNA (Händeler & Wägele 2007). *Elysiella* is synonym to *Elysia* (Gosliner 1995; Händeler & Wägele 2007), *Elysia* sp. 1 is *Elysia* "asbecki", *Ercolania* sp. 1 has been described as *Ercolania kencolesi* and *Ercolania* sp. 5 is *Ercolania* "*annelyleorum*".

1.2 Kleptoplasts in Sacoglossa: photosynthetic abilities among Sacoglossa

While plastid retention occurs in several unicellular organisms, e.g. in ciliates (Johnson et al. 2007), in Foraminifera (Lee 2006) and in Dinoflagellata (Gast et al. 2007), kleptoplasts in Metazoa are only known in Sacoglossa. Taylor (1970) mentioned kleptoplasts in the digestive system of the rotifer *Ascomorpha ecaudis*. However, these were not organelles of algae but whole algal organisms; zoochlorellae (de Manuel Barrabin 2000).

The first investigation of this phenomenon probably goes back to De Negri and De Negri (1876) who found the green pigment of *Elysia viridis* to be identical to chlorophyll. Brandt (1883) followed, interpreting the green bodies in *Elysia timida* as algae comparable to algae in *Hydra* or turbellarians. Kawaguti and Yamasu described functional chloroplasts in the "hepatic diverticula" of *Elysia atroviridis* by detailed investigation through electron microscopy as "probably derived from a food alga, *Codium fragile*" (1965, p. 84).

Eventually, evidence were provided that photosynthetic assimilates are released from the chloroplasts in the slug cells (Greene 1970b; Trench et al. 1972; Trench, R.K. et al. 1974; Marín & Ros 1989; Raven et al. 2001). Presence of chloroplasts was not only investigated through electron microscopy (Curtis et al. 2005; Hirose 2005; Kawaguti & Yamasu 1965; Kremer & Janke 1988; Taylor 1968), but also through evaluation of carbon fixation rate and O_2-production (Clark et al. 1981; Hinde & Smith 1974; Marín & Ros 1989, 1992; Taylor 1971a).

Plastids are incorporated by phagocytosis into the digestive cell of the slug, whether the slug starts immediately to digest it or is capable of keeping the plastid functional for a shorter or longer time (McLean 1976; Evertsen & Johnsen 2009) (see figure 1.8).

Recently, Casalduero and Muniain (2008) showed, that starving specimens of *Elysia timida*, that were kept under light conditions, survived better than those kept in the dark, and endosymbiosis of functional chloroplasts supplies the animals with additional energy. Teugels et al. (2008) showed that nitrogen acquisition in *Elysia viridis* is influenced by kleptoplasts.

The first compendious overview on different photosynthetic abilities among Sacoglossa is given by Trench (1975) and Clark et al. (1990). In 2001 Wägele and Johnsen used a Pulse Amplitude Modulated Fluorometer (PAM) for the first time to quantify photosynthesis rates in different sacoglossans and discovered variance over different species. Evertsen and colleagues expanded these studies (2007); *Plakobranchus ocellatus* and *Elysia timida* showed representative photosynthetic activity, other species lost activity within a few days. The record for chloroplast retention is 14 months for *Elysia chlorotica* (Rumpho et al. 2006).

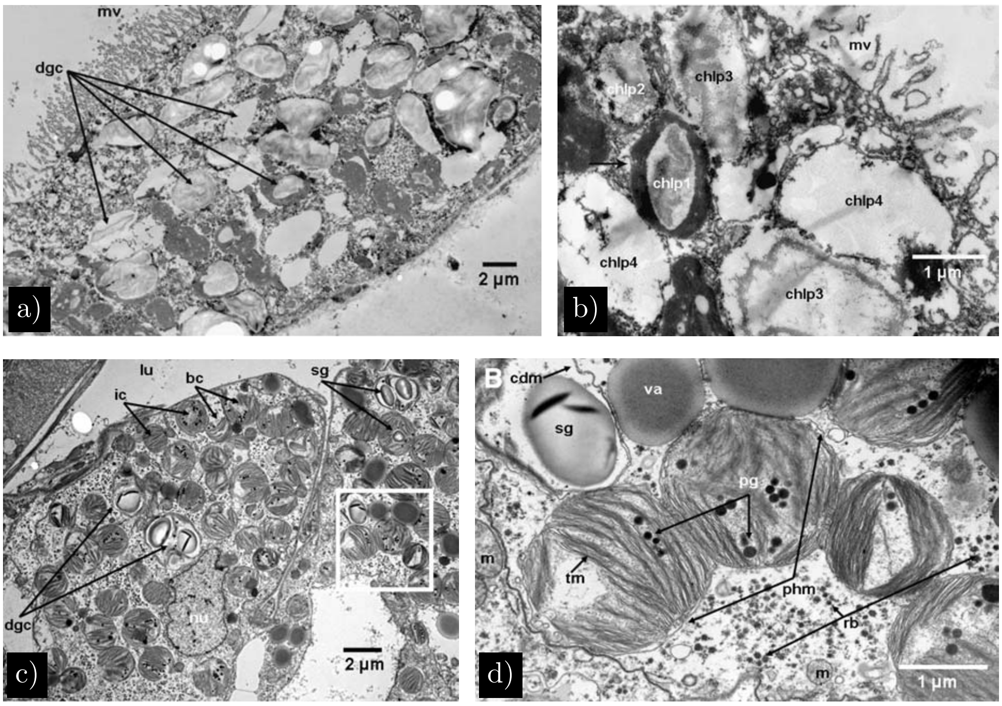


Figure 1.8 Chloroplasts of *Codium fragile* in the digestive cells of the limapontioidean *Placida dendritica*, that digests chloroplasts, and the plakobranchoidean *Elysia viridis*, that shows a functional chloroplast retention. In both of them chloroplasts are phagocytosed into the cells of the digestive gland. a) Chloroplasts in a cell of *Placida dendritica*; chloroplasts are degraded. b) Detailed view of latter picture. c) Chloroplasts in the digestive cell of *Elysia viridis*; chloroplasts are healthy. d) Detailed view of b). mv – microvilli, dgc – degrading chloroplasts, chlp1 to 4 – four stages of degradation, ic – intact chloropasts, bc – broken chloroplasts exposed to the digestive cell cytoplasm, sg – starch grain, nu – nucleus, lu – lumen, phm – phagosome membrane, cdm – double chloroplast membrane, tm – thylakoid membranes, pg – plastoglobuli. Pictures taken and modified (cut to fit format) from Evertsen & Johnsen (2009).

1.3 Food organisms of Sacoglossa

Sacoglossans mainly feed on green algae that belong to the taxon Ulvophyceae sensu Floyd & O'Kelly 1990. This taxon comprises quite diverse forms, that have been considered as separate groups by some authors (e.g. van den Hoek et al. 1995; Silva 1980), but is now reunited after Adl et al. 2005 in the sense of Mattox & Stewart 1984. They were the first who proposed the concept of this group, that they spelled Ulvaphyceae. Further descriptions were later given by Floyd & O'Kelly (1990).

Knowledge on algal food of Sacoglossa was assembled by Jensen (1980, 1997), Williams & Walker (1999) and most recently by Händeler & Wägele (2007). Table 1.2 lists the different groups of Ulvophyceae and which genera are known to be fed by sacoglossans. While food sources are well documented for some species, they are unknown for many others. The food source for the genus *Cylindrobulla* is unknown, although this is important in revealing the ancestral feeding state of Sacoglossa. All shelled sacoglossans feed exclusively on the genus *Caulerpa*. Out of the 24 described species of *Thuridilla* only for *Thuridilla hopei* food sources are known (Gavagnin et al. 1994b; Marín & Ros 1989, 2004). Chloroplast incorporation in the digestive gland of *Plakobranchus ocellatus* has been extensively investigated without clarifying the food source (Greene 1970a, 1970c as *P. ianthobapsus*; Hirose 2005).

To understand correlation of food and functional retention of chloroplasts as well as the evolution of slug/alga interaction, an unambiguous identification of the chloroplast origins is essential. Identification by studying genes (barcoding) has become a suitable method. *RbcL* and *matK* were constituted as barcode for landplants (Hollingsworth et al. 2009). While *matK* cannot be found in Ulvophyceae (Pombert et al. 2005, 2006), *rbcL* has already been used for identification of food organisms in *Elysia crispata* (Pierce et al. 2006).

Table 1.2 Genera of Ulvophyceae that are known to be fed by sacoglossans according to Händeler & Wägele (2007) and own unpublished data are listed. Classification after Floyd & O'Kelly 1990. Cp – Chloroplasts per cell; n – numerous.

Group	Organisation	Cp	Genera known to be fed by Sacoglossa
Ulotrichales	uni- or multinucleate cells	1	*Urospora*
Ulvales	uninucleate cell	1	*Ulva*
Cladophorales*	siphonocladous	n	*Boergesenia*, *Boodlea*, *Chaetomorpha*, *Cladophora*, *Cladophoropsis* *Dictyosphaeria*, *Rhizoclonium* *Valonia*
Dasycladales	siphonous	n	*Acetabularia*, *Batophora*, *Cymopolia*
Caulerpales	siphonous	n	*Avrainvillea*, *Bryopsis*, *Boodleoposis* *Caulerpa*, *Chlorodesmis*, *Codium* *Derbesia*, *Halimeda*, *Penicillus* *Tydemania*, *Udotea*

*=Siphonocladales

Figure 1.9 Ulvophyceae. Cladophorales: thalli consist of several multinucleate cells. a) *Chaetomorpha* sp., Normandy, France 2007. Diameter of filament is 40µm. Picture taken of ethanol preserved material. b) *Dictyosphaeria* cf. *cavernosa*, Lizard Island, Australia 2008. c) Dasycladales: *Acetabularia* cf. *parvula*, Guam, USA 2009. Caulerpales: thalli are siphonous. d) Egg masses (most probably from *Cyerce* sp. 3 (Gosliner et al. 2008)) on *Udotea geppii*, Guam, USA 2009. e) *Bryopsis* sp., USA, Guam 2009. f) *Tydemania expeditionis* from the Maledives. g) *Halimeda* cf. *tuna*, habitus, Guam, USA 2009 and detail of surface, Lizard Island, Australia 2008. While the entire thallus is higher than 10cm, the diameter of a single utricle can vary from only 34 to 100µm (Hillis-Colinveaux 1980). Between these utricles the alga calcifies. h) *Caulerpa* sp., Guam, USA 2009.


a)
b)
c)
d)
e)
f)
g)
h)

1.4 Notes on undescribed species

Numerous data have been collected on "nameless", i.e. undescribed species. The notes on undescribed species are summarized to save this information.

Two plakobranchacean species will be described shortly. They are included in this manuscript under the working titles *Ercolania "annelyleorum"* (Limapontiidae) and *Elysia* "asbecki" (Plakobranchidae).

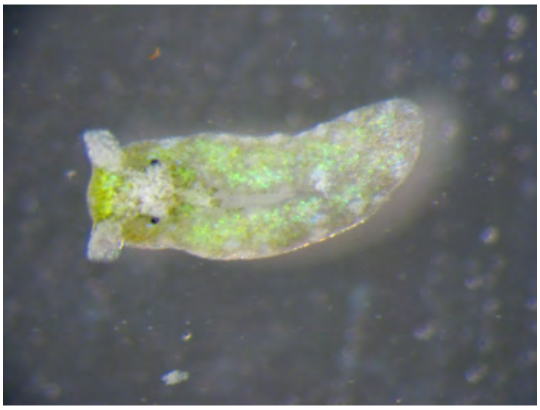
1.5 Aims of this thesis

The main goal of this thesis is to provide data that might lead to a better understanding of the evolution of Sacoglossa under special consideration of chloroplast retention. The following problems are addressed:

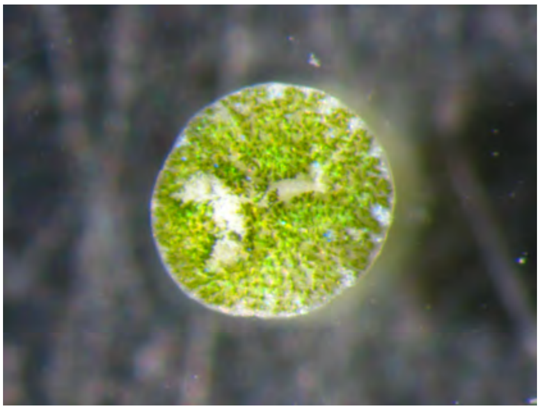
- Are there different qualities of chloroplast retention among Sacoglossa?
- Is functional chloroplast retention an apomorphic state for a specific group?
- Is functional long-term retention over several weeks an apomorphic state for a specific group?
- Is retention ability correlated to food organisms (plastid donator)?

To achieve this, information is needed on 1) the phylogeny of Sacoglossa 2) photosynthetic abilities among Sacoglossa and 3) the origin of the plastids.

A molecular approach is extended to three genes (partial): mitochondrial 16S rDNA, *coxI* and nuclear 28S rDNA (domains D1-D3) to obtain a phylogeny of Sacoglossa. Photosynthetic activity of several species from all different groups of Sacoglossa, i.e. Oxynoacea, Limapontioidea and Plakobranchacea, is investigated with the help of a Pulse Amplitude Modulated Fluorometer that measures chlorophyll a fluorescence as proof of the intactness of photosystem II of the incorporated chloroplasts. A reliable method of identifying the food organisms of slugs is needed; an additional marker to the already existing *rbcL* shall be established: *tufA*. Information on food organisms is desired, to test and confirm or extend known data from literature and reveal unknown food sources.



Sacoglossa are quite flexible. This young specimen of *Elysia pusilla* (Lizard Island, Australia 2008) had been speeding through the petri dish, annoying the desperate photographer, until it suddenly decided that it had found the perfect place to stay and took up its rest position. A comparable circular rest position can be found in *Bosellia mimetica*. Compare figures 1.6a) on p. 7 and 1.2b) on p. 3.



2 Material and Methods

2.1 Material

Depending on depth, slugs and algae were collected by reef-walking during low tide, snorkeling or scuba diving. Bigger animals, especially species of *Thuridilla* or *Plakobranchus*, that were never found on any alga, but crawling on the ground (figure 2.1a) and 1.2a), p. 3), were collected in situ. Otherwise food algae were collected (figure 2.1c)) and stored in aquaria without any fresh air or water (figure 2.1d)). Owing to the worsening conditions in the water the animals crawled to the water surface or on top of the alga and could be plucked off. Smaller animals showed up earlier than bigger ones.

Unfortunately, some species, e.g. shelled sacoglossans, prefer to stay attached to their food alga. Therefore, algal material had also been looked through under

Figure 2.1 Collection of material. a) Some species were usually found crawling on the ground as this *Elysia* sp. 11 (seaslugforum.net). MacGillivray Reef, Australia 2008. b) Single slugs were collected in small containers. Guam, USA 2009. c) Algal samples were transferred with sediment into plastic bags, Guam 2009. d) Algae were (kept) separated in small aquaria, Lizard Island Research Station, Australia 2008.

the microscope. Furthermore, sediment of algae were sampled and sifted through to find shelled species that live near to or in the sediment.

Animals were fixed for DNA extraction in absolute EtOH and stored as cold as possible (-20°C or -80°C). Algae were fixed in EtOH as well or dried or both. Algae were cleaned and epiphytes removed as far as possible. The slugs and algae that were used for DNA extraction are listed in table 2.1 and table 2.2 respectively.

Table 2.1 Species used for DNA extraction and internal no. of DNA sample. Collection place and collection date are given. Remarks: Sample code in column 4, e.g. Cyl-09GU-1, indicates internal signatur; ZSM – Animal can be found in the collection of the Zoologische Staatssammlung München; if animal has been collected by another person, this is indicated by his/her name or initials: HW+ – Heike Wägele and colleagues, YG – Yvonne P. Grzymbowski, CZ – Carmen Zinßmeister, IB – Ingo Burghardt. Internal number starting with S are DNA samples that were extracted by Gregor Christa.

Species	No.	Collection place	Collection date	Remarks
Oxynoacea				
Cylindrobullidae Thiele, 1931				
Cylindrobulla beauii P. Fischer, 1856	728	USA: Florida, Miami	00.04.2005	
Cylindrobulla sp.	847	USA: Guam: Cocos Lagoon, sediment sample of mixed algae	21.08.2009	Cyl-09GU-1
Volavatellidae Pilsbry, 1895				
Volvatella viridis Hamatani, 1976	789	Australia: Lizard Island South Island	12.07.2006	HW+
Volvatella cf. *ventricosa* (Jensen & Wells 1990)	S121	Australia: Lizard Island Casuarina Beach on *Caulerpa serrulata*	19.10.2008	Vv08LI-58
Volvatella sp. (juvenile)	858	USA: Guam: Piti *Caulerpa* sample	21.08.2009	V-09GU-1
Juliidae E. A. Smith, 1885				
Julia exquisita Gould, 1862	783	Australia: Lizard Island South Island	11.07.2006	HW+
	826	Australia: Lizard Island Coconut Beach	09.07.2006	HW+
Tamanovalva limax Kawaguti & Baba, 1959	792	Australia: Lizard Island Casuarina Beach	13.07.2006	HW+

OXYNOIDAE H. Adams & A. Adams, 1854

Lobiger viridis Pease, 1863	785	Australia: Lizard Island South Island	10.07.2006	HW+
	802	Australia: Lizard Island Coconut Beach	12.08.2006	HW+
	805	Fiji: Viti Levu: Laucala Bay, Nukumbutho channel west side of Nukumbutho Island	26.08.2006	ZSM2006150
Oxynoe antillarum Mörch, 1863	816	USA: Florida Big Pine Key	26.07.2007	YG
	821	USA: Florida Geiger Key	09.07.2007	YG
Oxynoe azuropunctata Jensen, 1980	828	Panama, caribbean	17.03.2007	CZ
Oxynoe viridis (Pease, 1861)	786	Australia: Lizard Island South Island	10.07.2006	HW+
	827	Australia: Lizard Island South Island	11.07.2006	HW+

Plakobranchacea

PLAKOBRANCHOIDEA

BOSELLIIDAE Marcus, 1982

Bosellia mimetica Trinchese, 1890	739	Spain: Mataro Baretta del Abre	23.05.2006	
	740	Spain: Mataro Baretta del Abre	23.05.2006	
	744	France: Banyuls-sur–Mer on *Halimeda*	11.05.2006	
	745	France: Banyuls-sur-Mer on *Halimeda*	11.05.2006	

PLAKOBRANCHIDAE Rang, 1829

Elysia amakusana Baba, 1955	703	Australia, Lizard Island	04.03.2005	K. Stemmer
Elysia ”asbecki”	722	Australia: Lizard Island	25.07.2005	IB
Elysia bennettae Thompson, 1973	797	Australia: Lizard Island South Island	11.07.2006	HW+
	779	Samoa: Upolu Island Apia, harbour	00.08.2005	ZSM20060293
Elysia chlorotica Gould, 1870	807		29.10.2007	M. Rumpho
Elysia cornigera Nuttall, 1989	810	USA: Florida Cudjoe Key	30.07.2007	YG
	812	USA: Florida: Summerland Key, Henry Street	29.07.2007	YG
Elysia crispata Mörch, 1863	837	USA: Florida: Summerland Key	24.06.2007	YG
	838	USA: Florida Summerland Key	24.06.2007	YG

Elysia cf. *grandifolia* Kelaart, 1858	849	USA: Guam: Pago Bay	14.08.2009	Eo-09GU-8
	851	USA: Guam: Pago Bay on *Bryopsis*	13.08.2009	Eo-09GU-5
Elysia macnaei Ev. Marcus, 1980	726	Indonesia: Sulawesi Bunaken Nationalpark, Gangga	23.07.2203	ZSM20033821
Elysia marcusi (Ev. Marcus, 1972)	835	USA: Florida Summerland Key	26.06.2007	YG
	836	USA: Florida Summerland Key	26.06.2007	YG
Elysia obtusa Baba, 1983	778	Samoa: Savaii Island Vaisala lagoon	20.08.2005	ZSM20060257
Elysia ornata (Pease, 1860)	798	Australia: Lizard Island North Point	14.07.2006	HW+
	734	Australia: Lizard Island	18.03.2005	K. Stemmer
Elysia patina Ev. Marcus, 1980	813	USA: Florida, Summerland Key, Henry Street	02.07.2007	YG
	825	USA: Florida Summerland Key, West	07.07.2008	YG
Elysia pratensis Ortea & Espinosa, 1996	811	USA: Florida Summerland Key	29.07.2007	YG
	824	USA: Florida Summerland Key	08.07.2007	YG
Elysia pusilla (Bergh, 1872)	764	Maledives	00.04.2006	
	794	Maledives	00.04.2006	
	796	Maledives	00.04.2006	
	799	Australia: Lizard Island South Island	10.07.2006	HW+
	A4	Maledives	29.03.2007	W. Loch
Elysia serca (Pease, 1871)	829	Bolivarian Republic of Venezuela: Isla de Margarita	00.04.2007	CZ
	830	Bolivarian Republic of Venezuela: Isla de Margarita	00.04.2007	CZ
Elysia cf. *setoensis* Hamatani, 1968	803	Australia: Lizard Island	13.07.2006	HW+
Elysia subornata Verrill, 1901	832	USA: Florida, Summerland Key/Mote Canal	30.07.2007	YG
	833	USA: Florida, Summerland Key/Mote Canal	30.07.2007	YG
Elysia timida (Risso, 1818)	761	France: Banyuls-sur-Mer, bay north of Plage des Elmes	11.05.2006	
	752	France: Banyuls-sur-Mer, le troc	02.05.2006	
	766	Spain: bay north of Cala Giverola	25.05.2006	
	769	Spain: Roses	22.05.2006	
	743	Spain: Cadaques: Plaja Caials	07.05.2006	
Elysia tomentosa Jensen, 1997	710	Australia: Lizard Island	13.09.2004	IB
	711	Australia: Lizard Island	21.03.2005	K. Stemmer

Elysia translucens Pruvot-Fol 1957	845	France: Banyuls-sur-Mer	01.05.2009	Etr09By-1
Elysia tuca Marcus & Marcus, 1967	819	USA: Florida Summerland Key	09.07.2007	YG
	823	Panama: Bocas del Toro, Isla Colon	26.03.2007	CZ
Elysia viridis (Montagu, 1804)	727	France: Roscoff	28.06.2005	A. Klussmann-Kolb
	737	Spain: Tossa de Mar	19.05.2006	
	738	France: Banyuls-sur-Mer on *Halimeda*	06.05.2006	
	S125	France: Banyuls-sur-Mer	28.04.2009	Ev09By-2
Elysia zuleicae Ortea-Rato & Espinosa, 2002	817	USA: Florida Sugarloaf Key	24.06.2007	YG
Elysia sp. 2	781	Samoa: Upolu Island: Namua Island	09.08.2005	ZSM20060179
Elysia sp. 5	806	Australia: Lizard Island South Island	24.07.2006	HW+
Elysia sp. Banyuls	841	France: Banyuls-sur-Mer	30.04.2009	Espec09By-3
	S143	France: Banyuls-sur-Mer on *Halimeda tuna*	01.05.2009	Ev09By-6
*Elysia*sp. 1GUAM	868	USA: Guam: Pago Bay on *Bryopsis*	13.08.2009	E1-09GU-1
	871	USA: Guam: Apra Harbour, Western Shoals	02.09.2009	E1-09GU-4
Elysia sp. 4GUAM	867	USA: Guam: Pago Bay on *Chlorodesmis hildebrandtii*	27.08.2009	E4-09GU-1
Elysia sp. 5GUAM	865	USA: Guam: Asan on *Tydemania expeditionis*	28.08.2009	E5-09GU-4
Elysia sp. 11 (seaslugforum.net)	S178	Australia: Lizard Island MacGillivray Reef	01.11.2008	E1108LI-179
	S200	USA: Guam: Cocos Lagoon on *Udotea geppii*	21.08.2009	E11-09GU-3
Elysia sp. 15 (Gosliner et al. 2008)	S197	USA: Guam: Cocos Lagoon on *Udotea geppii*	21.08.2009	E15-09GU-3
	869	USA: Guam: Cocos Lagoon on *Udotea geppii*	02.09.2009	E6-09GU-1a
Plakobranchus ocellatus van Hasselt, 1824	705	Australia: Lizard Island	21.03.2005	IB
	706	Australia: Lizard Island	25.07.2005	IB
	850	USA: Guam	00.08.2009	Po-09GU-4
	852	USA: Guam: Luminau	25.08.2009	P-09Gu-7
	854	USA: Guam Cocos Lagoon	18.08.2009	Po-09GU-6
	856	USA: Guam: Pago Bay	14.08.2009	P-09GU-2
Thuridilla albopustulosa Gosliner, 1995	782	Indonesia: Sulawesi Bunaken Nationalpark: S-Siladen	17.07.2003	ZSM20033615

Thuridilla bayeri (Marcus, 1965)	725	Indonesia: Sulawesi: Bunaken Nationalpark: S-Siladen	17.07.2003	ZSM20033612
Thuridilla carlsoni Gosliner, 1995	801	Australia: Lizard Island	25.06.2006	HW+
	714	Australia: Lizard Island	13.09.2004	IB
	S116	Australia: Lizard Island Loomis Reef	25.10.2008	Tc08LI-114
Thuridilla gracilis (Risbec, 1928)	704	Australia: Lizard Island	18.03.2005	IB
	712	Australia: Lizard Island	25.06.2005	IB
	713	Lizard Island	13.09.2004	IB
	S117	Australia: Lizard Island Casuarina Beach on *Halodule pinifolia*	19.10.2008	Tg08LI-48
	S118	Australia: Lizard Island Casuarina Beach on sediment	20.10.2008	Tg08LI-64 (b)
Thuridilla hoffae Gosliner, 1995	777	Samoa: Savaii Island Vaisala lagoon	16.08.2005	ZSM20060224
Thuridilla hopei (Verany, 1853)	120	Italy: Elba	00.07.2001	DNA only
	736	Spain: Blanes Cala St. Francesc	22.05.2006	
	746	Spain: Mataro Baretta del Abre	24.05.2006	
	808	Ialy: Isola del Giglio Campese, left side	26.06.2008	GI20
	809	Italy: Isola del Giglio Cala del Corvo	06.07.2008	GI87
	839	Iatly: Isola del Giglio Campese, left side	01.07.2008	GI45
Thuridilla kathae Gosliner, 1995	707	Australia: Lizard Island	13.09.2004	IB
	S120	Australia: Lizard Island	15.10.2008	Tk08LI-1
Thuridilla lineolata (Bergh, 1905)	715	Indonesia: Sulawesi		M. Schrödl
Thuridilla livida (Baba, 1955)	846	USA: Guam: Bile Bay	17.08.2009	Tl-09GU-1
Thuridilla vatae (Risbec, 1928)	780	Samoa: Savaii Island Vaisala lagoon	11.08.2005	ZSM20060088

LIMAPONTIOIDEA

Polybranchiidae O'Donoghue, 1929

Cyerce antillensis Engel, 1927	155	Portugal: Azores	00.09.2001	P. Wirtz
	815	USA: Florida Summerland Key	08.07.2007	YG
Cyerce elegans Bergh, 1870	724	Indonesia: Sulawesi Bunaken Island, Celah Celah	11.07.2003	ZSM20033600
Cyerce nigra (Pease, 1866)	853	USA: Guam: Cocos Lagoon on *Udotea geppii*	21.08.2009	Cyni-09GU4
	860	USA: Guam: Cocos Lagoon on *Udotea geppii*	21.08.2009	Cyni-09GU3

Cyerce nigricans (Pease, 1866)	732	Samoa: Savaii Island Apia, Palolo Deep Marine Reserve	05.08.2005	ZSM20060045
	701	Australia: Eagle Island	13.07.1999	L. Wägele
	790	Australia: Lizard Island Channel	13.07.2006	HW+
Cyerce sp. 1 (Wägele et al. 2006)	723	Australia: Lizard Island	25.07.2005	IB
Cyerce sp. 2 (Wägele et al. 2006)	721	Australia: Lizard Island	13.09.2004	IB
Cyerce sp. 3 (Gosliner et al. 2008)	848	USA: Guam: Cocos Lagoon on *Udotea geppii*	21.08.2009	Cy3-09GU-2
	859	USA: Guam: Cocos Lagoon on *Udotea geppii*	18.08.2009	Cy3-09GU-1
Mourgona osumi Hamatani, 1994	791	Australia: Lizard Island North Point	14.07.2006	HW+
Polybranchia orientalis Kelaart, 1858	709	Australia: Lizard Island	01.09.2004	IB, (cf.)
	772	Australia: Lizard Island	24.07.2002	W. Wägele
	S113	Australia: Lizard Island Coconut Beach	26.10.2008	Py08LI-140
COSTASIELLIDAE Clark, 1984				
Costasiella cf. *kuroshimae* Ichikawa 1993	804	Australia: Lizard Island Casuarina Beach on *Avrainvillea erecta*	12.07.2006	HW+
	S101	Australia: Lizard Island Casuarina Beach on *Avrainvillea erecta*	17.10.2008	Ck08LI-17
	S102	Australia: Lizard Island Casuarina Beach on *Avrainvillea erecta*	17.10.2008	Ck08LI-21
	S105	Australia: Lizard Island Casuarina Beach on *Avrainvillea erecta*	17.10.2008	Ck08LI-33
	S190	USA: Guam Cocos Lagoon on *Avrainvillea erecta*	24.08.2009	Ck-09GU-10
Costasiella nonatoi Marcus and Marcus 1960	814	USA: Florida: Summerland Key/Mote Key	26.07.2007	YG
	818	USA: Florida: Summerland Key/Mote Key	25.07.2007	YG
Costasiella ocellifera Simroth, 1895	820	USA: Florida Long Key	08.07.2007	YG
	822	USA: Florida Cudjoe Key	23.07.2007	YG
Costasiella sp. black	863	USA: Guam Cocos Lagoon on *Avrainvillea erecta*	21.08.2009	Cu-09GU-4
	870	USA: Guam Cocos Lagoon on *Avrainvillea erecta*	24.08.2009	Cu-09GU-7

Costasiella sp. 1 (seaslugforum.net)	864	USA: Guam Cocos Lagoon on *Avrainvillea erecta*	24.08.2009	Co1-09GU-7
	S196	USA: Guam Cocos Lagoon on *Avrainvillea erecta*	24.08.2009	Co1-09GU-4
Limapontiidae Gray, 1847				
Ercolania "*annelyleorum*"	787	Australia: Lizard Island Casuarina Beach in *Boodlea*	07.07.2006	HW+
Ercolania sp.	855	USA: Guam Apra Harbour, wall in *Boodlea*	19.08.2009	Er-09GU-1
Ercolania kencolesi Grzymbowski, Stemmer & Wägele, 2007	788	Australia: Lizard Island Casuarina Beach	02.07.2006	HW+
Ercolania viridis (A. Costa, 1866)	844	France: Banyuls-sur-Mer, tide pool in *Enteromorpha*	07.05.2009	
	857	France: Banyuls-sur-Mer, tide pool in *Enteromorpha*	07.05.2009	
	S150	France: Banyuls-sur-Mer, tide pool in *Enteromorpha*	07.05.2009	Ee09By-3
Limapontia senestra (Quatrefages, 1844)	834	Germany: Helgoland	24.04.2007	IB
Placida cremoniana (Trinchese, 1893)	862	USA: Guam edge of watertable	28.08.2009	Pc-09GU-1
Placida dendritica (Alder & Hancock, 1843)	768	Spain: Tossa de Mar	20.05.2006	
	795	Spain: Tossa de Mar	20.05.2006	
	793	Spain: Tossa de Mar	20.05.2006	
Placida verticillata Ortea, 1981	831	Bolivarian Republic of Venezuela Isla de Margarita	00.04.2007	CZ
Stiliger ornatus Ehrenberg, 1828	840	Egypt: Dahab	00.00.2008	S. Bleidißel
	842	Egypt: Dahab	00.00.2008	S. Bleidißel
	843	Egypt: Dahab	00.00.2008	S. Bleidißel

Table 2.2 Ulvophycean species and specimens used for DNA extraction and internal no. of DNA sample. Collection place and collection date are given. Remarks: Sample code of algae, e.g. B-09GU-8; if animal has been collected by another person, this is indicated by his/her name. DNA samples with internal no. above A100 were extracted by Gregor Christa.

Species	No.	Collection place	Collection date	Remarks
Bryopsidales				
BRYOPSIDACEAE				
Bryopsis plumosa (Hudson) C. Agardh , 1823	A15	France: Banyuls-sur-Mer, harbour	24.04.2007	S. Affeld
Bryopsis sp. J.V. Lamouroux, 1809	A102	France: Banyuls-sur-Mer, mole	28.04.2009	Bsp09By-5
Bryopsis sp. J.V. Lamouroux, 1809	A170	USA: Guam Pago Bay	16.08.2009	B-09GU-8
CAULERPACEAE				
Caulerpa cf. *racemosa* (Forsskål) J.Agardh, 1873	A110	Italy: Isola del Giglio near camping place	22.06.2208	GI2
	A121	Italy: Isola del Giglio lighthouse	29.06.2008	GI27
Caulerpa sertularioides (S.G. Gmelin) M.A.Howe, 1905	A159	Australia: Lizard Island, Blue Lagoon	27.10.2008	Csp08LI-154
Caulerpa cupressoides (West) C.Agardh 1817	A134	Australia: Lizard Island, Casuarina Beach	19.10.2008	Ca08LI-49
Halimeda cuneata (as sp. 1) K. Hering in Krauss 1846	A1	Maledives	29.03.2007	W. Loch
(as sp. 2)	A2	Maledives	29.03.2009	W. Loch
Halimeda tuna (J.Ellis & Solander) J.V.Lamouroux, 1816	A6	France Banyuls-sur-Mer	08.06.2006	
Halimeda macroloba Decaisne, 1841	A155	Australia Lizard Island	26.10.2008	H08LI-135
Halimeda melanesica Valet, 1966	A171	USA: Guam Cocos Lagoon	21.08.2209	H09GU-5
Halimeda minima (W.R.Taylor) Hillis-Colinvaux, 1968	A154	Australia: Lizard Is- Lizard Island Horseshoe Reef	23.10.2008	H08LI-89b
Halimeda sp. 3 J.V. Lamouroux, 1812	A7	Maledives	29.03.2009	W. Loch

CODIACEAE				
Codium effusum (Rafinesque) Delle Chiaje, 1829	A128	Italy: Isola del Giglio lighthouse	03.07.2008	GI59
Codium cf. *tomentosum* Stackhouse, 1797	A133	Spain: Ria de Ferrol	13.01.2009	Ct09Fe-1
Codium vermilara (Olivi) Chiaje, 182	A101	France Banyuls-sur-Mer	28.04.2009	Cv09By-4
	A130	Italy: Isola del Giglio lighthouse	03.07.2008	GI86
Codium sp. Stackhouse, 1797	A107	France Banyuls-sur-Mer	04.05.2009	Csp09By-8
UDOTEACEAE				
Avrainvillea erecta (Berkeley) A. Gepp & E.S. Gepp, 1911	A162	Australia: Lizard Island, Casuarina Beach	29.10.2008	Ae08LI-187
Flabellia petiolata (Turra) Nizamuddin, 1987	A14	France Banyuls-sur-Mer	24.04.2007	S. Affeld
Rhipilia crassa A.J.K. Millar & Kraft, 2001	A166	USA: Guam, Cocos Lagoon, outfront	25.08.2009	A-09GU-1
Tydemania expeditionis Weber-van Bosse, 1901	A3	Maledives	29.03.2009	
	A168	USA: Guam, Asan	28.08.2009	Te-09GU-1
Cladophorales				
CLADOPHORACEAE				
Chaetomorpha sp. Kützing, 1845	A27	France: St. Jouin	28.04.2007	
Ulvales				
ULVACEAE				
Ulva lactuca Linnaeus, 1753	A26	France: St. Jouin	09.05.2007	
Ulva sp. Linnaeus, 1753	A20	France Banyuls-sur-Mer	14.04.2007	S. Affeld

2.2 Phylogeny of Sacoglossa and specific groups of Sacoglossa

DNA was extracted as described in Händeler et al. 2009.

28S rDNA

Partial sequences of nuclear 28S rDNA gene were amplified in the Institut für Evolutionsbiologie und Ökologie as in Händeler et al. 2009 and in the ZFMK as follows: Amplification reactions for primer pair 28SC1 and 28SD3 (Vonnemann et al. 2005) (25µl) consisted of 7.5µl RNase-free water, 2.5µl Q-Solution, 12.5µl

2x QIAGEN Multiplex PCR Master Mix, 1µl of each Primer (10pmol/µl) and 0.5µl DNA. PCR was carried out under following conditions: 95°C for 15min, followed by 9 Touch Down cycles of 45s at 94°C, 45s at 62.5(–1)°C and 90s at 72°C, followed by 30 amplification cycles of 30s at 94°C, 90s at 52.5°C, 90s at 72°C and a final extension at 72°C for 10min followed by cooling down.

Amplification reactions for primer pair 28SC1 (Vonnemann et al. 2005) and 28SR1 (Morgan et al. 2002) (25µl) consisted of 7.5µl RNase-free water, 2.5µl Q-Solution, 12.5µl 2x QIAGEN Multiplex PCR Master Mix, 1µl of each Primer (10pmol/µl) and 0.5µl DNA. PCR was carried out under following conditions: 95°C for 15min, followed by 9 Touch Down cycles of 45s at 94°C, 45s at 65(–1)°C and 90s at 72°C, followed by 30 amplification cycles of 30s at 94°C, 90s at 56°C, 90s at 72°C and a final extension at 72°C for 10min followed by cooling down.

Internal primers 28SC2F(C2')*, 28SD2F, 28SD2R and 28SC2R(C2)* (Vonnemann et al. 2005) were used if necessary to gain both strains of 28S. For sequencing this longer part of 28S, primer D3F 5'-TGA CGT GCA AAT CGA TCG TC-3', the reverse complement of 28SD3, has been used as internal primer.

Due to amplification problems, a new primer, 28SK, is proposed: 5'-AKG ATK TCC CCA GTA ACG GCG AGT GAA G-3' instead of 28SC1. Amplification conditions have not been tested yet for reasons of limited time.

Sometimes the algal food had been amplified instead of or additionally to the slug's sequence. These results are included in the chapter 3.3.3 Food organisms of Sacoglossa.

16S rDNA

Partial sequences of the mitochondrial 16S rDNA were amplified in the Institut für Evolutionsbiologie und Ökologie as in Händeler and Wägele 2007 respectively in the ZFMK as follows: partial sequences of the 16S rDNA gene were amplified with primers LR-J-12887 and LR-N-13398 (Händeler & Wägele 2007). Amplification reactions (20µl) consisted of 5.5µl RNase-free water, 2µl Q-Solution, 10µl 2x QIAGEN Multiplex PCR Master Mix, 1µl of each Primer (10 pmol/µl) and 0.5µl DNA.

Complete mitochondrial genomes of *Ascobulla fragilis* (NC_012428) (Grande et al. 2008) and *Elysia chlorotica* (NC_010567) (Rumpho et al. 2008) became available and allowed a correction of the existing primer pair; S_16Sup, 5'-CCT YAM CTG TTT AHC AAA AAC ATA G-3', and S_16Sdo, 5'-CCT ACA TGA TCT GAG TTC ARA CCG G-3', were created. Amplification reactions (20µl) for this primer pair consisted of 5.2µl RNase-free water, 2µl Q-Solution, 10µl 2x QIAGEN Multiplex PCR Master Mix, 1µl of primer S_16Sup (10pmol/µl), 1.3µl of primer S_16Sup (10 pmol/µl) and 0.5µl DNA.

PCR was carried out under the following conditions: 95°C for 15min, followed by 9 Touch Down cycles of 45s at 94°C, 45s at 56(-1)°C and 90s at 72°C, followed by 25 amplification cycles of 30s at 94°C, 90s at 48°C, 90s at 72°C and a final extension at 72°C for 10 min followed by cooling down.

coxI

Partial *coxI* was amplified in the Institut für Evolutionsbiologie und Ökologie as in Händeler and Wägele 2007 respectively in the ZFMK as in Händeler et al. 2009 or as follows: Partial sequences of the mitochondrial *coxI* gene was amplified with primers LCO1490 and HCO2198 (Bass & Karl 2006 after Folmer et al. 1994). Amplification reactions (20µl) consisted of 5.5µl RNase-free water, 2µl Q-Solution, 10µl 2x QIAGEN Multiplex PCR Master Mix, 1µl of each primer (10pmol/µl) and 0.5µl DNA. PCR was carried out under the following conditions: 95°C for 15min, followed by 9 Touch Down cycles of 45 s at 94°C, 45s at 56(-1)°C and 90s at 72°C, followed by 25 amplification cycles of 30s at 94°C, 90s at 48°C, 90s at 72°C and a final extension at 72°C for 10 min followed by cooling down.

Complete mitochondrial genomes of *Ascobulla fragilis* (NC_012428) (Grande et al. 2008) and *Elysia chlorotica* (NC_010567) (Rumpho et al. 2008) became available and allowed a correction of the existing primer pair; coxFS, 5'-TTT CAA CAA ACC ATA ARG ATA TTG G-3', and coxRS, 5'-TAY ACT TCW GGG TGW CCA AAA AAY CA-3', were created. Amplification reactions (20µl) for this primer pair consisted of 5.4µl RNase-free water, 2µl Q-Solution, 10µl 2x QIAGEN Multiplex PCR Master Mix, 1µl of primer coxFS (10pmol/µl), 1.1µl of primer coxIRS (10 pmol/µl) and 0.5µl DNA.

PCR was carried out under the following conditions: 95°C for 15min, followed by 9 Touch Down cycles of 45s at 94°C, 45s at 56(-1)°C and 90s at 72°C, followed by 25 amplification cycles of 30s at 94°C, 90s at 48°C, 90s at 72°C and a final extension at 72°C for 10 min followed by cooling down.

Amplicons were purified by means of QIAquick PCR Purification Kit by Qiagen, guided by the enclosed protocol and sequenced with service of Macrogen Inc., Korea. Additional sequences were downloaded from GenBank.

Sequences were aligned using the web server of MAFFT (http://mafft.cbrc.jp/alignment/server/). The algorithm E-INS-i (Katoh et al. 2005), that is recommended for multiple conserved domains and long gaps, was chosen. Other parameters were used as they are in the default settings: gap opening penalty was 1.53 and Offset value 0.

Phylogenetic analysis by Maximum Likelihood research was performed with the help of the web server of RaXML (http://phylobench.vital-it.ch/raxml-bb/) (Stamatakis et al. 2008). Data set was partitioned according to the three different partial gene sequences. RaXML was told to estimate model parameters separately for each partition but to leave out estimation of proportion of invariable sites. 100 bootstrap references were executed. A ML-search was performed, bootstrap support values are shown on the trees.

2.3 Kleptoplasts in Sacoglossa: photosynthetic abilities among Sacoglossa

The maximum quantum yield value of chlorophyll a fluorescence in photosystem II was measured with the help of a Pulse Amplitude Modulated Fluorometer (DIVING PAM, Walz, Germany). This non-invasive method was used because it can be easily applied in the field, and leave the animals for further investigations like phylogeny or barcoding of food.

Every reaction centre that is present and "open", can absorb photons. The PAM emits an actinic light, that stimulates photosystem II, i.e. photons are absorbed and the reaction centres "close", chlorophyll a emits fluorescence. Measurements were taken with chloroplasts acclimated to darkness, thus with all reaction centres open and a minimal fluorescence emission. Before emitting the actinic flash the fluorescence yield F_0 is measured with the help of a non-actinic light source. After emission of the actinic light the maximal yield F_m is measured by the PAM. F_m is the maximum emission of fluorescence, since the chloroplasts were acclimated to darkness and all reaction centers open. ΔF is calculated as $\Delta F = F_m - F_0$. The photosynthesis yield $Y = \Delta F/F_m$ is automatically calculated, the obtained data displayed and stored for later analysis.

Figure 2.2 Measurements with a Diving PAM. a) Animals are kept separated from their food algae right after capture. b) PAM instrument. Photo: Heike Wägele.

PAM measurements were performed as described in Händeler et al. 2009. Data obtained on Isola del Giglio, Italy from *Bosellia mimetica* and *Thuridilla hopei*, Lizard Island, Australia from *Lobiger viridis*, *Costasiella* cf. *kuroshimae*, *Polybranchia orientalis*, *Elysia pusilla*, *Elysia tomentosa*, *Plakobranchus ocellatus*, *Thuridilla carlsoni*, *Thuridilla gracilis* and *Thuridilla kathae*, purchased samples from the Philippines (*Plakobranchus ocellatus*) in 2008 and in Banyuls-sur-Mer in 2009 from *Bosellia mimetica* are included in Händeler et al. 2009. New data obtained after this publication are listed in table 2.3.

Table 2.3 Species and specimens whose PAM data are not included in Händeler et al. 2009. Origin and collection date are given. # – number of investigated specimens.

Species	#	Origin	Collection date
Oxynoacea			
CYLINDROBULLIDAE			
Cylindrobulla sp.	4	USA: Guam, Cocos Lagoon	2009
Plakobranchacea			
PLAKOBRANCHOIDEA			
PLAKOBRANCHIDAE			
Elysia translucens	1	France: Banyuls-sur-Mer	2009
Elysia viridis	3	France: Banyuls-sur-Mer	2009
Elysia cf. *grandifolia*	5	USA: Guam, Pago Bay	2009
Elysia cf. *tomentosa*	1	USA: Guam, Pago Bay	2009
Elysia sp. 2GUAM	1	USA: Guam, Pago Bay	2009
Elysia sp. 3GUAM	1	USA: Guam, *Caulerpa*-spot near Clay Carlson's farm, on *C. cupressoides*	2009
Elysia sp. 4GUAM	1	USA: Guam, Pago Bay	2009
Elysia sp. 5GUAM	3	USA: Guam, Asan on *Tydemania expeditionis*	2009
Elysia sp. Banyuls	2	France: Banyuls-sur-Mer	2009
Elysia sp. 15 (Gosliner et al. 2008)	3	USA: Guam, Cocos Lagoon on *Udotea geppii*	2009
Elysia sp. 11 (seaslugforum.net)	2	Australia: Lizard Island, MacGillivray Reef / USA: Guam, Bile Bay	2008 / 2009
Plakobranchus ocellatus	3	USA: Guam, Pago Bay	2009
Thuridilla bayeri	2	USA: Guam, outfront Cocos Lagoon	2009
Thuridilla livida	2	USA: Guam, Bile Bay	2009
LIMAPONTIOIDEA			
POLYBRANCHIIDAE			
Cyerce nigra	4	USA: GUAM, Cocos Lagoon	2009
Cyerce sp. 3 (Gosliner et al. 2008)	5	USA: GUAM, Cocos Lagoon	2009
COSTASIELLIDAE			
Costasiella cf. *kuroshimae*	4	USA: GUAM, Cocos Lagoon on *Avrainvillea erecta*	2009
Costasiella sp. 1 (seaslugforum.net)	2	USA: GUAM, Cocos Lagoon on *Avrainvillea erecta*	2009
LIMAPONTIIDAE			
Ercolania "*annelyleorum*"	4	Australia: Lizard Island	2008
Ercolania viridis	4	France: Banyuls-sur-Mer	2009

Feeding experiments with Thuridilla hopei PAM measurements were also used to identify possible food algae. Feeding experiments have been performed with several specimens of *Thuridilla hopei* on the Isola del Giglio, Italy. All specimens were starved until no photosynthetic activity could be measured by means of the PAM. Algae were collected in the according habitat, cleaned, especially from epiphytes, and given to starved animals. Potential photosynthetic activity was monitored and health of algae checked daily with the help of a PAM. If necessary, algae were replaced.

2.4 Food organisms of Sacoglossa: barcoding

Several primers were designed and tested. Primers are listed in table 2.4, those which were eventually successful are printed white on black. Finally *tufA*, encoding protein synthesis elongation factor Tu, had been chosen to serve as a second barcode additional to *rbcL*. It is part of the chloroplast genome according to the known cp genomes of ulvophycean algae (Pombert et al. 2005, 2006; complete cp genome of *Bryopsis hypnoides* stored under NC_013359 in GenBank, unpublished) and several *tufA* sequences of all groups of Ulvophyceae are represented in GenBank.

tufA

DNA extraction and PCR for the *tufA* gene amplified with primer pair tufA_alg_up and tufA_alg_do were performed like in Händeler et al. 2010. For DNA extraction in the ZFMK the Qiagen DNeasy Plant kit was used. Algae were fixed in EtOH and stored at -20°C dried in a Speed Vac and pulverised with the help of liquid nitrogen. Amplification reactions for *tufA* obtained with primer pair tufAF and tufAR (20µL) consisted of 4.9µL RNAse free H_2O, 2µL Q-Solution, 10µL 2x QIAGEN Multiplex PCR Master Mix, 1.3µL of each primer (10pmol/µL) and 0.5µL DNA. The PCR was carried out under following conditions: 95°C for 15 minutes (activation of Hotstart-Taq), followed by 9 Touchdown-cycles of 45s at 94°C, 90s at 61(-1)°C, 90s at 72°C, followed by 25 amplification-cycles of 45s at 94°C, 90s at 52°C, 90s at 72°C and a final extension at 72°C for 10 min. To gain PCR-products of difficult amplifying samples, e.g. genetic cp material in small amounts in Sacoglossa, a modification of a less specific existing program (Fama et al. 2002) was used: 95°C for 15 minutes (activation of Hotstart-Taq), followed by 40 amplification-cycles of 45s at 94°C, 90s at 45°C, 90s at 72°C and a final extension at 72°C for 10 min.

rbcL

Amplification reactions for *rbcL* obtained with primer pair rbcL 1 and rbcL R (20µL) consisted of 3.1µL RNAse free H_2O, 2µL Q-Solution, 10µL 2x QIAGEN Multiplex PCR Master Mix, 3µL of primer rbcL 1 (5pmol/µL), 1.4µL of primer rbcL R and 0.5µL DNA. The PCR was carried out under following conditions: 95°C for 15 minutes (activation of Hotstart-Taq), followed by 9 Touch-down-cycles of 45s at 94°C, 45s at 53(-1)°C, 90s at 72°C, followed by 25 amplification-cycles of 45s at 94°C, 90s at 45°C, 90s at 72°C and a final extension at 72°C for 10 min.

Cloning and determination of origin

Amplicons were size fractionated in an 1% agarose gel and purified by means of the NucleoSpin Extract II Kit by Machery-Nagel, guided by the enclosed protocol or as is described in Händeler et al. 2010.
At least cp sequences obtained from slugs were cloned as performed in Händeler et al. 2010, to be able to differentiate varying sequences from one individual slug. Sequencing was performed by Macrogen Inc. Korea. Sequence identity was verified with BLAST algorithm by GenBank. Affinity of sequences to reference sequences was determined by Neighbornet analyses with the help of SpiltsTree (Huson and Bryant 2006). For details see Händeler et al. 2010.

Gregor Christa was introduced into this method and supervised during the compilation of his diploma thesis on the supplied material. Cloning was performed in the Institut für Pharmazeutische Biologie in the laboratory of Prof. Dr. Gabriele König according to the methods elaborated in this laboratory.

Table 2.4 All tested primer pairs that could potentially be used for barcoding of cp genes of DNA extracted from Sacoglossa. Those which were successful and eventually used as barcodes are printed white on black. IZMB - Institut für Zelluläre und Molekulare Botanik, ZFMK – Zoologisches Forschungsmuseum Alexander Koenig.

Locus	Name	Sequence 5' ⟶ 3'	Reference	Lab
tufA	tufA_alg_up	ATG ATW ACN GGH GCN GCW CAA ATG G	U. Wahrmund	IZMB
	tufA_alg_do	TTG TTC KAA CAT AAA ATT GWG GTC	U. Wahrmund	IZMB
tufA	tufAF	TGA AAC AGA AMA WCG TCA TTA TGC	Fama et al. 2002	ZFMK
	tufAR	CCT TCN CGA ATM GCR AAW CGC	Fama et al. 2002	ZFMK
rbcL	rbcL 1	AAA GCN GGK GTW AAA GAY TA	Pierce et al. 2006	ZFMK
	rbcL R	CCA WCG CAT ARA NGG TTG HGA	modified after rbcL 2 (Pierce et al. 2006)	ZFMK
rbcL	rbcL_alg_up	CAM GTA WHA GAW ACN GAY ATT CTT GC	U. Wahrmund	IZMB
	rbcL_alg_do	GCR AGA GAA CGT CCT TCR TTA CG	U. Wahrmund	IZMB
intergeneric spacer rpoB - rpoC1	rpoB_up	GAR GTW TGG GCY YTR GAR GGH TTY GGA GC	U. Wahrmund	IZMB
	rpoC1_do	TTC ACA AAA YAA WCC HYC TTT YTC MGG TTT T	U. Wahrmund	IZMB
intergeneric spacer rpoB - rpoC1	rpoBupv2	GAR ATG GAR GTN TGG GCN YTN GAR GGN TTY GG	self-designed	IZMB
	rpoC1dov2	TCR CAR AAN ARN CCN CCY TTY TTY TCN GGY TT	self-designed	IZMB
intergeneric spacer rpoC1 - rpoC2	rpoC1_up	TTT ACC YAG YCA AGA TAT GVT WTT AGG	U. Wahrmund	IZMB
	rpoC2_do	AGC AGG WRT TTC WYT WGG KAT TGA TGA	U. Wahrmund	IZMB
rpoC2	rpoC2up	ATG GCN TTY TCN GGN GCN MGN GG	self-designed	IZMB
	rpoC2do	GGY TCN CCD ATN GAY TGN GCN GC	self-designed	IZMB

Species of the genus *Thuridilla* (Plakobranchidae) are not found on their food algae, but crawling on the ground being brightly coloured. Often their colouration varies intraspecific; *Thuridilla hopei* shows a rosy (above) and a bluish form (below). Both forms were found on the Isola del Giglio (Italy) in 2008.

3 Results

Partly, results were already published in Händeler et al. 2009 and Händeler et al. 2010, which are included as separated hard copies. Additional results that are unpublished or used in other publications that are still in progress or handed in or are published without being the first author are summarized in the chapter "Unpublished results".

3.1 K. Händeler, Y. P. Grzymbowski, P. J. Krug and H. Wägele (2009) Functional chloroplasts in metazoan cells - a unique evolutionary strategy in animal life

Frontiers in Zoology 2009, 6:28doi:10.1186/1742-9994-6-28
Open Access: http://www.frontiersinzoology.com/content/6/1/28

Since several authors have been involved in this publication, the following explanation of my parts is given here. 89 sequences were produced by myself, 56 by P. Krug and 58 taken from GenBank. Details are given in table 6.1 on p. 99 in the appendix. Alignment was performed by myself, phylogenetic analysis were started by myself in Bonn but eventually proceeded in Los Angeles by P. Krug due to computer capacity. PAM measurements of 53 specimens were taken by myself with the help of H. Wägele and of 125 specimens by Y. Grzymbowski. Details are given in table 6.2 on p. 102 in the appendix. PAM data of all 178 specimens were analysed by myself. The manuscript was drafted by myself with the help of P. Krug and H. Wägele.

3.2 K. Händeler, H. Wägele, U. Wahrmund, M. Rüdinger, V. Knoop (2010) Slugs' last meals: Molecular identification of sequestered chloroplasts from different algal origins in Sacoglossa (Opisthobranchia, Gastropoda)

Molecular Ecology Resources, 10 (6), 968-978.

All data were produced and analysed by myself. U. Wahrmund designed primer, M. Rüdinger introduced and helped in the lab of Prof. Dr. V. Knoop in the IZMB. The manuscript was drafted by myself with the help of V. Knoop and H. Wägele.

3.3 "Unpublished results"

Parts included here have been published now in Wägele et al. 2010 but are included in this chapter since the author of the thesis is not the first author of the mentioned publication. A survey of all data, published and unpublished, is given for the food organisms of Sacoglossa.

3.3.1 Phylogeny of Sacoglossa and specific groups of Sacoglossa

The two sequences 16S and *coxI* of *Elysia* sp. 11 (seaslugforum.net) are identical respectively. All sequences (28S rDNA, 16S rDNA and *coxI*) of *Thuridilla gracilis* 1 (704) and 2 (712) are identical. The sequences 28S, 16S rDNA and first and second position of *coxI* are identical for: *Bosellia mimetica* 1 and 2; *Ercolania viridis* 1 and 2; *Cyerce* sp. 3 (Gosliner et al. 2008) 1 (848) and 2 (859); *Stiliger ornatus* 1 (843) and 3 (842); *Costasiella* sp. GUAM (black) 1 (870) and 2 (863); *Plakobranchus ocellatus* GUAM 1 (850) and 4 (856).

Several phylogenetic analyses have been perfomed for different publications. Partial 28S rDNA, partial 16S rDNA and 1st and 2nd position of partial *coxI* were concatenated as is shown in table 6.3 in the appendix. Since the 3rd position of the partial *coxI* sequence is strongly saturated, it was excluded from the analysis.

In contrast to sequences obtained from GenBank, sequences that were produced by myself are indicated by my internal numbers; if these sequences have an accession number, the internal number is given in brackets behind the accession number.

The 28S rDNA sequence of *Cylindrobulla beauii* (EF489371) was produced in the lab of Annette Klussmann-Kolb (Frankfurt), but were obtained from my DNA sample 728.

The different data sets are indicated in table 6.3 on p. 103 (appendix) as follows:

- **Preliminary phylogeny of Sacoglossa** Data set consists of all sequences except for those that are in a grey box and concatenated as they occur in the table. An analysis of the phylogeny of Sacoglossa additional to the analysis published in Händeler et al. 2009 became necessary since many additional species/specimens became available and new sequences were produced after the phylogeny of Sacoglossa had been published in 2009. Sequences of more than 40 specimens, including 16 new species, were added. Furthermore, gene sets, i.e. 28S rDNA, 16S rDNA and *coxI*, were completed for single specimens; e.g. the 28S rDNA sequence of *Elysia serca* 829 has been concatened in Händeler et al. 2009 with a 16S rDNA and *coxI* sequence extracted from GenBank. Now, all three sequences are from specimen 829. Comparing with the data set used in Händeler et al. 2009 this data set includes 27 new sequences of 28S rDNA, 46 new 16S rDNA sequences and 46 new *coxI* sequences.

  The analysis is considered to be preliminary since important sequences/ species are still missing; sequences of 25 specimens have not yet been included, since sequences, mainly 28S rDNA, are missing. It shall give a compendium of the most recent phylogeny of Sacoglossa available at the point of the completion of this thesis. Result is shown in figure 3.1, p. 43.

- **Phylogeny of Sacoglossa** that is implemented in Händeler et al. 2009: data set consists of sequences that are printed in bold type.
- **Phylogeny of Plakobranchoidea** included in Wägele et al. 2010. Data set consists of all sequences that are printed in italics. Sequences that were submitted to GenBank for this publication received accession numbers that start with "HM". The *coxI* sequence of a specimen of *Plakobranchus ocellatus* (856) with the accession number HM187635 has not been used in this analysis but got an accession number by mistake. The publication of Wägele et al. 2010 focuses on sacoglossan species that are able to perform a functional retention over several weeks up to months. These sacoglossans belong to the taxon Plakobranchoidea. Result is shown in figure 3.2, p. 44.
- **Phylogeny of the genus *Elysia*** Since the genus *Elysia* is monophyletic (Händeler et al. 2009, preliminary phylogeny of Sacoglossa, figure 3.1 and phylogeny of Plakobranchoidea, figure 3.2), a separate analysis is possible and is implemented in Wägele et al. (in press) to point out the phylogenetic position of the new species *Elysia* "*asbecki*". This species will be described within this publication. All sequences of the genus were used that had been used in the data set "Plakobranchoidea" except for the Mediterranean *Elysia translucens*, since this species is the only one, where the 28S rDNA sequence is missing. *Plakobranchus ocellatus* 1 and 2, *Thuridilla carlsoni*, *Thuridilla hoffae* and *Thuridilla kathae* were used as outgroup taxa.
- **Phylogeny of Limapontiidae** Since this family is monophyletic (preliminary phylogeny of Sacoglossa, figure 3.1; Händeler et al. 2009), a separate analysis is possible and is implemented in Wägele et al. (in press) to point out the phylogenetic position of the new species *Ercolania* "*annelyleorum*". This species will be described within this publication. All sequences of the family Limapontiidae were used except for: 855, an undescribed species missing the partial 28S rDNA sequence; *Ercolania viridis* S150 and 844, since all positions of the concatenated sequences of 857 (included in the analysis) and S150 are identical and there is only the partial 16S rDNA sequence of specimen 844; *Placida dendritica* 793, since already two other specimens are included and partial 28S rDNA sequence of 793 is missing; *Stiliger ornatus* 842, since concatenated sequences are identical to those of specimen 843 (included). The two specimens of *Stiliger ornatus* (843 and 840) are included, although the partial 28S rDNA sequence is missing, because these are the only sequences that exist for the genus *Stiliger*. *Mourgona osumi* and *Cyerce nigricans* were used as outgroup taxa.

Sacoglossa

The data set comprised 120 specimens, including 88 species. Final length of aligned and concatenated sequences was 2131 bp: 28S rDNA gene, positions 1-1210, 16S rDNA gene, positions 1211-1695 and *coxI* (1st and 2nd position), positions 1696-2131. The phylogeny of Sacoglossa is shown in figure 3.1. Analysis of the three gene regions strongly support monophyly for Sacoglossa including *Cylindrobulla* and the two major sacoglossan lineages: the Oxynoacea, comprising all shelled Sacoglossa with *Cylindrobulla* being part of it, and the shell-less Plakobranchacea, comprising Plakobranchoidea and Limapontioidea.

The group Plakobranchoidea has a low support value (57). However, the families Boselliidae (monogeneric) and Plakobranchidae are well supported. Within the Plakobranchidae, the three genera *Elysia*, *Thuridilla* and *Plakobranchus* are monophyletic and well supported. *Elysia* is sistertaxon to a monophylum including *Thuridilla* and *Plakobranchus.*

Relationships of the main subgroups of the Limapontioidea are not resolved. However, most families are monophyletic as Limapontiidae, Costasiellidae and Hermaeidae. There are two monophyletic groups of members of the family Polybranchiidae; the genus *Cyerce* and the group (*Caliphylla mediterranea*, (*Mourgona*, *Polybranchia*)).

Families within the shelled Oxynoacea are not monophyletic. The family Volvatellidae (*Volvatella* and *Ascobulla*) appears not to be monophyletic, since the as *Ascobulla* sp. identified specimen is found as sister-taxon to the monophyletic Cylindrobullidae. The family Juliidae is polyphyletic, since *Julia exquisita* is sistertaxon to Volvatellidae ex *Ascobulla* sp. and *Tamanovalva limax* sistertaxon to all other Oxynoacea. *Lobiger viridis* (Oxynoidae) and the monophyletic genus *Oxynoe* (Oxynoidae) remain unresolved together with the group (Volvatellidae ex *Ascobulla* sp. /*Julia exquisita*). *Oxynoe antillarum* appears paraphyletic, since *Oxynoe azuropunctata* can be found within the two specimens of *Oxynoe antillarum.*

Plakobranchoidea

The data set comprised 62 specimens, including 41 species. Final length of aligned and concatenated sequences was 1986 bp: 28S rDNA gene, positions 1-1082, 16S rDNA gene, positions 1083-1550, and *coxI* (1st and 2nd position), positions 1551-1986. Phylogeny of Plakobranchoidea on species level is shown in figure 3.2. The monophyletic family Boselliidae is sistertaxon to the monophyletic family Plakobranchidae. Each of the three genera of the Plakobranchidae (*Elysia*, *Thuridilla* and *Plakobranchus*) are monophyletic. *Elysia* is sistertaxon to a taxon comprising the genera *Plakobranchus* and *Thuridilla.*

Different to the result of the phylogeny of Sacoglossa (figure 3.1) is the sistertaxa-relationship of *Elysia viridis* and *Elysia chlorotica.* The analysis of Sacoglossa reveals *Elysia* "*asbecki*" (*Elysia* sp. 1) as sistertaxon to *Elysia* sp. 5. The relationship of the taxa *Elysia* "*asbecki*", *Elysia* sp. 5. and *Elysia macnaei* remains unresolved, considering that all nodes in figure 3.2 that show no support values were not supported with a support value higher than 50. (Nodes

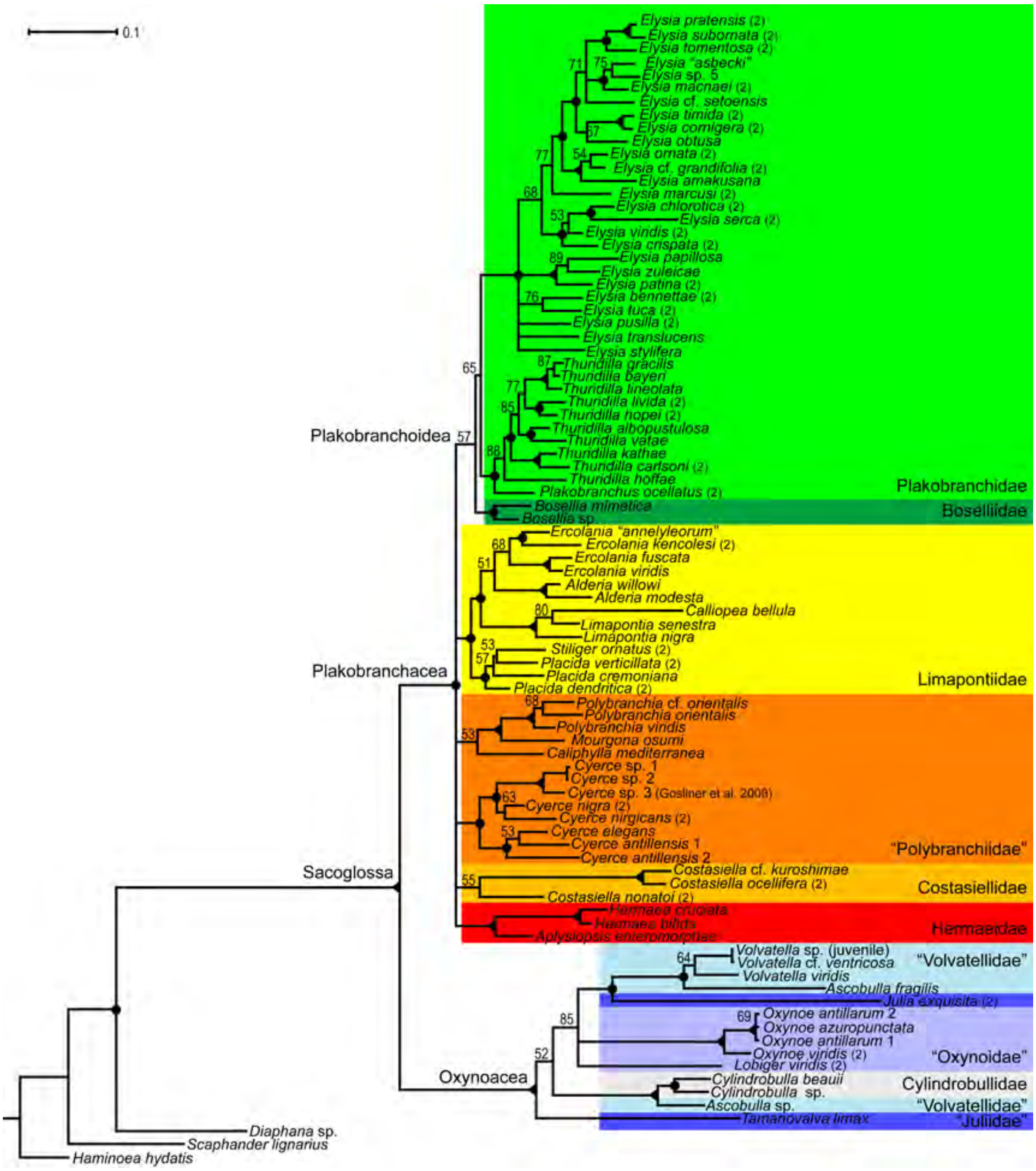


Figure 3.1 Results of a ML bootstrap analysis with 100 replicates of the Sacoglossa by means of the partial molecular markers 28S rDNA, 16S rDNA and 1st and 2nd position of partial *coxI*. Support values are given; black dot - support value is 100, black triangle – support value is between 90 and 99. (2) – node comprising two specimens of same species was collapsed. Nodes with support values lower than 50 were collapsed.

with support values lower than 50 were collapsed in figure 3.1). In the analysis of Sacoglossa is the clade (*Elysia ornata* (*Elysia cornigera* + *Elysia timida*)) sistertaxon to (((*Elysia pratensis* + *Elysia subornata*) + *Elysia tomentosa*), ((*Elysia asbecki* + *Elysia* sp. 5) + *Elysia macnaei*), *Elysia* cf. *setoensis*) and not included in this group as in the analysis of the Plakobranchoidea.

Phylogenetic relationships of the species of the genus *Thuridilla* are the same in both analyses. Monophyletic *Thuridilla gracilis* and *Thuridilla bayeri* are sistertaxon to *Thuridilla lineolata*. Sistertaxon to this clade is the monophyletic group composed of *Thuridilla livida* and *Thuridilla hopei*. This clade is sistertaxon to the monophyletic group of *Thuridilla albopustulosa* and *Thuridilla vatae*. Sistertaxon to this clade is (*Thuridilla kathae* + *Thuridilla carlsoni*). Sistertaxon to all other species is *Thuridilla hoffae*.

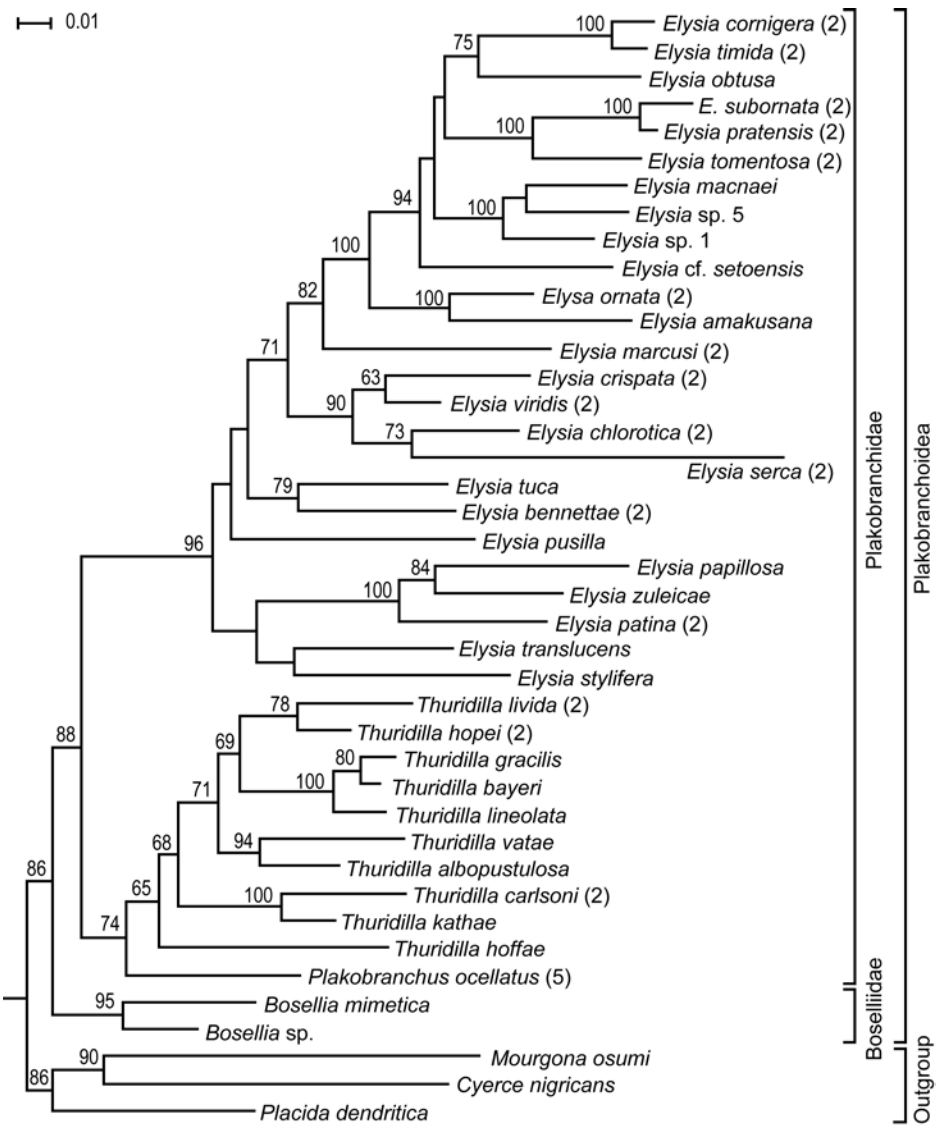


Figure 3.2 Results of a ML bootstrap analysis with 100 replicates of the Plakobranchoidea by means of the partial molecular markers 28S rDNA, 16S rDNA and 1st and 2nd position of partial *coxI*. Support values larger than 50 are shown. (2) – node comprising two specimens of same species was collapsed. *Elysia* sp. 1 is *Elysia* "*asbecki*".

The genus Elysia

The data set comprised 42 specimens, including 28 species. Phylogeny of *Elysia* is shown in figure 3.3. Final length of aligned and concatenated sequences was 1967 bp: 28S rDNA gene, positions 1-1069, 16S rDNA gene, positions 1070-1531 and *coxI* (1st and 2nd position), positions 1532-1967. The monophyly of the genus *Elysia* is well supported (100). The position of some species and species groups is not resolved, i.e. *Elysia stylifera*, *Elysia pusilla*, (*Elysia tuca* + *Elysia bennettae*) and (*Elysia patina* + (*Elysia zuleicae* + *Elysia papillosa*)).

Elysia "*asbecki*" groups with *Elysia macnaei* and *Elysia* sp. 5. This group forms, together with *Elysia* cf. *setoensis* and (*Elysia tomentosa* + (*Elysia pratensis* + *Elysia subornata*)), an unresolved polytomy that is sistertaxon to (*Elysia obtusa* + (*Elysia timida* + *Elysia cornigera*)) as in the analysis of Sacoglossa (figure 3.1). This group is sistertaxon to a monophyletic group of *Elysia ornata* and *Elysia amakusana*. While the latter node is well supported (100), the monophyly of this group and *Elysia marcusi* is not (56). This group is sistertaxon to ((*Elysia chlorotica* + *Elysia serca*) + (*Elysia crispata* + *Elysia viridis*)). The monophyly of *Elysia crispata* and *Elysia viridis* is consistent with analysis of Plakobranchoidea but not with that of Sacoglossa.

Limapontiidae

The data set comprised 22 specimens, including 18 species. Phylogeny of the taxon Limapontiidae is shown in figure 3.4. Final length of aligned and concatenated sequences was 1953 bp: 28S rDNA gene, positions 1-1069, 16S rDNA gene, positions 1070-1517 and *coxI* (1st and 2nd position), positions 1518-1953. *Ercolania* sp. can be assigned to *Ercolania kencolesi* based on pairwise sequence comparison of *coxI*; the p-distance of *coxI* is smaller than 2%. Four species of the genus *Ercolania* form a monophyletic clade, i.e. *Ercolania kencolesi*, *Ercolania* "*annelyleorum*", *Ercolania viridis* and *Ercolania fuscata*. *Ercolania felina* groups with the two species of *Limapontia* and *Calliopea bellula*. Position of *Ercolania boodleae* is unresolved as is the position of the species of *Placida* and *Stiliger ornatus*. The two species of *Alderia* are monophyletic and are sistertaxon to the monophyletic group of *Ercolania*.

Ercolania felina, *Ercolania boodleae* and *Placida kingstoni* were not included in the preliminary analysis of Sacoglossa owing to their dubious genus affiliation according to the result of the analysis of Limapontiidae and the result obtained by Händeler et al. 2009. The remaining species of *Placida* and *Stiliger ornatus* appear in the analysis of Sacoglossa as monophyletic group as sistertaxon to all other species of Limapontiidae whose relationship to each other is the same as in the analysis of Limapontiidae.

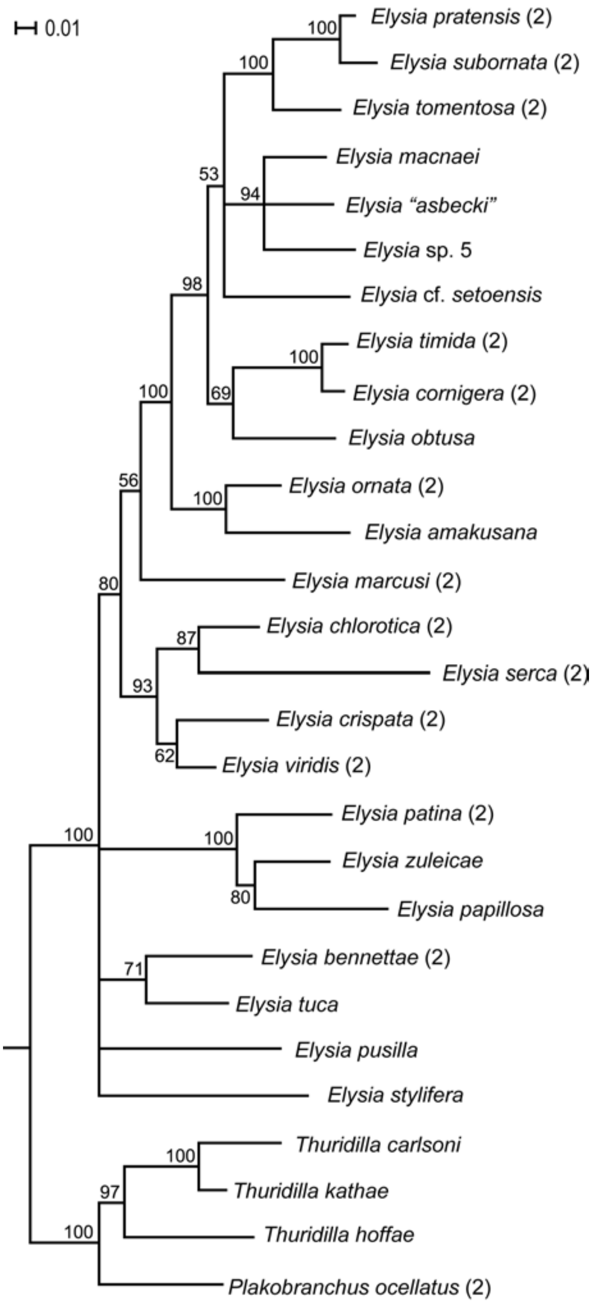


Figure 3.3 Results of a ML bootstrap analysis with 100 replicates of the genus *Elysia* by means of the partial molecular markers 28S rDNA, 16S rDNA and 1st and 2nd position of partial *coxI*. Support values are given. (2) – node comprising two specimens of same species was collapsed.

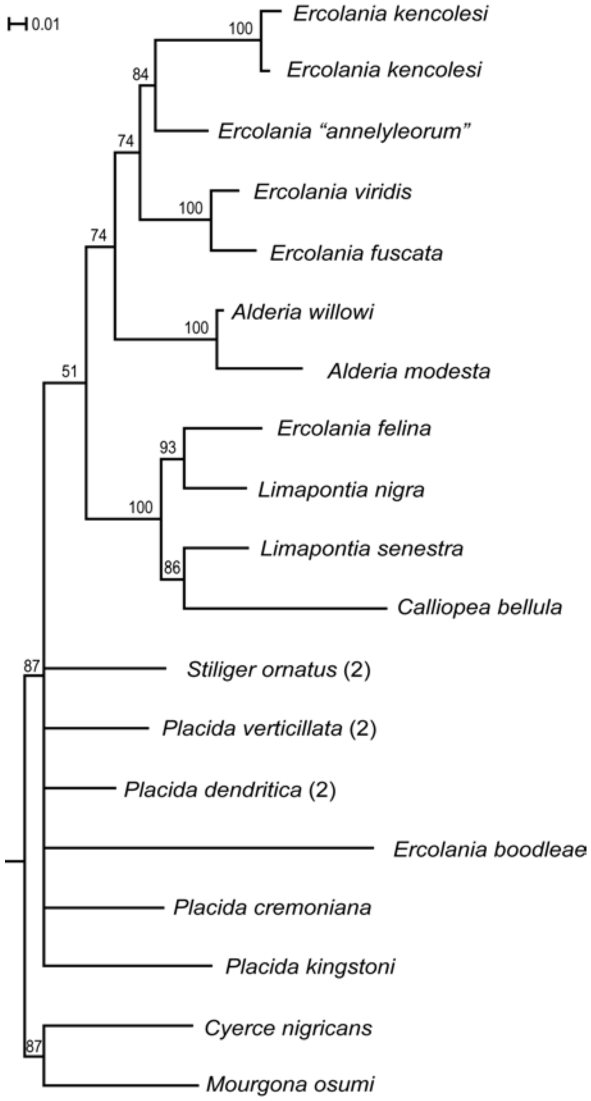


Figure 3.4 Results of a ML bootstrap analysis with 100 replicates of Limapontiidae by means of the partial molecular markers 28S rDNA, 16S rDNA and 1st and 2nd position of partial *coxI*. Support values are given. (2) – node comprising two specimens of same species was collapsed.

3.3.2 Kleptoplasts in Sacoglossa: photosynthetic abilities among Sacoglossa

Results on photosynthetic abilities that are not published in Händeler et al. 2009 are summarized in table 3.1. Three different levels of photosynthetic abilities are distinguished: (a) no functional retention, (b) short-term retention: chloroplasts endure within the slug's cell for a few up to ten days, (c) long-term retention: chloroplasts persist for several weeks up to months (Händeler et al. 2009).

No functional retention could be observed in the shelled *Cylindrobulla* sp., the polybranchid *Cyerce nigra*, *Cyerce* sp. 3 (Gosliner et al. 2008) and the limapontiid *Ercolania viridis* and *Ercolania* "*annelyleorum*".
Data of short-term and long-term forms are visualised in figure 3.5 to 3.8; yield values are plotted against days of starvation, blue trendlines refer to the observed species with a functional short-term retention. Data and trendline in black represent the long-term retention form *Plakobranchus ocellatus*, that are given as reference. These data are published in Händeler et al. 2009. Species with a functional long-term retention are marked with a green trendline.

All studied species of *Elysia* (figures 3.5 to 3.7) and *Thuridilla* (figure 3.7c) and d)) showed short-term retention. Long-term retention of *Plakobranchus ocellatus* as it has been described in Händeler et al. (2009) could be confirmed (figure 3.8a)). Two undetermined species of the genus *Costasiella* from Guam (USA), *Costasiella* cf. *kuroshimae* and *Costasiella* sp. 1 (seaslugforum.net), seem to be capable of functional retention (figure 3.8b) and c), p. 53); final yield values were 0.4 after 15 and 0.6 after eight days of starvation. Surprisingly, the initial yield values of both species were lower: 0.3 and 0.55 respectively. Photosynthetic activity over time did not decline, but an incipient increase of yield values was followed by a decrease within one week. The final yield value after that decrease was higher than the yield value right after capture. It was possible to investigate *Costasiella* cf. *kuroshimae* for almost double the time as *Costasiella* sp. 1 (seaslugforum.net): 15 days. In that case the "cycle" of yield progression could be observed to be run through two times.

Data obtained from *Plakobranchus ocellatus* are published in Wägele et al. 2010, and those from *Ercolania* "*annelyleorum*" are included in Wägele et al. (in press).

Table 3.1 Results of PAM-measurements that are not included in Händeler et al. 2009. Data of several specimens were pooled. * – Only one specimen was available. Yield values printed in bold type – only initial yield value was available, animal was fixed afterwards. Days are days of starvation.

Species	Initial yield	Days	Final yield	Retention
Oxynoacea				
CYLINDROBULLIDAE				
Cylindrobulla sp.	0	–	0	non-functional
Plakobranchacea				
PLAKOBRANCHOIDEA				
PLAKOBRANCHIDAE				
Elysia translucens	**0.74**	–	–	short-term
Elysia viridis	0.58	3	0.33	short-term
Elysia cf. *grandifolia*	0.64	4	0.20	short-term
Elysia cf. *tomentosa*	0.49	9	0	short-term
Elysia sp. 2GUAM*	0.62	3	0.03	short-term
Elysia sp. 3GUAM*	0.45	8	0.09	short-term
Elysia sp. 4GUAM*	0.69	2	0.45	short-term
Elysia sp. 5GUAM*	0.55	2	0.05	short-term
Elysia sp. BANYULS	0.59	7	0.14	short-term
Elysia sp. 15 (Gosliner et al. 2008)	0.40	1	0.28	short-term
Elysia sp. 11 (seaslugforum.net)	0.52	2	0.24	short-term
Plakobranchus ocellatus	0.76	19	0.68	long-term
Thuridilla bayeri	0.68	4	0	short-term
Thuridilla livida	0.57	3	0.20	short-term
LIMAPONTIOIDEA				
POLYBRANCHIIDAE				
Cyerce nigra	0	–	0	non-functional
Cyerce sp. 3 (Gosliner et al. 2008)	0	–	0	non-functional
COSTASIELLIDAE				
Costasiella cf. *kuroshimae*	0.30	15	0.4	functional
	(with maximum yield 0.59)			
Costasiella sp. 1 (seaslugforum.net)	0.55	8	0.6	functional
	(with maximum yield 0.72)			
LIMAPONTIIDAE				
Ercolania ”annelyleorum”	0	–	0	non-functional
Ercolania viridis	0	–	0	non-functional

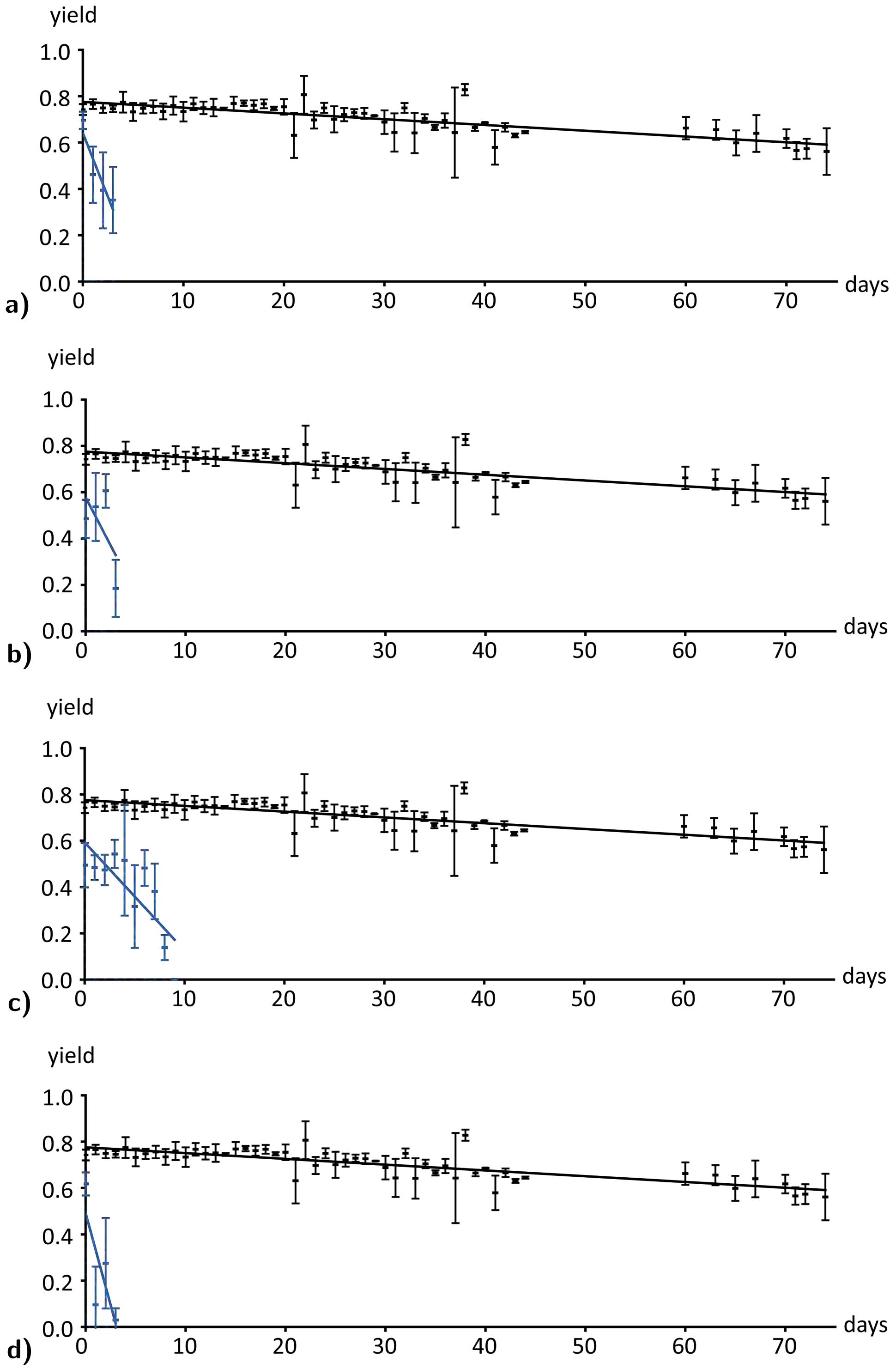


Figure 3.5 Photosynthetic activity of the short-term forms a) *Elysia* cf. *grandifolia*, b) *Elysia viridis*, c) *Elysia* cf. *tomentosa* and d) *Elysia* sp. 2GUAM (blue lines). Yield values are plotted against starvation days after capture. *Plakobranchus ocellatus* (black line) is given as reference for a long-term retention species. Trendlines estimated by means of Excel.

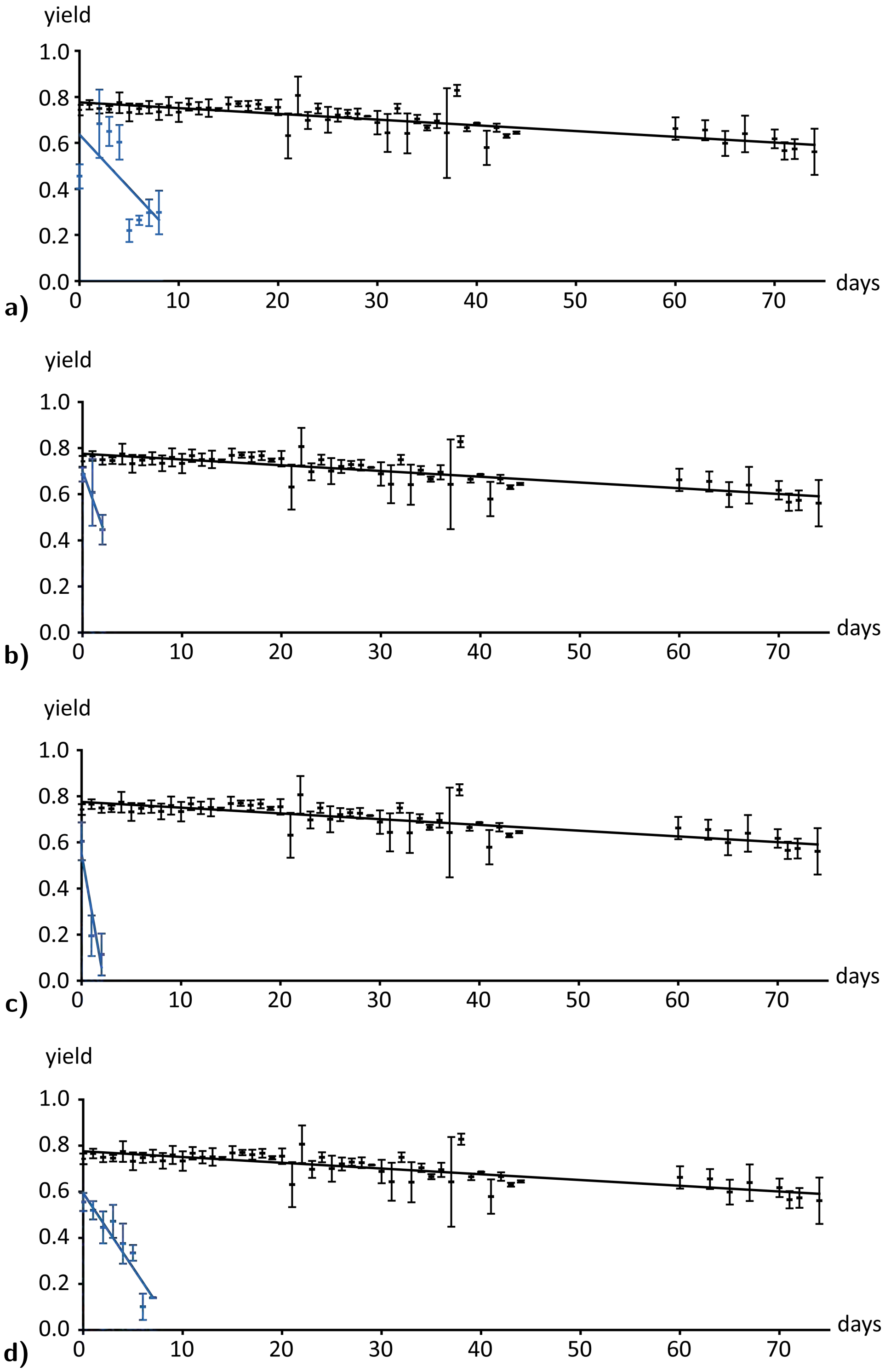


Figure 3.6 Photosynthetic activity of the short-term forms a) *Elysia* sp. 3GUAM, b) *Elysia* sp. 4GUAM, and c) *Elysia* sp. 5GUAM and d) *Elysia* sp. BANYULS (blue lines). Yield values are plotted against starvation days after capture. *Plakobranchus ocellatus* (black line) is given as reference for a long-term retention species. Trendlines estimated by means of Excel.

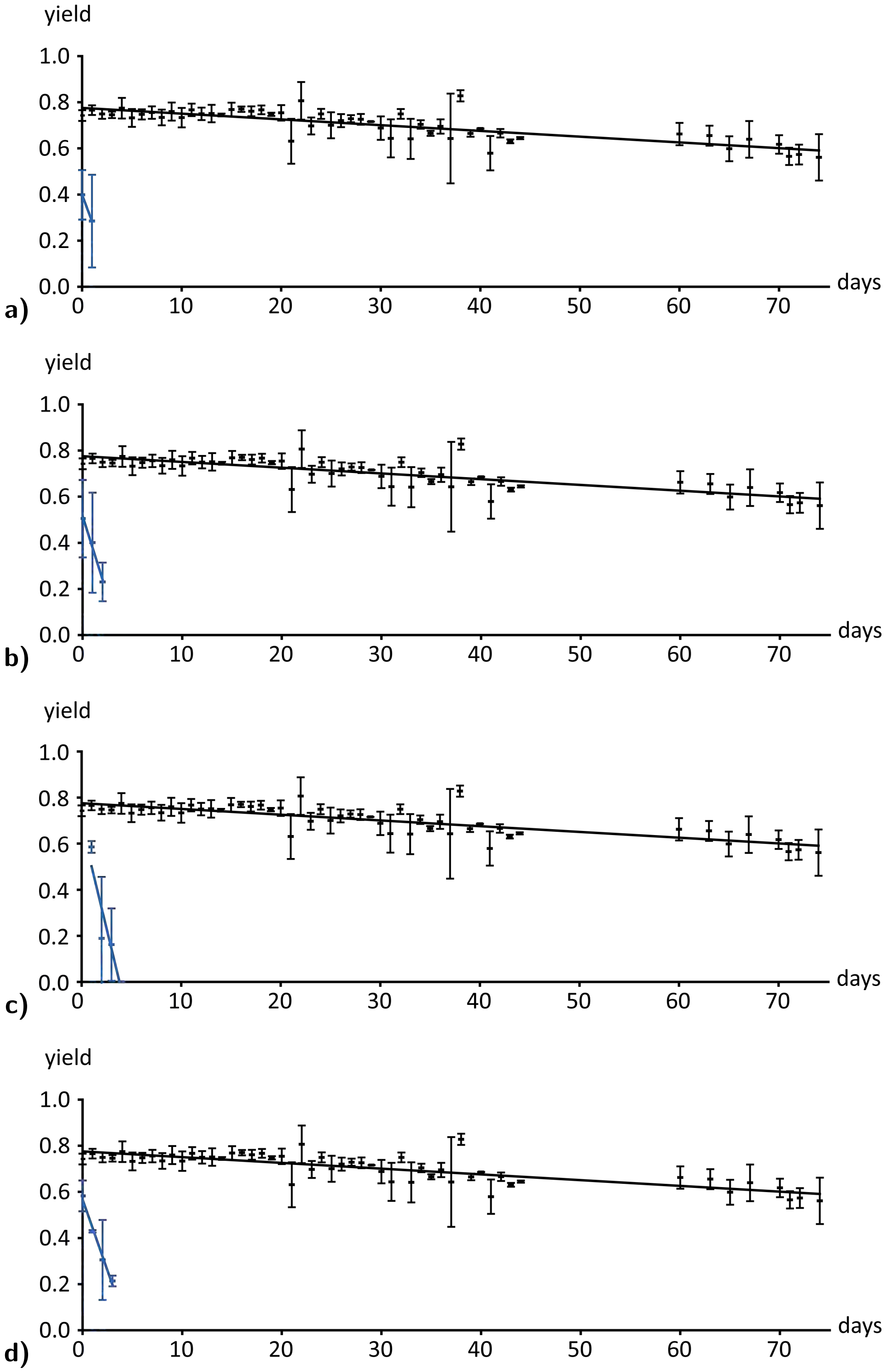


Figure 3.7 Photosynthetic activity of a) *Elysia* sp. 15 (Gosliner et al. 2008), b) *Elysia* sp. 11 (seaslugforum.net), c) *Thuridilla bayeri* and d) *Thuridilla livida* (blue lines). Yield values are plotted against starvation days after capture. *Plakobranchus ocellatus* (black line) is given as reference for a long-term retention species. Trendlines estimated by means of Excel.

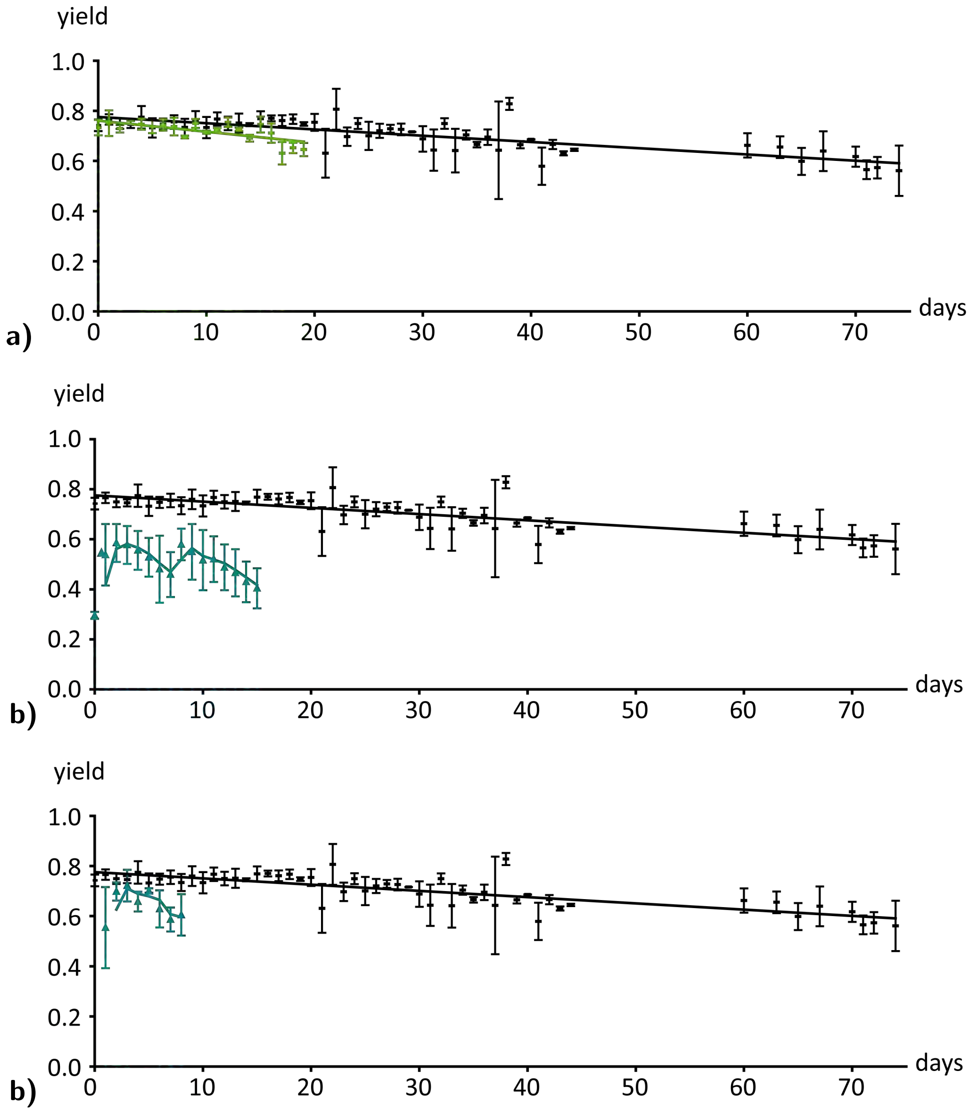


Figure 3.8 a) Photosynthetic activity of the long-term form *Plakobranchus ocellatus*. Data obtained from specimens collected on Guam, USA. b) Photosynthetic activity of *Costasiella* cf. *kuroshimae* and c) *Costasiella* sp. 1 (seaslugforum.net). Yield values are plotted against starvation days after capture. *Plakobranchus ocellatus* (black line) is given as reference for a long-term retention species. Trendlines estimated by means of Excel.

Feeding experiments Thuridilla hopei Feeding experiments were conducted to obtain information about the feeding source of *Thuridilla hopei*. Results of feeding experiments are summarized in table 3.2. Neither specimens of the genera *Codium*, *Udotea*, *Caulerpa* nor *Halimeda* led to an increase in photosynthetic activity within the slug, and therefore are not the food source. A rhizom of *Posidonia oceanica*, overgrown with smallest algae, did not result in feeding. Only an unidentified filamentous green alga had a positive effect on the rate of photosynthesis. Yield development is shown for the two specimens Th08GI-35 and Th08GI-33 and their offered algae in figure 3.9.

Table 3.2 Results of feeding experiment. Different specimens of *Thuridilla hopei* were supplied with different algal species; Day indicates the day after capture on which the animal were offered the alga. The animals were starved until no fluorescence could be measured with the help of the PAM. ↑ - yield value increased again.

Specimen	Day	Alga species	Yield value
Th08GI-33	7	filamentous green alga (GI110)	↑
Th08GI-35	6	filamentous green alga (GI109)	↑
Th08GI-38	4	filamentous green alga (GI75)	↑
Th08GI-34	6	*Codium*	–
Th08GI-36	6	*Halimeda tuna*	–
Th08GI-39	3	*Halimeda tuna*	–
Th08GI-37	6	*Udotea petiolata*	–
Th08GI-40	6	*Caulerpa* cf. *racemosa*	–
Th08GI-41	7	*Caulerpa* cf. *racemosa*	–
Th08GI-39b	6	rhizom of *Posidonia oceanica*	–

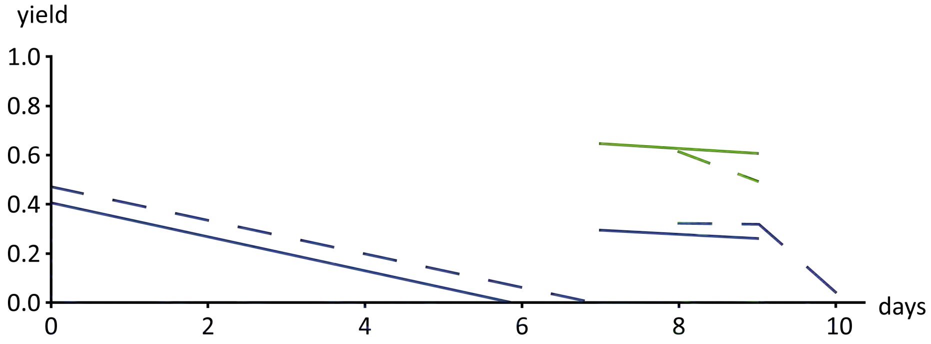


Figure 3.9 *Thuridilla hopei* feeding experiment: blue line – specimen Th08GI-35; dashed blue line – specimen Th08GI-33; green line – photosynthetic activity of filamentous green alga GI109, that was given to Th08GI-35, dashed green line – photosynthetic activity of filamentous green alga GI110, that was given to Th08GI-33. Algae were removed after day nine; yield values of Th08GI-33 declined again (Th08GI-35 was fixed on the ninth day). Trendlines estimated by means of Excel.

3.3.3 Food organisms of Sacoglossa

Partial *tufA* sequences obtained with primer pair tufA_alg_up and tufA_alg_do are maximal 714bp long (IZMB), with primer pair tufAF and tufAR (ZFMK) about 900bp. Lengths can differ within Ulvophyceae. All obtained *tufA* sequences belong to the Ulvophyceae identified by the BLAST algorithm of GenBank.

TufA sequences that were obtained from DNA extracted from sacoglossans are listed in table 3.3. Those obtained from Ulvophyceae are listed in table 3.5. If clones obtained from one PCR product were considered to have different origins, this is indicated by capital letters following the internal numbers. Capital letters can refer to a single sequence or a consensus sequence. Consensus sequences were obtained from several sequences that obviously belong to the very same individual due to a minor genetic distance that can be explained by sequencing errors. Therefore accession numbers may refer to consensus sequences (for details see Händeler et al. 2010). E.g., there are three consensus sequences generated from the clones obtained from *Elysia* "*asbecki*": 722 A, 722 B and 722 C. 722 A comprises eight clones, 722 B and C two clones respectively.

Noticeable is, that the four *tufA* sequences of *Tydemania expeditionis* from the Maledives (A3), Guam (USA) (A168) and the Philippines (FJ432661, AM049971) are completely identical.

I established the amplification of *rbcL* with primer pair rbcL 1 and rbcL R; partial *rbcL* sequences are about 560bp long. Further sequences are produced by G. Christa especially to confirm results obtained by means of *tufA*.
Cylindrobulla sp. fed on *Halimeda macroloba* (see figure 3.10, p. 58). Different species of the shelled genus *Volvatella* fed on different species of *Caulerpa* (see figure 3.10, p. 58; Händeler et al. 2010). Known food sources for *Bosellia mimetica* (Händeler et al. 2010), *Elysia timida* and *Costasiella* cf. *kuroshimae* (see figure 3.10, p. 58) could be confirmed (*Halimeda tuna*, *Acetabularia acetabulum* and *Avrainvillea erecta* respectively). *Elysia macnaei* and *pusilla* fed on different species belonging to the genus *Halimeda* (Händeler et al. 2010).

The two different specimens of *Elysia tomentosa* (710 and 711) fed on algae of different genera: specimen 710 fed on a species of the genus *Bryopsis* (see figure 3.10, p. 58), 711 on two different species of *Caulerpa* (see details in Händeler et al. 2010).

The three specimens of *Elysia viridis* fed on algae belonging to the genus *Bryopsis* (see figure 3.10, p. 58 and Händeler et al. 2010). A neighbornet analysis of the sequences obtained from the specimens of *Elysia viridis* and all available sequences of *Bryopsis* is shown in figure 3.11. Food organism B of *Elysia viridis*

Table 3.3 *TufA* sequences obtained from DNA extracted from sacoglossans: *tufA* short – amplification was performed with primer pairs tufA_alg_up and tufA_alg_do, length 714bp; *tufA* long – amplification was performed with primer pairs tufAF and tufAR, length 900bp. * – performed by G. Christa within his diploma thesis. Sequences written in bold type were published in Händeler et al. 2010. Sequences that have an accession number starting with HM are published in Wägele et al. 2010. Internal numbers can be found in brackets behind the accession number. Capital letters refer to different (consensus) sequences obtained from one and the same individual.

Species	*tufA* short	*tufA* long
Oxynoacea		
Cylindrobullidae		
Cylindrobulla sp.		847*
Volavatellidae		
Volvatella viridis	**GU592606 (789)**	
Volvatella cf. *ventriosa*		S121*
Plakobranchacea		
PLAKOBRANCHOIDEA		
Boselliidae		
Bosellia mimetica	**GU592584 (740)**	
	GU592590 (739)	
Plakobranchidae		
Elysia ”*asbecki*”	**GU592558 (722 A)**	
	GU592567 (722 B)	
	GU592570 (722 C)	
Elysia macnaei	**GU592595 (726)**	
Elysia pusilla	**GU592553 (A4)**	
Elysia timida		743*
Elysia tomentosa	**GU592610 (711 A)**	710*
	GU592617 (711 B)	
Elysia viridis	**GU592635 (737 A)**	S125*
	GU592641 (737 B)	
	GU592642 (737 C)	
	GU592623 (738 A)	
	GU592629 (738 B)	
Plakobranchus ocellatus	HM140187 (705 A)	HM140225 (852 A)*
	HM140191 (705 B)	HM140228 (852 B)*
	HM140210 (705 C)	HM140235 (852 C)*
	HM140188 (705 D)	HM140245 (852 D)*
	HM140189 (705 E)	HM140234 (852 E)*
	HM140212 (706 A)	HM140238 (854 A)*
	HM140193 (706 B)	HM140231 (854 B)*
		HM140236 (854 C)*
Thuridilla carlsoni		S116 A, B*
Thuridilla gracilis		S117*
		S118 A, B*
Thuridilla hopei		HM140214 (809)*
Thuridilla kathae		S120*
Thuridilla livida		846 A, B*
LIMAPONTIOIDEA		
Costasiellidae		
Costasiella cf. *kuroshimae*		S190*
Limapontiidae		
Ercolania viridis		S150*

Table 3.5 *TufA* sequences obtained from Ulvophyceae: *tufA* short – amplification was performed with primer pairs tufA_alg_up and tufA_alg_do, length 714bp; *tufA* long – amplification was performed with primer pairs tufAF and tufAR, length 900bp. * – performed by G. Christa within his diploma thesis. Sequences written in bold type were published in Händeler et al. 2010. The sequence of *Halimeda macroloba* HM140244 (A155) is published in Wägele et al. 2010.

Species	*tufA* short	*tufA* long
Bryopsidales		
BRYOPSIDACEAE		
Bryopsis plumosa		A15*
Bryopsis sp.		A102*
Bryopsis sp.		A170*
CAULERPACEAE		
Caulerpa cupressoidess		A134*
Caulerpa cf. *racemosa*		A110*
Caulerpa cf. *racemosa*		A121*
Caulerpa sertularioides		A159*
Caulerpa sp.		A134*
Halimeda cuneata (as sp. 1)	**GU592539 (A1)**	A1
(as sp. 2)	**GU592547 (A2)**	
Halimeda tuna	**GU592573 (A6)**	
Halimeda macroloba		HM140244 (A155)*
Halimeda melanesica		A171*
Halimeda minima		A154*
Halimeda sp. 3	**GU592599 (A7)**	
CODIACEAE		
Codium effusum		A128*
Codium cf. *tomentosum*		A133*
Codium vermilara		A101*
		A130*
Codium sp.		A107*
UDOTEACEAE		
Avrainvillea		A162*
Flabellia petiolata	**GU592533 (A14)**	
Rhipilia crassa		A166*
Tydemania expeditionis	**GU592605 (A3)**	A168*
Cladophorales		
CLADOPHORACEAE		
Chaetomorpha sp.		A27
Ulvales		
ULVACEAE		
Ulva lactuca		A26*
Ulva sp.	**GU592643 (A20)**	

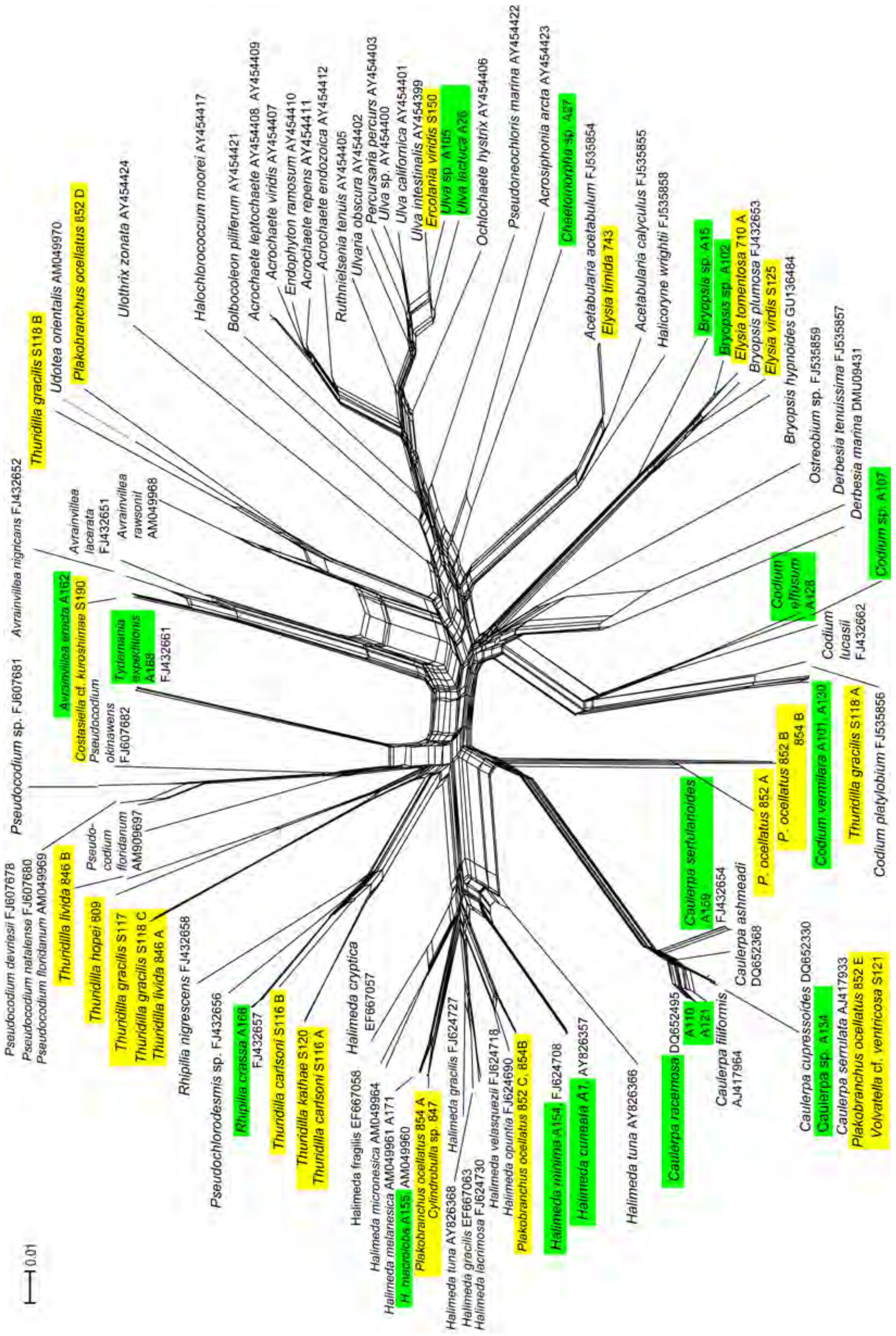


Figure 3.10 Neighbornet analysis of partial *tufA* (852bp) performed by G. Christa within his diploma thesis. Organisms marked in green refer to new sequences of Ulvophyceae, those marked in yellow to sequences obtained from DNA extracted from sacoglossans. All other sequences were extracted from GenBank, accession numbers are given. Derived from a figure that was kindly provided by G. Christa.

738 (GU592629), collected in Banyuls-sur-Mer in 2006, seems to be the same species as, or at least is closely related to, the undetermined species *Bryopsis* sp. A15, collected in Banyuls-sur-Mer in 2007 (sequence difference: 0.99%). Food organism A of *Elysia viridis* 738 is the same as the one of *Elysia viridis* S125, collected in Banyuls-sur-Mer in 2009 from an algal sample dominated by *Halimeda tuna*. Food organisms of *Elysia viridis* 737, collected in Tossa de Mar (Spain) cannot be determined to species level yet.

Many different ulvophycean *tufA* sequences could be obtained from various species of *Thuridilla* (figure 3.10), but only one could be identified as *Codium vermilara* in the case of *Thuridilla gracilis* S118. A second food source of S118 is similar to that of *Thuridilla gracilis* S117, *Thuridilla livida* 846 and closely related to another food source of *Thuridilla livida* 846 and *Thuridilla hopei* 809, but this group cannot be assigned to any other represented group of Ulvophyceae. The two different food organisms of *Thuridilla carlsoni* and *Thuridilla kathae* belong to a group including *Pseudochlorodesmis* and *Rhipilia*, but cannot be assigned to species level. For half of the investigated specimens of *Thuridilla* (3) at least two different food sources could be determined, i.e. *Thuridilla carlsoni*, *Thuridilla gracilis* specimen S118 and *Thuridilla livida*. One *tufA* sequence obtained from *Thuridilla gracilis* S118 and one from *Plakobranchus ocellatus* 852 group with *Udotea*.

Plakobranchus ocellatus fed on several types of algae (figure 3.12). Specimens 852 and 854, collected on Guam, fed on *Halimeda opuntia*, 854 additionally on *Halimeda macroloba*, and 852 additionally on *Caulerpa* cf. *serrulata*. Furthermore, both of them fed on at least one other ulvophycean species that could not be determined. Also, food organisms of 705 und 706 are ulvophycean but

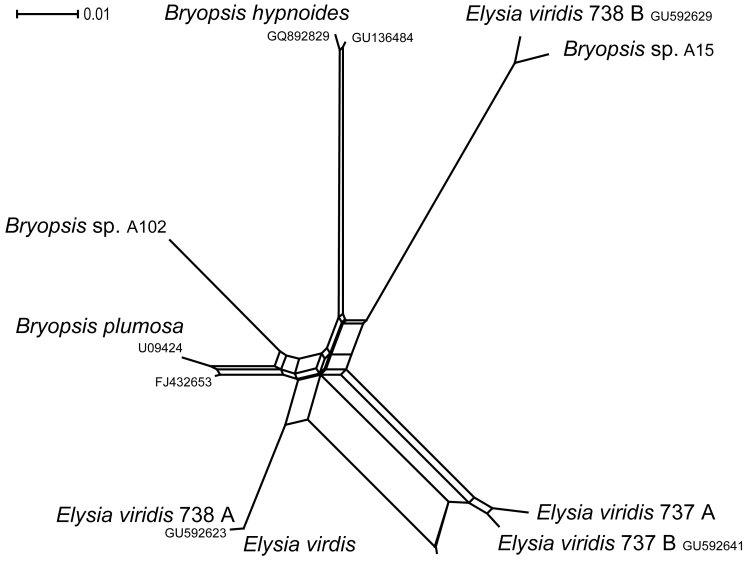


Figure 3.11 NeighborNet analysis of partial *tufA* (708bp) including sequences gained from three specimens of *Elysia viridis* (737, 738 and S125) and all sequences of the genus *Bryopsis*.

cannot be identified to species nor genus level due to missing reference sequences.

Results on food organisms and data from literature are summarized in table 3.6.

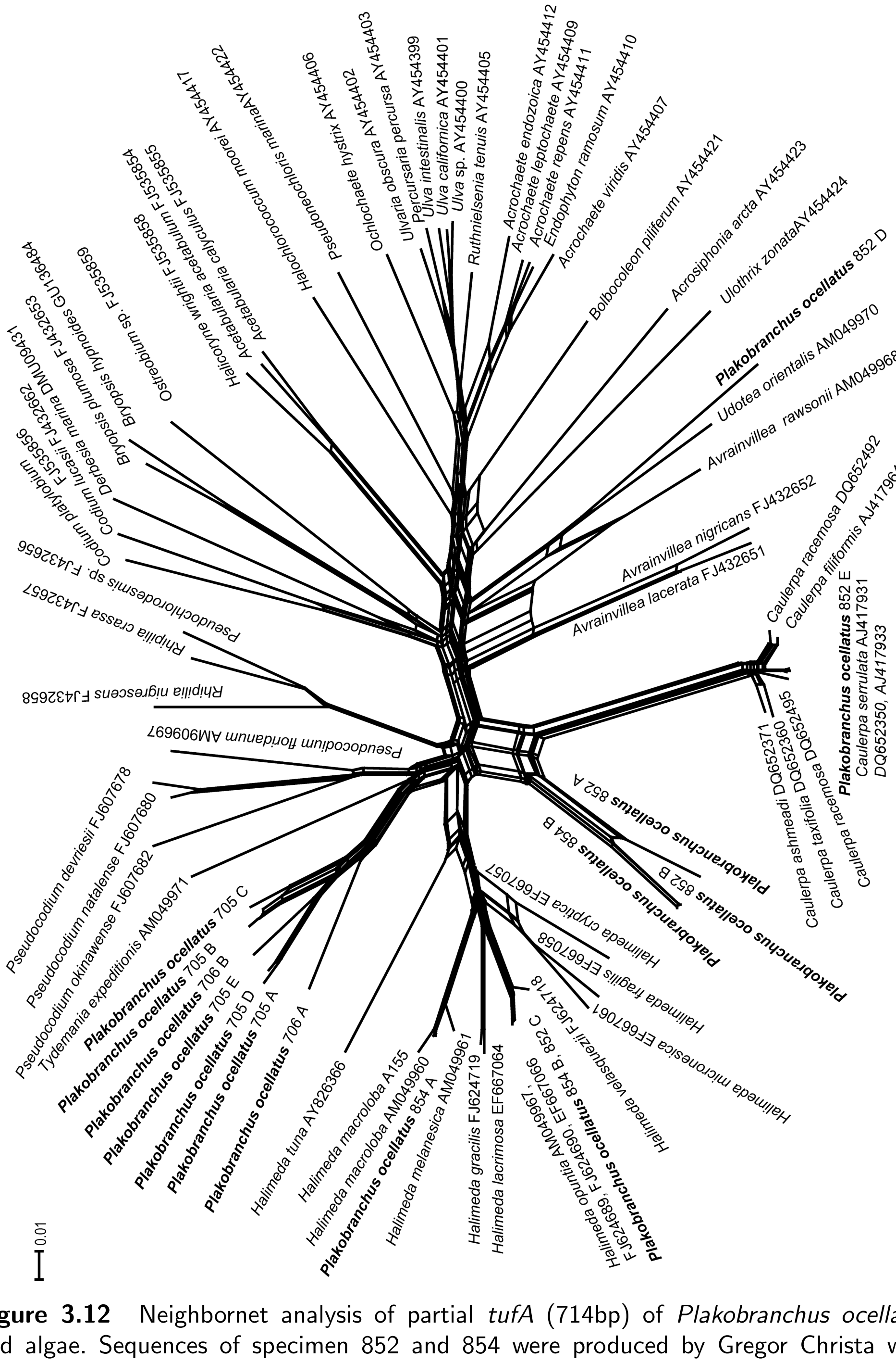


Figure 3.12 Neighbornet analysis of partial *tufA* (714bp) of *Plakobranchus ocellatus* and algae. Sequences of specimen 852 and 854 were produced by Gregor Christa with primer pair tufAF and tufAR and cut to the shorter length of sequences of specimen 705 and 706 produced with primer pair tufA_alg_up and tufA_alg_do. Sequences obtained from *Plakobranchus ocellatus* are printed in bold type.

Table 3.6 Survey on the results of food of several sacoglossan species, that has been barcoded by means of *tufA*, in comparison with previously available data; Results written in bold type have been published in Händeler et al. 2010, data shown in a grey box were obtained by Gregor Christa in his diploma thesis. Food data from literature are taken from the food review in Sacoglossa in Händeler & Wägele 2007, otherwise data are separated by ; and reference is given. ⋆ indicates, that reference for barcoded sequence(s) is missing, number in brackets refers to number of differing sequences indicating different food species, that could not be determined further.

Species	Food according to literature	No.	Barcoded food
Oxynoacea			
CYLINDROBULLIDAE			
Cylindrobulla sp.	–	847	*Halimeda macroloba*
VOLAVATELLIDAE			
Volvatella viridis	–	**789**	**cf. *Caulerpa racemosa* no. 1**
Volvatella cf. *ventricosa*		S121	*Caulerpa* cf. *serrulata*
Plakobranchacea			
PLAKOBRANCHOIDEA			
BOSELLIIDAE			
Bosellia mimetica	*Halimeda*, *H. tuna*, *Udotea*	**740** **739**	***Halimeda tuna***
PLAKOBRANCHIDAE			
Elysia "asbecki"	–	**722**	**⋆ (3)**
Elysia macnaei	*Halimeda cuneata* *H. macroloba*	**726**	***Halimeda* Section HALIMEDA, cf. *H. macrophysa***
Elysia pusilla	*Halimeda* spp.	**A4**	***Halimeda opuntia***
Elysia timida	*Acetabularia acetabulum*	743	*Acetabularia acetabulum*
Elysia tomentosa	–	710 **711**	*Bryopsis* sp. **cf. *Caulerpa racemosa* no. 1,2**
Elysia viridis	*Codium* spp., *C. fragile* *C. tomentosum* *Bryopsis* spp. *B. plumosa* *Chaetomorpha*, *C. linum* *Cladophora rupestris*; *Dasysiphonia* sp. RHODOPHYTA (Teugels et al. 2008)	**737** **738**	**cf. *Bryopsis plumosa* no. 1-3** **cf. *B. plumosa* no. 4-5**
		S125	*Bryopsis* sp.

Plakobranchus ocellatus	–	852	*Halimeda opuntia* *Caulerpa* cf. *serrulata* ⋆ (3)
		854	*Halimeda opuntia* *Halimeda macroloba* ⋆
		705	⋆ (up to 5)
		706	⋆ (2)
Thuridilla carlsoni	–	S116	⋆ (2)
Thuridilla gracilis	–	S117	⋆
		S118	*Codium vermilara* ⋆
Thuridilla hopei	*Derbesia tenuissima* *Cladophora vagabunda*	809	⋆
Thuridilla kathae	–	S120	⋆
Thuridilla livida	–	846	⋆ (2)
LIMAPONTIOIDEA			
Costasiellidae			
Costasiella cf. *kuroshimae*	*Avrainvillea erecta* (own observation)	S190	*Avrainvillea erecta*
Limapontiidae			
Ercolania viridis	*Chaetomorpha* spp. *C. linum*, *C. capillaris* *Cladophora* spp. *Cladophoropsis*	S150	*Ulva intestinalis*

Data on food organisms of sacoglossans obtained from other methods than barcoding of cp genes Two partial 28S rDNA sequences of Ulvophyceae were obtained from sacoglossans; *Volvatella* cf. *ventricosa* (S121) fed on a species of *Caulerpa* and *Elysia pusilla* (799) on a species of *Halimeda* (for details see figure 6.1, p. 108).

Undetermined filamentous green algae were fed by *Thuridilla hopei* (see chapter 3.3.2 Kleptoplasts in Sacoglossa). Sequencing of these algae for identification is still in progress.

3.3.4 Notes on undescribed sacoglossan species

Notes on specimens of 14 undescribed sacoglossans, that were found on expeditions to the Indo – Pacific Lizard Island (Australia, Great Barrier Reef), the Pacific island Guam (USA) and to Banyuls-sur-Mer at the Mediterranean coast (France) (figure 3.13), are given. The numbers used for the species are partly internal numbers, or those taken from literature or the seaslugforum, when illustrated there.

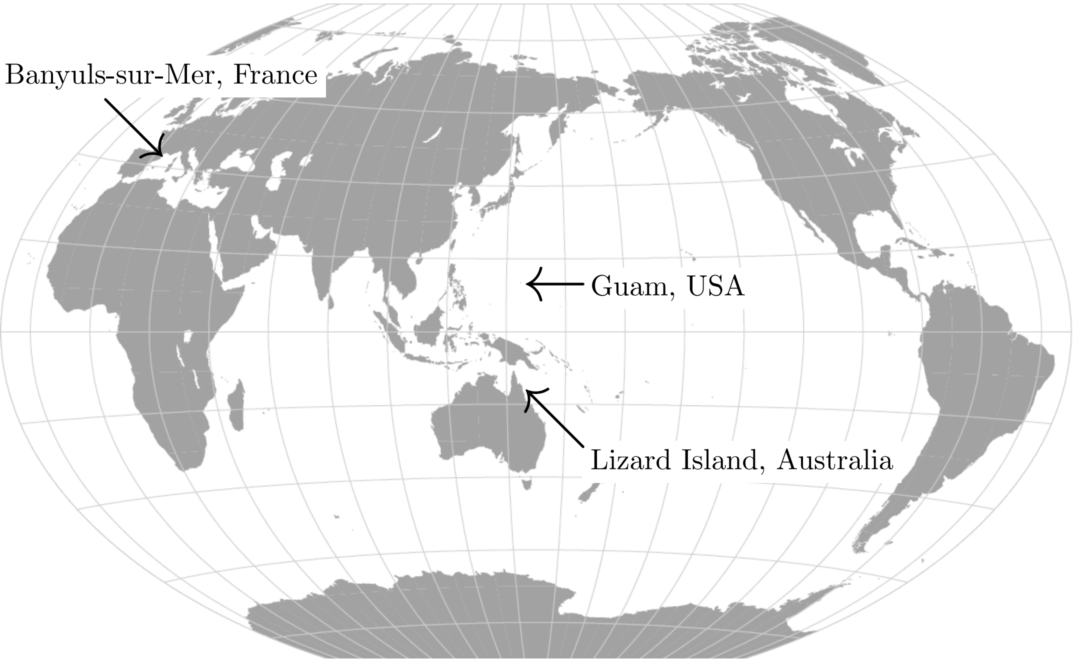


Figure 3.13 Collection places, where unsdescribed sacoglossan species were found. Map: http://www.mygeo.info/weltkarten.html.

Cylindrobulla sp., Cylindrobullidae

collection place	USA: Guam, Cocos Lagoon
collection date	substratum 21./24.08.2009 slug: 22./26.08.2009
substratum	mixed algae, mainly *Halimeda* + sediment
size	0.5-1cm
specimens	Cyl-09GU-1 to Cyl-09GU-5

A sample of mixed algae was taken together with sediment which was looked through during the following days. Specimens appeared partly green right after capture (figure 3.14a)), but colour vanished more and more over the time of starvation (figure 3.14b)). Animals secret white mucus when disturbed (figure 3.14c)). Relation of head shield to shell is one. Animal is able to retreat into its shell completely. The radula is shown in figure 3.14e); the ascending limb consists of 37, the descending limb at least of 61 teeth, including leading

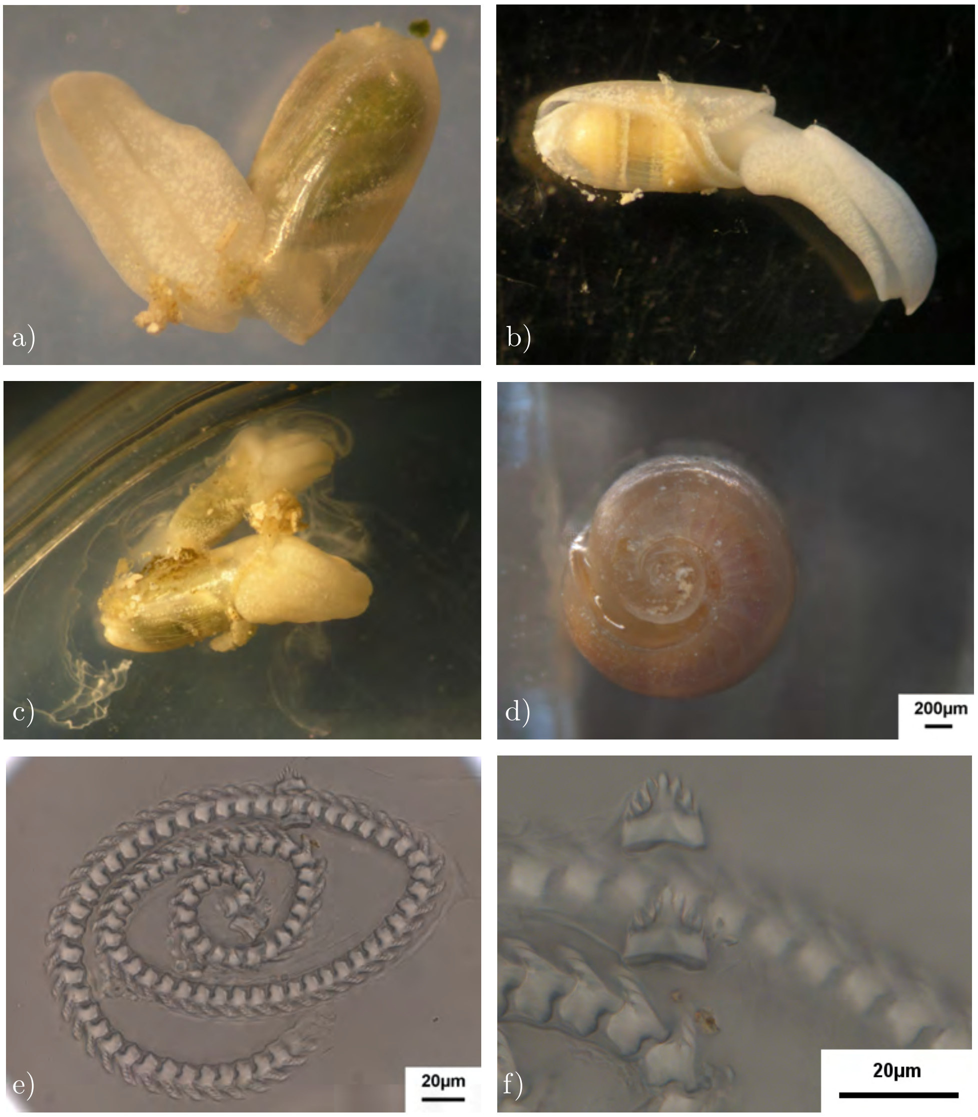


Figure 3.14 *Cylindrobulla* sp. found on Guam (USA) in 2009; a) right after capture, b) starved for 5 days. c) Two individuals secret white mucus. d) Apex. e) Radula. f) The tooth is broad with a central cusp and is flanked by 3 to 4 denticles on each side.

tooth. Some teeth were lost during preparation (figure 3.14e)). Single teeth are enlarged in figure 3.14f). The tooth is broad and has about seven denticles.

Several *Halimeda* and *Caulerpa* species were offered to the specimens to test food choice. Unfortunately, animals would use everything as cover or try to bury into it and showed no intention of feeding at all. Nevertheless, food could be detected by barcoding: *Cylindrobulla* sp. feeds on *Halimeda macroloba* (see table 3.6, p. 61). It has no functional chloroplast retention.

One specimen was embedded for histological preparation, but histological slices could not be made because of limited time.

Cyerce sp. 3 (Gosliner et al. 2008), Polybranchiidae

collection place	USA: Guam, Cocos Lagoon
collection date	18./21./24.08.2009
substratum	*Udotea geppii*
size	1-2.5cm
specimens	Cy3-09GU-1 to Cy3-09GU-13

This species has been portrayed before as *Cyerce* sp. 3 by Gosliner and colleagues (2008).

The animal has a shining and fluorescent appearence. Cerata broaden from the base with a maximum width in the last third. Then they reduce in a sharp angle to widen again and end in a heart-like structure with the two bows at the outer part (figure 3.15b)). The two sides of the flat cerata are different in colour and structure (figure 3.15a) to c)). The anterior part of the cerata is mainly greenish yellow with a pattern of meandering lines. There is a colour gradient from dark at the top to transparent at the base. The outer edge in the upper third is clearly differentiated by a darker rim, coloured from pink to violet. The posterior part (figure 3.15b)) is more whitish. It has a dark rim at the upper third as well, that is not so colourful, but more brownish yellow. The surface of the posterior part is covered by spheric papillae which are surrounded by a thin brown circle at the base. Animals move their cerata back and forth while crawling (figure 3.15c)). When disturbed they bend their whole body along an anterior-posterior line, and are able to perform some kind of swimming in that way. The dorsal head region is shown in figure 3.15d); the rhinophores and oral tentacle show a semitransparent grey to violet colour sprinkled with light and fluorescent spots.

This species has no functional chloroplast retention.

Costasiella sp. 1 (seaslugforum.net), Costasiellidae

collection place	USA: Guam, Cocos Lagoon
collection date	24.08.2009
substratum	*Avrainvillea erecta*
size	about 5mm
specimens	Co1-09GU-1 to Co1-09GU-7

Costasiella sp. 1 (figure 3.15e) and f)) has been portrayed before in the seaslugforum (seaslugforum.net). Rhinophores and head region are completely white except for two black lines, that initiate parallel directly behind the eyes and continue curved till they end almost lateral at the end of the head region. The cerata appear primarily white, and taper in thin tips. There are darker spots on the cerata that look if there were holes in the surface. The more lateral and smaller the cerata are, the more they apppear greenish. The lowest rows of cerata are filled with a lot of green pigment together with some bluish and fluorescent pigments.

This species showed a "cyclic" photosynthetic activity (see table 3.1, p. 49 and figure 3.8, p. 53).

Costasiella cf. kuroshimae, Costasiellidae

collection place	USA: Guam, Cocos Lagoon
collection date	18./24.08.2009
substratum	*Avrainvillea erecta*
size	about 5mm
specimens	Ck-09GU-2 to Cu-09GU-4, Ck-09GU-7 Ck-09GU-10 to Ck-09GU-13

Costasiella cf. *kuroshimae* (figure 3.15g)) has white rhinophores. The tips of the rhinophores are not completely black, but have black dots on the dorsal side. The base of the rhinophores are sprinkled with orange. The head region behind the eyes is coloured brownish orange, a more yellow spot can be found directly following the eyes but not broader than these. In front of the eyes is another orange spot. Mainly cerata are green with a lot of white, partly fluorescent, pigments. They end in tapering whitish tips, which are surrounded beneath by a ring of brownish-orange pigments.

This species showed a ”cyclic” photosynthetic activity (see table 3.1, p. 49 and figure 3.8, p. 53).

Avrainvillea erecta was confirmed as food via barcoding in the specimen S190 (see table 3.6, p. 61).

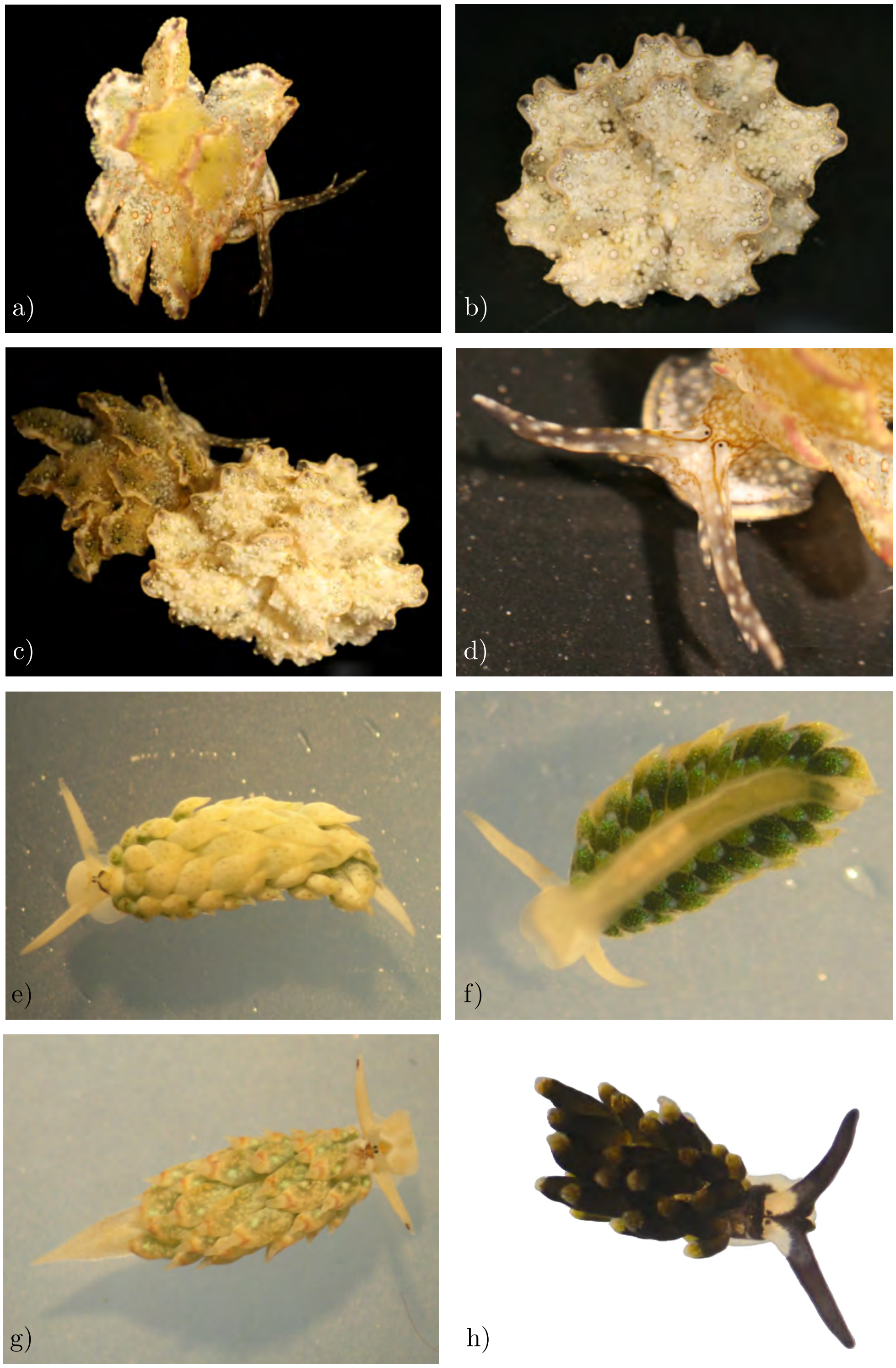
Costasiella sp. black, Costasiellidae

collection place	USA: Guam: Cocos Lagoon
collection date	21./24.08.2009
substratum	*Avrainvillea erecta*
size	about 1mm
specimens	Cu-09GU-1 to Cu-09GU-7

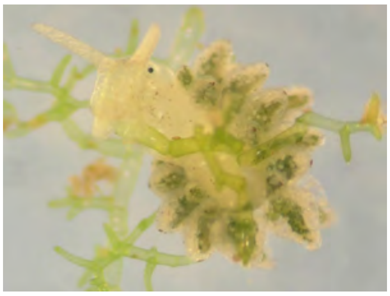
This very small species is mainly black coloured (figure 3.15h)). The digitiform black cerata end in greenish dirty yellow tips. The head region is partly white; two white spots arranged in pairs start right behind the eyes and continue to the base of the black rhinophores. These spots are separated by a black line that originates from a black triangle connecting the black rhinophores in the anterior head region, running between the eyes to the black posterior part of the head region.

Unfortunately, this species is too small to measure its potential photosynthetic activity with the help of a PAM.

Figure 3.15 *Cyerce* sp. 3 (Gosliner et al. 2008) found on Guam (USA) in 2009: a) habitus, b) dorsal view on forswifted cerata; posterior part can be seen, c) two individuals crawling; the cerata are moved back and forth, d) head region with rhinophores and oral tentacles. *Costasiella* sp. 1 (seaslugfourm.net) found on Guam (USA) in 2009; e) dorsal, f) ventral. Pictures were taken on 02.09.2009, on the ninth day of starvation. Size about 5mm. g) *Costasiella* cf. *kuroshimae* found on Guam (USA) in 2009. Picture taken on 02.09.2009, after 15 days of starvation. Size about 5mm. h) *Costasiella* sp. found on Guam (USA) in 2009. Size about 1mm.


a)
b)
c)
d)
e)
f)
g)
h)

Ercolania sp., Limapontiidae



collection place	USA: Guam, Upper Harbour, wall
collection date	19.08.2009
substratum	*Boodlea*
size	about 2mm
specimen	Er1-09GU-1

Figure 3.16 *Ercolania* sp. found on Guam (USA) in 2009 surrounded by *Boodlea*.

Elysia, Plakobranchidae

A Neighbornet analysis has been performed with SplitsTree (Huson & Bryant 2006) of a partial 16S rDNA sequence of the undescribed *Elysia* species. Taxa sampling has been chosen due to a previous Neighbornet analysis (not shown). Only those sequences were included that are related to sequences of the undescribed species to aid clarity. The result is shown in figure 3.17.

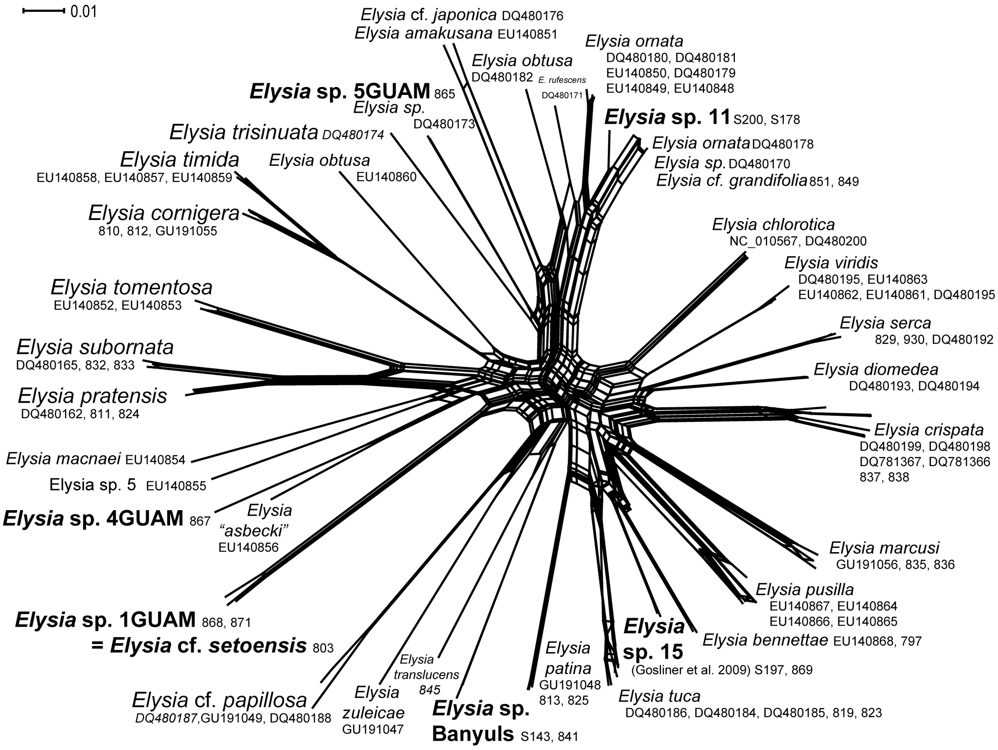


Figure 3.17 Neighbornet analysis of partial 16S rDNA (463bp) performed with Splits–Tree (Huson and Bryant 2006).

Elysia sp. 1GUAM/Elysia cf. setoensis

`collection place`	`Australia: Lizard Island / USA: Guam`
`collection date`	`13.07.2006/08. and 09.2009`
`substratum`	`see table 3.7`
`size`	`about 1 to 3mm`
`specimens`	`E1-09GU-1 to E1-09GU-5`

Characteristic for this species is the appearance of the rhinophores: they are decorated by dark ribbons on the anterior and posterior side, which leads to the impression the rhinophores would be annulated. One specimen was found on Lizard Island (Australia) in 2006 and labeled as *Elysia* cf. *setoensis* (803). Many more specimens were found on Guam (USA) in 2009. Molecular analysis revealed, that these specimens belong to one species (figure 3.17). DNA sample 868 refers to specimen E1-09GU-1 and 871 to one of two specimens of sample E1-09GU-4. Re-examination of a picture showed that the Australian animal (803) had the same dark spots on its rhinophores. There are no closer relations to any other *Elysia* species.

The animals were found on quite different algae as *Bryopsis* or *Halimeda* (table 3.7). Animals from *Bryopsis* had a darker colour than those from *Halimeda* (see figure 3.18 a) and b)). The specimen from Lizard Island was collected from a mixed algal sample. The specimen E1-09GU-1 was transferred from *Bryopsis* to *Halimeda*, which it fed on without any problems. Animals were too small to perform PAM measurements.

Table 3.7 Specimens of *Elysia* sp. 1GUAM found on Guam (USA) in 2009 and their different substrata.

Specimen	Origin	Substratum	Collection date
E1-09GU-1	Pago Bay	*Bryopsis*	13.08.09
E1-09GU-2	Bile Bay	*Halimeda*	17.08.2009
E1-09GU-3	Asan	algal mix (*Caulerpa verticillata*, *Halimeda*, *Bornetella*)	algae: 28.08.09 slug: 31.08.09
E1-09GU-4	Apra Harbour: Western Shoals	mix of *Caulerpa* and *Halimeda*	02.09.2009
E1-09GU-5	?	*Halimeda*	02.09.2009

Elysia sp. 2GUAM

`collection place`	`USA: Guam, Pago Bay`
`collection date`	`15.08.2009`
`size`	`about 3cm`
`specimen`	`E2-09GU-1`

This species (figure 3.18c), d)) has the appearance of an intermediate type of *Elysia ornata*, *Elysia grandifolia* and *Elysia* sp. 11 (seaslugforum.net). The parapodia are folded in three lobes as in *Elysia* sp. 11 (seaslugforum.net), but are not so thick. This species is a short-term retention form (figure 3.5, p. 50).

Figure 3.18 *Elysia* sp. 1GUAM found on Guam (USA) in 2009; a) dark green specimen from *Bryopsis*, b) light green specimen from *Halimeda*. *Elysia* sp. 2GUAM found on Guam (USA) in 2009; c) closed parapodia, d) parapodia are open. *Elysia* sp. 3GUAM found on Guam (USA) in 2009: e) habitus, f) *Elysia* sp. 3GUAM has thorn-shaped papillae, that are located laterally on the parapodia.

Elysia sp. 3GUAM

collection place	USA: Guam, *Caulerpa*-spot near C. Carlson's farm
collection date	17.08.2009
substratum	*Caulerpa cupressoides*
size	about 2cm
specimen	E3-09GU-1

This species is coloured green with a lot of black pigments and papillae on the lateral side of the parapodia and the rhinophores (figure 3.18e), f)). The rhinophores have a greenish dirty yellow colour that continues in a noticeable rim along the parapodia edges. This species is a short-term retention form (figure 3.6, p. 51). The only specimen stays with P. Krug (Los Angeles), who found this animal.

Elysia sp. 4GUAM

collection place	USA: Guam, Pago Bay
collection date	27.08.2009
substratum	*Chlorodesmis hildebrandtii*
size	about 1cm
specimen	E4-09GU

This specimen crawled out from an algal sample of *Chlorodesmis*, on which it fed, after several days. This species is almost invisible within its host. It reminds one of the description of *Elysia* sp. 8 from Sulawesi (sealugforum.net). My specimen is missing the anterior part of its right parapodium (figure 3.19a)). The closest related species is *Elysia* ”*asbecki*” (figure 3.17).

Elysia sp. 5GUAM

collection place	USA: Guam, Asan
collection date	28.08.2009
substratum	*Tydemania expeditionis*
size	0.5 to 2cm
specimen	E5-09GU-1 to E5-09GU-5 and E5-09GU-7

Specimens of this species arose numerously from the alga *Tydemania expeditionis*, on which they are cryptic (figure 3.19b), c)). This species is a short-term retention form (figure 3.6, p. 51). The neighbornet analysis (figure 3.17) shows that a specimen of this species had been found before by A.L. Bass; its 16S rDNA sequence is deposited in GenBank under DQ480173.

Elysia sp. 11 (seaslugforum.net)

collection place	Australia: Lizard Island, MacGillivray Reef USA: Guam, Bile Bay and outfront Cocos Lagoon
collection date	01.11.2008/17. and 24.08.2009
size	up to 5cm
specimens	E1108LI-179 and E11-09GU-1 to E11-09GU-3

According to the neighbornet analysis this species belongs to the species complex of *Elysia ornata* – *Elysia grandifolia* (figure 3.17) and has been portrayed before in the seaslugforum. It is a big animal with quite thick parapodia. This species is a short-term retention form (figure 3.7, p. 52).

Figure 3.19 a) *Elysia* sp. 4GUAM found on Guam (USA) in 2009. The anterior part of the right parapodium is missing. *Elysia* sp. 5GUAM found on Guam (USA) in 2009; b) habitus, c) the species is cryptic on its host *Tydemania expeditionis*. d) *Elysia* sp. 11 (seaslugforum.net) found on Lizard Island (Australia) in 2008.

Elysia sp. 15 (Gosliner et al. 2008)

collection place	USA: Guam, Cocos Lagoon
collection date	18./21.08.2009
substratum	*Udotea geppii*
size	up to 2.5cm
specimens	E15-09GU-1 to E15-09GU-5 and E6-09GU-1

This species has been portrayed before as *Elysia* sp. 15 by Gosliner et al. (2008). Their picture shows a juvenile "spheric" form (figure 3.20e)).

Right after collection many small specimens were discovered. Eventually, one specimen (E6-09GU-1 [DNA 869]) with a length of about 2.5 cm showed up on the 02.09.2009, i.e. more than ten days later, from the same algal sample. This specimen was thin and elongated (figure 3.20a)-d)). Smaller specimens had a more compact appearance with large curved parapodia (figure 3.20e)). The smaller the animals were, the smoother was the edge of the parapodia. However, the big specimen has a noticeable formation: the edges of the parapodia were formed like a zip that enables the animal to close its parapodia completely (compare figure 3.20c) and d)). The adults have a small tail (figure 3.20b)) and an uncovered triangle behind the head region (figure 3.20a)). While the adult shows no particular way of movement, the juveniles stretch forward with their anterior part (figure 3.20f)) and drag the rest of their body to the head position (figure 3.20 e)).

The closest known relative seems to be *Elysia bennettae* (figure 3.17).

This species is a short-term retention form (figure 3.7, p. 52).

Elysia sp. BANYULS

collection place	France: Banyuls-sur-Mer
collection date	30.04.2009
substratum	*Halimeda tuna*
size	up to 2.5cm
specimen	Espec09By-1 to Espec09By-3 and Ev09By-6

According to the neighbornet analysis (figure 3.17) this species seems to be related to another Mediterranean species: *Elysia translucens*. Unfortunately, there is no picture of this species. The animal is dark green and has strong thick parapodia, but no peculiar features.

This species is a short-term retention form (figure 3.7a)).

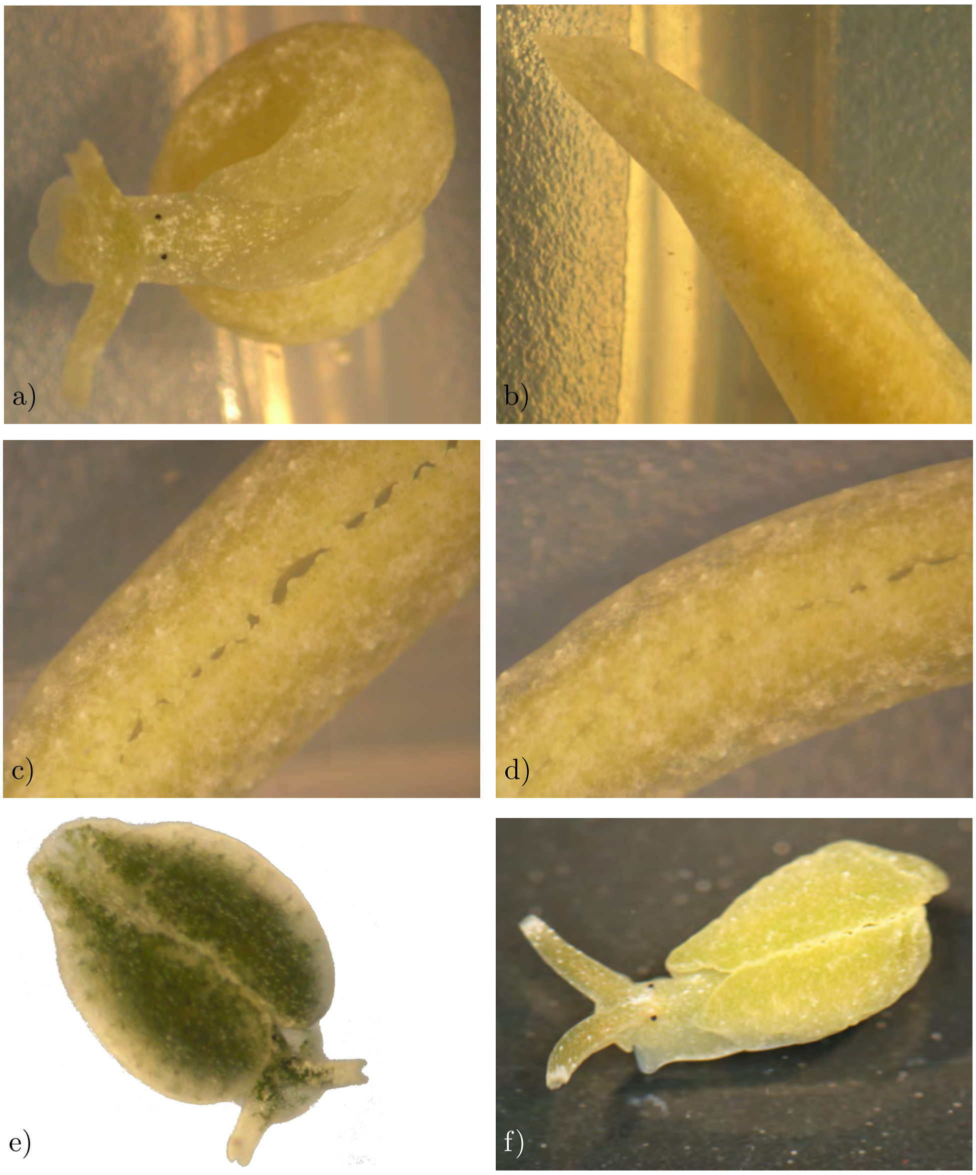
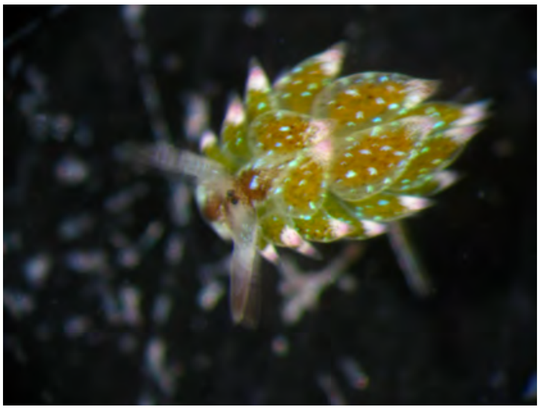


Figure 3.20 *Elysia* sp. 15 (Gosliner et al. 2008) found on Guam (USA) in 2009: adults are elongated; a) an uncovered triangle behind the head region can be found, b) pointed tail, c) zip-like structure at the edge of the parapodia: open and d) closed. Juveniles are not so elongated, but can appear more spherical in e) rest position. f) Movement is initiated by stretching the head region into target course.



Sacoglossa are quite photogenic. But they have two properties which can drive any photographer crazy; they speed along as this *Costasiella* on Lizard Island, Australia (above) or they surf upside down along the water surface as this *Thuridilla livida* on Guam, USA (below).

4 Discussion

4.1 Phylogeny of Sacoglossa

While the monophyly of Sacoglossa is well supported, their phylogenetic position relating to other groups of Opisthobranchia/Heterobranchia is still not resolved (Grande et al. 2004; Dinapoli & Klussmann-Kolb 2010; Klussmann-Kolb et al. 2008; Vonnemann et al. 2005; Wägele & Klussmann-Kolb 2005). Therefore *Cylindrobulla*, discussed as sistertaxon to Sacoglossa (Jensen 1996) or primordial sacoglossan (Mikkelsen 1998), has been used as the outgroup for morphological or molecular analysis of phylogeny of Sacoglossa (Händeler & Wägele 2007; Händeler et al. 2009; Jensen 1996). Some authors included Cylindrobullidae in the Sacoglossa (Clark 1994; Gosliner 1987; Schmekel & Portmann 1982). Jensen separated them but assigned Cylindrobullidae to be sistertaxon to Sacoglossa (1996).

Within this thesis the opisthobranch species *Haminoea hydatis*, *Diaphana* sp. and *Scaphander lignarius*, considered to be primordial (Klussmann-Kolb et al. 2008), have been used as outgroup to reveal the relation of *Cylindrobulla* and Sacoglossa. This is the first molecular analysis of Sacoglossa which shows that *Cylindrobulla* groups within the monophyletic Oxynoacea.

Synapomorphies of the Sacoglossa sensu Jensen are the epithelium lined ascus and adaptations of the alimentary system (e.g. pharyngeal musculature) to suctorial feeding (Jensen 1996). Therefore Jensen excluded the monogeneric family Cylindrobullidae, which was missing these features, from the Sacoglossa. Externally *Cylindrobulla* looks very similar to the genus *Ascobulla*. Hence, it does not surprise, that all species that are assigned to the genus *Ascobulla* today, i.e. *Ascobulla fischeri* (Adams & Angas, 1864), *Ascobulla fragilis* (Jeffreys, 1856), *Ascobulla japonica* (Hamatani, 1969), *Ascobulla souverbiei* (Montrouzier, 1874), *Ascobulla ulla* (Marcus & Marcus, E. d. B. R., 1970), *Ascobulla pusilla* (Nevill & Nevill, 1869) and *Ascobulla californica* (Hamatani, 1971), were originally described as *Cylindrobulla*. Species of both genera live within the sediment, are white, have a bulloid-shaped shell, no rhinophores but a head shield (figure 1.4a) and c), p. 5). Marcus, E. d. B. R. (1972) constituted the genus *Ascobulla* and transferred all former species of *Cylindrobulla* that showed dagger-shaped teeth, as it is typically for Sacoglossa, to it. The teeth of *Cylindrobulla* are broad and have a central cusp flanked by some denticles (figure 3.14f), p. 64).

Synapomorphies for Sacoglossa, including *Cylindrobulla* or a group composed of Sacoglossa and *Cylindrobulla* as sistertaxa, are a lamellate gill and one diagonal shell adductor as it can be found in Volvatellidae (the shell of Oxynoidae is reduced and shell adductor absent, in Juliidae the shell is strongly modified) (Jensen 1996). However, Mikkelsen (1998) reunited them, considering *Cylindrobulla* to have an ascus.

Oxynoacea

While *Ascobulla* sp. appears as sistertaxon to Cylindrobullidae, *Ascobulla fragilis* is sistertaxon to several specimens of *Volvatella.* The position of *Ascobulla fragilis* has to be seen as preliminary since 28S is missing for this species. The specimen from which the sequences of *Ascobulla* sp. were obtained (Malaquias et al. 2008) was used completely for DNA extraction and is not available for further analysis, e.g. investigation of the radula (personal communication Manuel Malaquias), to reveal or exclude a potential affinity to the genus *Cylindrobulla.* Jensen (1996) did not find any synapomorphies for Volvatellidae (*Ascobulla* + *Volvatella*).

According to Jensen's result (Jensen 1996), living in the sediment is a plesiomorphic character of Sacoglossa. According to the results of this study, burrowing has evolved at least twice independently, or the epifaunal life trait has evolved at least twice, when the plesiomorphic state is considered to be infaunal. Considering living in the sediment as apomorphic within Sacoglossa, i.e. Oxynoacea, the externally similarities of *Cylindrobulla* and *Ascobulla* can be explained as convergent adaptations to a new burrowing lifestyle.

While the body of *Volvatella* is white as in *Cylindrobulla* and *Ascobulla*, the transparent shell of some species, i.e. *Volvatella viridis* Hamatani, 1976, *Volvatella bermudae* Clark, 1982 and *Volvatella ventricosa* Jensen & Wells, 1990, appears green due to the green mantle (figure 1.4d)). *Volvatella viridis* was described from Japan. Jensen and Wells (1990) considered *Volvatella ventricosa*, found in Western Australia, to be a different species due to the existence of a small penial stylet that has not been described in *Volvatella viridis.* However, other remarkable morphological differences have not yet been found. Jensen assumes specimen S121, according to a photo, to be perhaps *Volvatella ventricosa*, but comments that the spout is longer than in the Western Australian specimens (Jensen, personal communication).

Uncorrected p-distances of 16S rDNA and *coxI* between *Volvatella* cf. *ventricosa* (S121) from Lizard Island (Australia) (figure 1.4d), p. 5) and the juvenile specimen (858) from Guam (figure 4.1) consider those to represent the same species (almost zero, rounded to two decimal points, see table 6.4, p. 109). The distances between *Volvatella viridis* 1 (789) and the latter two range from 0.10 to 0.15%. Further investigations, considering morphology and molecular data from one and the same specimen, are needed to clarify species affiliations in green forms of *Volvatella* in the Indo – Pacific.

Species of Oxynoidae have a reduced shell and are not able to retreat into it. This is correlated with a loss of the shell adductor muscle (Jensen 1996). The genus *Lobiger* is characterised by its lateral parapodia: two on each side (figure 1.4e)). It has a prominent tail as well as the genus *Oxynoe*, which covers its shell with its paired parapodia (figure 1.4f)). The genus Oxynoe is monophyletic but the Caribbean *Oxynoe azuropunctata* Jensen, 1980b appears within the two specimens of *Oxynoe antillarum* Mörch, 1863. *Oxynoe azuropunctata* has been described from Florida and separated from *Oxynoe antillarum* by a larger number and size of the papillae on the parapodia and tail in *Oxynoe azuropunctata* (Jensen, 1980b). Genetic distances in 16S rDNA sequences do

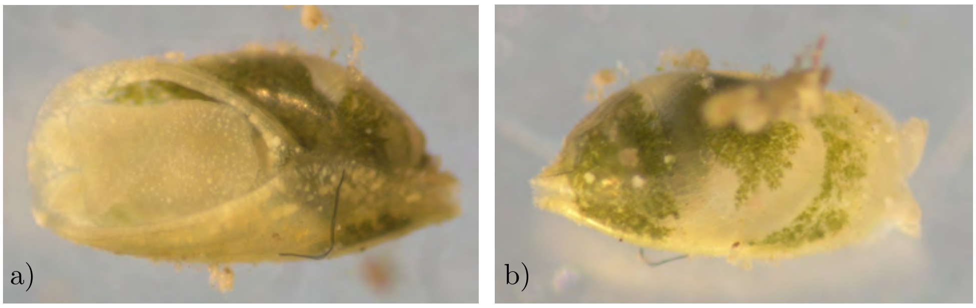


Figure 4.1 *Volvatella* sp., juvenile specimen found on Guam (USA) in 2009; a) ventral, b) dorsal. Approximately 2mm long.

not exceed 0.01% (table 6.4, p. 109) and all specimens of *Oxynoe antillarum* and *Oxynoe azuropunctata* belong to the same species. Therefore, they are synonymous or specimen(s) were misidentified. Clark and Goetzfried (1978) found planktotrophic larvae in *Oxynoe antillarum*, while *Oxynoe azuropunctata* produced lecithotrophic larvae (Clark & Jensen 1981; Jensen 1980b). However, this could be due to poecilogony as it occurs in sacoglossans (Ckark 1994; Krug 1998, 2009) and in gastropods in general (Bouchet 1989).

Roburnella and *Lophopleurella* were not available for analysis. Having lobigerid parapodial processes *Roburnella wilsoni* was formerly described as Lobiger, but separated by Marcus, E. d. B. R. (1982) due to its oxynoid shell. *Lophopleurella* shows a lobigerid shell and oxynoid parapodia.

A peculiar group is the family Juliidae: a bivalved shell evolved, which reminds one at first sight of the shells of Bivalvia (figure 1.4b)). Species of Juliidae, i.e. *Tamanovalva limax* and *Julia exquisita*, appear not to be monophyletic according to the result of the molecular phylogenetic analysis. Evolution of a bivalved shell is only known from Juliidae within Gastropoda. Therefore the hypothesis of manifold evolution within Oxynoacea has to be rejected.

Oxynoacean sequences of 28S rDNA, 16S rDNA and *coxI* are extremly derived compared to sequences obtained from other sacoglossan species including oxynoaceans. Therefore the investigated markers are not sufficient to resolve phylogenetic relationships within the Oxynoacea. Nevertheless, monophyly of Oxynoacea, including the genus *Cylindrobulla* as it has been obtained by Mikkelsen (1998) as a result of a morphological analysis, is confirmed.

Plakobranchacea

Most sacoglossan species belong to the group Plakobranchacea. Two main groups are distinguished: the Limapontioidea and the Plakobranchoidea.

Limapontioidean slugs bear dorsal appendices (figure 1.5), the so-called cerata. Plakobranchoidean slugs are characterised by lateral parapodia (figure 1.6). The latter are monophyletic and are discussed below.

Limapontioidea

While Limapontioidea appears not to be monophyletic due to missing resolution, the families Hermaeidae, Costasiellidae and Limapontiidae are monophyletic. Limapontiidae are discussed below.

Hermaeidae, i.e. *Hermaea* and *Aplysiopsis*, have also been found to be monophyletic by Jensen (1996). The exceptional genus *Hermaea* feeds on red algae. *Aplysiopsis* can have a red colouration as well, although it feeds on green algae. Both genera of Hermaeidae have fusiform cerata and their rhinophores end with a flap.

Species of the monogeneric family Costasiellidae have digitiform cerata and can be clearly differentiated from all others by the close position of their eyes (figure 1.5e), p. 6, figure 3.15, p. 66, see also p. 77). According to the results of this analysis *Costasiella* is considered to stay in its own family and not to be included in Limapontiidae as proposed by Jensen (1996). A sistergroup relationship of Costasiellidae and Limapontiidae cannot be rejected.

Polybranchiidae do not appear to be monophyletic, although monophyly cannot be rejected due to missing resolution. Members of the Polybranchiidae have broad flat cerata, bifurcate rhinophores and mostly oral tentacles. The digestive gland reaches into the cerata except for *Cyerce* and the monotypic genus *Sohgenia*. *Cyerce* is separated in the molecular analysis from the remaining genera. (*Sohgenia palauensis* was not available for analysis). Further investigations will hopefully result in a higher resolution that will reveal whether the taxon Polybranchiidae exists or not.

Since cerata evolved independently several times within opisthobranchiate slugs, I would consider the presence of cerata (taxon "Limapontioidea") and also the presence of broad flat cerata (taxon "Polybranchiidae") as weak apomorphies to support the taxa in question sufficiently.

Assuming the monophyly of Limapontioidea, forming a monophyletic clade with Plakobranchoidea, that is sistertaxon to Oxynoacea, raises the question of what the ancestor of Plakobranchacea looked like, since it would neither possess parapodia, cerata nor a shell.

Limapontiidae

Limapontiidae bear digitiform cerata and include genera such as *Limapontia* without any cerata, dwelling in tide pools, *Alderia*, that lives in patches of its host alga *Vaucheria* in mudflats, or the oophagous *Calliopea bellula*. Jensen (1996) found Limapontiidae monophyletic, but supported mainly by character reversals, and the genus *Costasiella* was included (see above).

Four of the six included species of the genus *Ercolania* (*Ercolania kencolesi*, *Ercolania "annelyleorum"*, *Ercolania viridis* and *Ercolania fuscata*) form a monophyletic clade, that is sistertaxon to the genus *Alderia*. Therefore, the result of Jensen (1996) is confirmed and *Alderia* considered to be included in the Limapontiidae. The sistertaxa *Ercolania kencolesi* and *Ercolania "annelyle-*

orum" can be found in the Indo – Pacific and the sistertaxa *Ercolania viridis* and *Ercolania fuscata* are Atlantic/Mediterranean species.

However, the position of *Ercolania boodleae* on a long branch has not been resolved and *Ercolania felina* groups with *Limapontia nigra* in the analysis of Limapontiidae. This considers the genus *Limapontia* polyphyletic, *Limapontia senestra* clustering with the oophagous *Calliopea bellula*. Considering the long branch, sequences of *Ercolania boodleae* are extremly derived and may cause a wrong or no phylogenetic position.

Remarkable is that *Ercolania felina*, that was described as *Calliopea felina* by Hutton (1882), and *Ercolania boodleae* live in tide pools as the species of *Limapontia* do. According to the hypothesis presented here the absence of cerata and partly rhinophores in the polyphyletic genus *Limapontia* (analysis of Limapontiidae) has evolved convergently due to living in intertidal pools and salt-marshes.

Phylogenetic positions of *Stiliger* and the four species of *Placida* remain uncertain due to different results in the different analyses. Considering morphological aspects, validity of different genera in Limapontiidae and affiliation to it has been discussed several times (e.g. Jensen 1985, 1996; Marcus, E. d. B. R. 1982). Additionally, the original species and genus description are often sketchy. Synonymisation of the genera *Ercolania* and *Stiliger*, as proposed by Marcus, E. d. B. R. (1982), is not possible according to the results of this study. In the analysis of Sacoglossa, in which the *Ercolania felina*, *Ercolania boodleae* and *Placida kingstoni* were removed, *Stiliger ornatus* appears within in the monophyletic genus *Placida*. A thorough morphological investigation on species level within Limapontiidae is necessary to clarify status of the genera and affiliation of species.

Plakobranchoidea

The group of Plakobranchoidea (figure 1.6) compromises only eight genera (see table 1.1, p. 4) but more than one third of all described sacoglossan species. *Bosellia* (figure 1.6a)) has been kept in its own family due to its uncertain position within Plakobranchoidea caused by its unusual morphology; the lateral flaps are not parapodia (Marcus, E. d. B. R. 1982). It appears in this study as sistertaxon to the family Plakobranchidae composed of the genera *Plakobranchus*, *Thuridilla* and *Elysia*.

Unfortunately none of the four species of the Platyhedylidae were available. They are morphologically derived to a worm-like habitus. *Platyhedyle denudata* is mesopsammic. According to Jensen's result (1996) they were included in the Plakobranchoidea, but evaluation of characters of these, most likely highly derived animals, is difficult (Jensen 1996; Marcus, E. d. B. R. 1982).

Several species of *Plakobranchus* described were synonymised to one (Jensen 2007). Nevertheless, the existence of different species seems to be obvious (personal communication P.J. Krug, see discussions on seaslugforum.net). Unfortunately they were not available for this study. *Plakobranchus* differs to *Elysia* and *Thuridilla* in its dorsal position of the anus (Jensen 1992), in having longitudinal dorsal lamellae and a flat head with lateral rhinophores.

The phylogenetic relationships of the genera within Plakobranchidae ((*Plakobranchus* + *Thuridilla*) + *Elysia*) obtained in this study confirm findings of Gosliner (1995) and Bass & Karl (2006) but contradict results of Jensen (1996) and may need a thorough morphological investigation including new data.

Phylogenetic relationships within the genus *Thuridilla* mainly differ from the results of Gosliner (1995) based on a morphological analysis. E.g. Gosliner (1995) found *Thuridilla hoffae* and *Thuridilla livida* monophyletic. Apomorphic character was the black body colour. According to my results, *Thuridilla livida* is sistertaxon to *Thuridilla hopei* and *Thuridilla hoffae* sistertaxon to all other species of *Thuridilla*. The use of colour as character is difficult, especially since colouration in the genus *Thuridilla* varies intraspecfically (see p. 37). However, the sistertaxa-relationship of *Thuridilla vatae* and *Thuridilla albopustulosa*, based on the connection between vessels described by Gosliner (1995), is confirmed by my results. Many species that are included in Gosliner's analysis (1995) have not been available for molecular analysis. Completion of taxon sampling is required.

Elysia

The genera *Tridachia*, *Tridachiella*, *Elysiella* and *Pattyclaya* have been transferred to the genus *Elysia* (Gosliner 1995). Jensen (1996) treated them still as separated genera. Händeler & Wägele (2007) showed that *Elysia pusilla* (described as *Elysiella pusilla*, Bergh) belongs to the genus *Elysia* by means of a molecular marker. This and the transfer of *Elysia crispata* (described as *Tridachia crispata*, Mörch) is confirmed by this study. Apomorphies of the genus *Elysia* according to Gosliner (1995) are: a blade-shaped tooth, absence of pharyngeal pouches and a long ascus muscle. Absence of pharyngeal pouches can be found in many sacoglossan genera, e.g. Oxynoidae and Juliidae, but not within Plakobranchoidea (Jensen 1996).

Never before has a data set been compiled including so many species of *Elysia*. Monophyly of the genus seems to be certain and some sistertaxa relationships described by Händeler & Wägele (2007) are confirmed. Phylogenetic positions of several species remain unresolved, or uncertain, due to different results from different data sets (e.g. sistertaxa-relationship of *Elysia chlorotica* and *Elysia viridis*). Continued taxon sampling and enlarging of the data set will certainly improve the resolution, since many "lone" species may miss their counterpart in the most recent data set.

4.2 Kleptoplasts in Sacoglossa: photosynthetic abilities among Sacoglossa

The aim of this study was to gain an overall survey on photosynthetic abilities among Sacoglossa throughout all their different groups. Measurements of fluorescence of chlorophyll a indicating intactness of chloroplast is a method that can be easily applied in the field and leaves animals for further investigation (phylogeny, barcoding).

The result of Händeler et al. (2009) was confirmed by additional studies in Banyuls-sur-Mer (France) and on Guam (USA) on 12 species: shelled and limapontioidean species show no functional chloroplast retention. Species of the plakobranchoidean genera *Thuridilla* and *Elysia* showed a functional short-term retention and *Plakobranchus ocellatus* showed a functional long-term retention. However, two undetermined species of the limapontiodean genus *Costasiella*, i.e. *Costasiella* cf. *kuroshimae* and *Costasiella* sp. 1 (seaslugforum.net), showed a functional retention. Clark and colleagues (1981) investigated *Costasiella ocellifera* (as *lilianae*) and found that symbiotic plastids continued to fix carbon after 65 days starvation, though efficiency of fixation declined by about 87%. Astonishing is the "cyclic" photosynthetic activity. It has not been observed in any other sacoglossan except for the genus *Costasiella*.

Other limapontioidean sacoglossans that were considered to be capable of a functional retention are *Hermaea bifida* (Kremer 1977; Taylor 1971b) and *Limapontia depressa* (Hinde & Smith 1974; Trench 1975). Unfortunately, specimens from the red algae feeding genus *Hermaea* were not available for PAM-measurements.

It has to be taken into consideration that feeding status of freshly caught animals is unknown. Further, photosynthesis in one and the same individual can vary, e.g. seasonally or geographically: Waugh and Clark (1986) found seasonal and geographical variation in chlorophyll level of *Elysia tuca*; Marín and Ros (1992) found seasonally carbon fixation rates in *Elysia timida*; Brandley (1984) showed that *Elysia* cf. *furvacauda* incorporated at any time at least three different types of chloroplasts but percentage of different types varied seasonally. Furthermore, chloroplast origin may depend on the life cycle, e.g. Curtis and colleagues (2007) found different sequestered chloroplasts in juvenile and adult specimens of *Elysia clarki*, because juveniles preferred to feed on algae species with a smaller diameter than adults. Chloroplasts of different food sources may cause different qualities of potential functional retention. *Elysia clarki* sequestered up to four different types of chloroplasts in one cell (*Penicillus lamourouxii*, *P. capitatus*, *Halimeda incrassata* and *H. monile*) (Curtis et al. 2006).

Further, photosynthetic abilities depend on external circumstances, e.g. Marín and Ros showed that carbon fixation rate in *Elysia timida* is reliant on light intensity and temperature (1989, 1992).

Unfortunately, *Elysia chlorotica* was not available for PAM-measurements. But it has been investigated extensively for the last 30 years. First reports on its photosynthetic ability were given by West (1977, 1980). Detailed investigations followed while searching for expression of chloroplast-encoded genes and protein synthesis (Green et al. 2000; Mujer et al. 1996; Pierce et al. 1996) and for a horizontal gene transfer from the algal to the slug's nucleus (Pierce et al. 2007; Rumpho et al. 2008, 2009; Pierce et al. 2009; Schwartz et al. 2010).

The data collected on photosynthetic abilities of different sacoglossans in this study is a starting point and background for detailed investigations and depict those species that have to be investigated in addition to *Elysia chlorotica* to understand a functional chloroplast retention over several weeks up to month, i.e. *Elysia timida*, *Elysia crispata* and *Plakobranchus ocellatus*.

4.3 Food organisms of Sacoglossa: barcoding

Amplification with primer pairs as e.g. rpoB/rpoC1 were not successful. Composition and arrangement of chloroplast genes vary within algal taxa, fragmentation and rearrangements of fragments of one gene is also possible (e.g. Brouard et al. 2008, 2010; Cambiaire et al. 2006; Pombert et al. 2006). E.g. the spacer rpoB/rpoC1 does exist in *Leptosira terrestris* and *Chlorella vulgaris* (Trebouxiophyceae) (Cambiaire et al. 2007; Wakasugi et al. 1997), but not in *Helicosporidium* (Trebouxiophyceae) (de Koning & Keeling 2006). Therefore different gene arrangements in different species of Ulvophyceae cannot be excluded. Although *tufA* has been transferred to the nucleus in other algae and landplants (see e.g., Baldauf et al. 1990; Bonny & Stutz 1993), it is present in the chloroplast genome of Ulvophyceae as far as known (Pombert et al. 2005, 2006) and therefore suitable for barcoding.

The new sequences of *Tydemania expeditionis* and *Rhipilia crassa* verify the existing sequences in GenBank; sequences of *Avrainvillea erecta*, *Codium vermilara*, *Codium effusum* are the first sequences of these species. Some could only be included as *Codium* sp., *Bryopsis* sp. or *Chaetomorpha* due to not being expert in alga identification. Nevertheless, these sequences enlarged the collection of these molecular poorly investigated genera.

Kawaguti (Greene 1970c after Kawaguti 1941) mentioned the frequent proximity of *Plakobranchus ocellatus* to *Halimeda*, but the proof that *Plakobranchus ocellatus* does feed on species of this genus had to wait – for almost 70 years. The knowledge obtained on the food organisms of the genera *Cylindrobulla* and *Plakobranchus* gives the best example to show that the system of barcoding established here has been extremly successful. *Plakobranchus ocellatus* has been investigated deeply because of its ability to incorporate chloroplasts (Evertsen et al. 2007; Greene 1970a, b, c; Hirose 2005; Kawaguti & Yamasu 1965) without discovering the food alga(e). Additionally, egg masses of *Plakobranchus ocellatus* were never found on any alga, but on a holothurian (Mercier & Hamel 2005). The only feeding observation was made in the laboratory: *Plakobranchus ocellatus* fed on *Udotea* and *Chlorodesmis* (Jensen 1980a after Switzer-Dunlap 1975). According to my results, the food spectrum of *Plakobranchus ocellatus* is even broader (*Halimeda opuntia*, *Halimeda macroloba*, *Caulerpa* cf. *serrulata* and several up to now unidentified species).

A narrow food spectrum shows *Cylindrobulla* sp. with just one species: *Halimeda macroloba.* Jensen assumed (1989b) that *Cylindrobulla phuketi* feeds on *Halimeda*, but she could not observe feeding. Clark (1994) described the habitat of *Cylindrobulla* as a "coarse *Halimeda* sand with mixed Siphonales" (p. 902). However, *Cylindrobulla xishaensis* was collected from *Caulerpa* (Lin, 1978). Barcoding clearly showed that at least *Cylindrobulla* sp., collected on Guam, fed on *Halimeda macroloba.*

Also, food data obtained on the plakobranchidean *Elysia tomentosa*, *Elysia "asbecki"*, *Thuridilla carlsoni*, *Thuridilla gracilis*, *Thuridilla kathae* and *Thuridilla livida* are new. Nothing has been known on the food of this species before. Unfortunately, for *Elysia "asbecki"* and the above-mentioned *Thuridilla* species the according algae have not yet been included in the reference library. Food organisms of *Thuridilla* species and *Elysia "asbecki"*, which are found crawling on the ground not obviously on any alga, may be unknown due to the fact that these algae are diminutive filaments. Unfortunately, amplification of *tufA* of the specimens of *Thuridilla hopei* investigated in the food experiment has not yet been successful. *Thuridilla hopei* 809, collected on the Isola del Giglio/Italy as the specimens mentioned above, fed on something related to food organisms of other, Indopacific species of *Thuridilla.* The food of *Thuridilla hopei* according to literature is *Derbesia tenuissima* (Gavagnin et al. 1994b; Marín & Ros 2004) and *Cladophora vagabunda* (Marín & Ros 1989). The amplified sequences do not refer to *Derbesia tenuissima*, that is included in the analysis. A *tufA* sequence of *Cladophora vagabunda* is unfortunately not available. The alga chosen in the feeding experiment could not be identified as *Cladophora vagabunda*, since these algae did not ramify.

However, one of the two different food sources of *Thuridilla gracilis* specimen S118 was identified as *Codium vermilara.* This appears to be most unlikely, since *Codium vermilara* is not known to be distributed in the Pacific (www.algaebase.com). However, knowledge on algae biodiversity is restricted since only few people work on algae and only specialists are able to identify species of a specific group. E.g. *Codium platylobium* described from South Africa (Areschoug, 1854) has been found recently in Asia (Korea) (www.algaebase.com after Lee 2008). Nevertheless results should be confirmed before pronouncing this the first record of *Codium vermilara* at the Great Barrier Reef.

Further recorded food sources are confirmed by barcoding, which shows the strength of the method of barcoding; *Volvatella viridis*, *Volvatella* cf. *ventricosa*, *Bosellia mimetica*, *Elysia pusilla*, *Elysia timida* and *Costasiella* cf. *kuroshimae.*

Knowledge on the food spectrum of *Elysia macnaei* could be enlarged; it has been recorded to feed on *Halimeda cuneata* and *Halimeda macroloba* (Macnae 1954; Paul & Alstyne 1988). The specimen investigated in the present study fed on *Halimeda macrophysa* and another species of this genus.

There are several food sources for *Elysia viridis* according to literature: *Bryopsis* (Jensen 1989a; Trowbridge & Todd 2001), *Bryopsis plumosa* (Jensen 1980a); *Codium* (Bouchet 1984; Jensen 1989a), *Codium tomentosum* (Taylor 1968; Thompson & Jaklin 1988; Trowbridge & Todd 2001), *Codium fragile* (Trench et al. 1973); *Cladophora rupestris* (Trowbridge & Todd 2001); *Chaetomorpha linum* (Jensen 1980a, 1989a) and the red alga *Dasysiphonia* (Teugels et al. 2008). Three Mediterranean species could be investigated and they all fed on (different) species of *Bryopsis*. Therefore the specific food of single specimens cannot be derived from a diverse food spectrum listed for this species. Rather, the food has to be determined carefully for any specimen that is used for research correlating food. There have been food choice experiments especially concerning *Elysia viridis* (e.g. Jensen 1989a; Trowbridge & Todd 2001) that describe biological effects on *Elysia viridis* on different hosts and food preference.

According to published results *Ercolania viridis* feeds on: *Chaetomorpha* (Jensen 1980a after Trinchese 1872; as *Ercolania funerea*, Jensen 1981, 1993a), *Chaetomorpha aerea* and *linum* (as *Ercolania funerea* Marín & Ros 1988), *Chaetomorpha capillaris* (as *Ercolania funerea* Di Marzo et al. 1993); *Cladophora* and *Cladophoropsis* (as *Ercolania funerea* Jensen 1981, Jensen 1993). But the investigated specimen from the Mediterranean fed on *Ulva intestinalis*. *Ulva* is only known as sacoglossan food in *Placida dendritica* (Brüel 1904 after Trinchese 1876b), *Limapontia capitata* (Gascoigne 1952), *Elysia australis* (Jensen 1991b, 1993) and maybe *Elysia serca* (Hosoe 1956). *Placida dendritica* was never observed feeding on *Ulva* again after Trinchese's publication in the 19th century. Gascoigne (1952) found only one population of *Limapontia capitata* feeding on *Ulva* (as *Enteromorpha*) but many others that fed on *Cladophora arcta*. *Elysia serca* is usually known to feed on seagrasses, e.g. *Halodule wrightii* (Jensen 1980a, 1982b), *Halophila engelmanni* and *Thalassia testudinum* (Jensen 1982b).

Thus, *Ulva* is an exceptional sacoglossan food and the only of the taxon Ulvales (see table 1.2, p. 12). Probably algae of Ulvales are not attractive for sacoglossans, since they consist of uninucleate cells which contain only one chloroplast. The only other ulvophycean alga consisting of cells with only one chloroplast, that is fed on by a sacoglossan, i.e. *Aplysiopsis enteromorphae*, is *Urospora* (Trowbridge 1993). All other genera belong to siphonous or siphonocladous groups.

The specimen of *Ercolania viridis* was found in a tide pool in which mainly *Ulva* grew. Whether *Ercolania viridis* feeds only occasionally (e.g. caught in a tide pool, that does not offer anything else) on *Ulva* and usually prefers other food sources can only be clarified by further analysis based on a broad taxon sampling.

Although an identification of the fed algae to species level has not been possible in any case yet, it is considered that 1. at least the sequenced food algae belong to the Ulvophyceae and 2. often one sacoglossan species fed on different algae species or genera.

4.4 Evolution of Sacoglossa

Figure 4.2 shows the phylogeny of Sacoglossa including data on photosynthetic activity and food.

Cylindrobulla, a species of Sacoglossa

The main question, whether *Cylindrobulla* possess an ascus (synapomorphy of Sacoglossa sensu Jensen 1996) or not, remains. Jensen considered it not to be an "ascus", since she did not find an epithelium-lined sac but a heap of loose teeth that are periodically shed (Jensen 1989b, 1996). Mikkelsen considered it to be an epithelium-lined ascus (1998). This problem will be addressed with a thorough examination of a new species of *Cylindrobulla* and can not be answered yet.

The original food source of a sacoglossan ancestor does not have to be *Caulerpa* as proposed by Händeler and Wägele (2007), but remains uncertain. The phylogenetic position of *Tamanovalva limax* as it is revealed in this study is most likely wrong. *Tamanovalva limax* is expected to cluster with the other bivalved sacoglossan *Julia exquisita*. Assuming *Cylindrobulla* to be the sistertaxon to all other shelled sacoglossans offers two options: a) the original food source was *Halimeda* as proposed by Jensen (1997) and all other Oxynoacea switched to *Caulerpa*; b) *Cylindrobulla* switched from *Caulerpa* to *Halimeda*. Further investigations have to show whether the position of *Cylindrobulla* within the Sacoglossa can be confirmed, especially based on morphological characters, and if so, what the position of *Cylindrobulla* is in Oxynoacea and which implications about original food source and habitat (infaunal or epifaunal) occur from that.

Chloroplast retention and the evolution of Sacoglossa

Short-term retention seems to be an apomorphic character of Plakobranchoidea. McLean (1976) was the first who showed that chloroplasts are phagocytosed in the limapontioidean *Placida dendritica*, but digested within 4 days (confirmed by Evertsen & Johnsen 2009). This seems to be a necessary preadaption from which plakobranchoidean species started development of functional retention possibly by simple "non-digestion" of chloroplasts. In fact, chloroplasts of ulvophycean algae and the siphonalean *Vaucheria* are quite robust and long-living (Grant & Borowitzka 1984; Green et al. 2005).

Further photosynthetic activity was observed in species of the genus *Costasiella*. It differed from all other known cases of functional retention in its cyclic appearance. Assuming that *Costasiella* is capable of a functional retention, the unique evolution of short-term retention in the stemline of Plakobranchoidea is questioned. However, convergent evolution is possible and whether *Costasiella* might be the sistertaxon to Plakobranchoidea cannot be ruled out at the moment.

Astonishingly, the four species of long-term retention, i.e. *Plakobranchus ocellatus*, *Elysia chlorotica*, *Elysia crispata* and *Elysia timida* are not monophyletic.

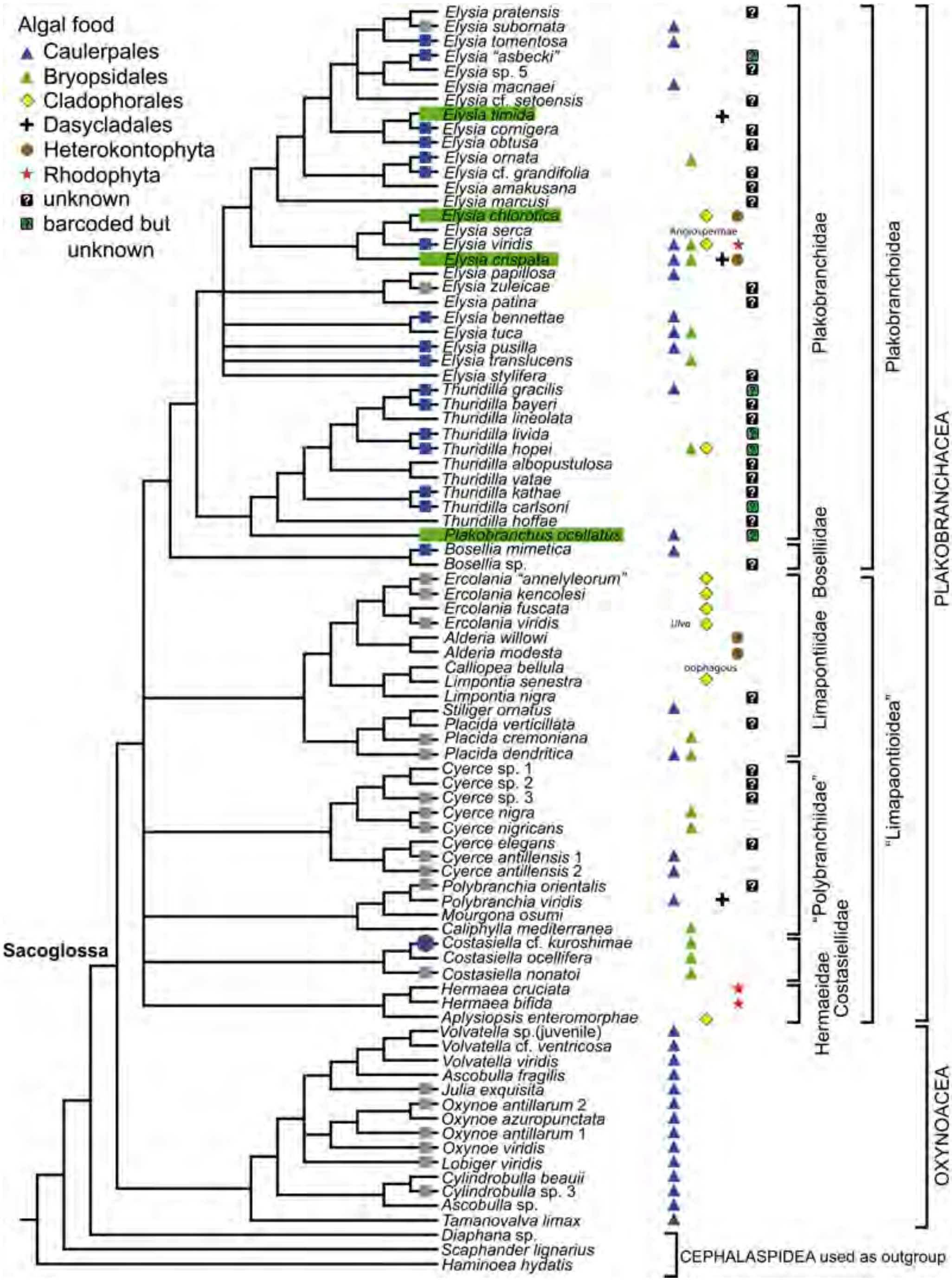


Figure 4.2 Data on food and photosynthetic activity mapped on the phylogeny of Sacoglossa. Information on host algae was taken from Krug et al. 2007, Händeler & Wägele 2007, Händeler et al. 2010, Teugels et al. 2008, Trowbridge 1994, Wägele et al. 2010 and data obtained in this study. Empty square: no-functional chloroplast retention, blue square: functional short-term chloroplast retention, green rectangle: functional long-term chloroplast retention. blue circle: "cyclic" functional retention in *Costasiella*.

According to the phylogeny presented here, long-term chloroplast retention evolved within the genus *Elysia* independently three times. The long-term forms are quite big sacoglossans of several cm length except for *Elysia timida*. However, not all big species of Plakobranchoidea perform a functional long-term retention, e.g. *Elysia ornata* is a short-term form. If the size of the animals is correlated to, or supported the development of, a long-term retention, the small size of *Elysia timida* (< 2cm) could be explained by a constraint that *Elysia timida* undergoes feeding exclusively on the small *Acetabularia acetabulum*.

Chloroplast retention and the chloroplasts's origin

Photosynthetic abilities of chloroplasts inside the cell of the sacoglossans do not seem to be correlated to the chloroplasts' origin. Both Limapontioidea and Plakobranchoidea have a broad food spectrum (figure 4.2). Short-term retention does not seem to rely on certain food species or genera, e.g. *Elysia ornata* feeding on *Bryopsis* (Jensen 1981; Horgen et al. 2000), *Elysia tomentosa* feeding on *Caulerpa* and *Bosellia mimetica* feeding on *Halimeda tuna* (Händeler & Wägele 2010) all show short-term retention.

While the Mediterranean long-term form *Elysia timida* is restricted to *Acetabularia acetabulum*, the Atlantic *Elysia crispata* and *Elysia chlorotica* and the Indo – Pacific *Plakobranchus ocellatus* show peculiar diverse food spectra (table 4.1). According to literature, juveniles of *Elysia timida* feed on *Cladophora* (Marín & Ros 1993). However, no hints of chloroplasts of *Cladophora* were found via barcoding and the feeding period on *Cladophora* seems not to be necessary since hatched juveniles can be reared on *Acetabularia acetabulum* alone (unpublished results Valérie Schmitt).

Elysia chlorotica and *Elysia crispata* feed on several ulvophycean algae and the heterokontophyte *Vaucheria*. *Vaucheria* is host algae of other plakobranchacean slugs, that have "exotic" food sources, often coevally to ulvophycean algae (table 6.5, p. 110). Belonging to the Vaucheriales (Xanthophyceae), this algal genus is composed of siphonous filaments containing numerous plastids as most ulvophycean genera that are fed on by sacoglossans.

The broad food spectra of the long-term forms *Elysia chlorotica*, *Elysia crispata* and *Plakobranchus ocellatus* raise the question of whether all types of chloroplasts take part in the long-term photosynthesis or only some or even one. Additionally, chloroplasts sequestered by long-term species are fed by species that are not capable of performing a long-term or functional retention at all (table 4.1). Therefore, the longevity of chloroplasts in plakobranchoidean long-term forms cannot be explained by the "robustness" of the plastids alone.

Table 4.1 Species performing a long-term functional retention and their food algae. The 3rd column lists the species that feed on the same food source and perform at least a short-term retention since they belong to the Plakobranchoidea. The 4th column lists those species that do not belong to Plakobranchoidea and do not perform a functional retention according to the result of this study and feed on the same algae as long-term species. Data on food according to the results of this study or taken from Händeler & Wägele 2007.

Long-term species	Food	Short-term species feeding on this food	Species feeding on this food and being incapable of performing functional retention
Elysia timida	*Acetabularia acetabulum*	–	–
Elysia chlorotica	*Vaucheria*	–	*Alderia modesta*, *Alderia willowi* *Costasiella pallida*, *Limapontia depressa*
	Cladophora	*Elysia australis*, *Elysia gordanae* *Elysia viridis*, *Thuridilla hopei*	*Aplysiopsis enteromorphae*, *A. formosa*, *A. smithi* *A. zebra*, *Costasiella ocellifera*, *Ercolania boodleae* *E. fuscata*, *E. viridis*, *Limapontia capitata* *Limapontia senestra*, *Placida kingstoni*
	Chaetomorpha	*Elysia australis*, *E. canguzua* *Elysia japonica*, *Elysia viridis*	*Aplysiopsis enteromorphae*, *A. smithi*, *A. zebra* *Ercolania boodleae*, *E. emarginata*, *E. fuscata* *E. gopalai*, *E. nigra*, *E. viridis*, *Limapontia capitata*
Elysia crispata	*Vaucheria*	–	see above
	Halimeda	*Bosellia mimetica*, *B. corinnae*, *Elysia macnaei**E. marcusi*, *E. papillosa* *E. patina*, *E. tuca*, *E. pusilla*	*Cylindrobulla* sp., *Cyerce antillensis*
	Caulerpa	*Elysia expansa*, *E. filicauda* *E. subornata*, *E. thompsoni*, *E. tuca* *E. tomentosa*, *Pattyclaya brucei*	Oxynoacea ex *Cylindrobulla* , *Polybranchia viridis*
	Bryopsis	*Elysia canguzua*, *E. hedgpethi* *E. ornata*, *E. patagonica*, *E rufescens* *E. viridis*	*Caliphylla mediterranea*, *Limapontia capitata* *Placida aoteana*, *P. daguilarensis*, *P. dendritica* *P. kingstoni*, *P. viridis*
	Derbesia	*Thuridilla hopei*	*Placida daguilarensis*
	Chaetomorpha	see above	see above
	Penicillus	*Elysia papillosa*, *E. patina*	*Cyerce antillensis*
	Batophora	–	–
Plakobranchus ocellatus	*Halimeda*	see above	see above
	Caulerpa	see above	see above
	?*Udotea*	*Bosellia mimetica*, *Elysia degeneri* *E. papillosa*, *E. patina*, *E. tuca*	*Cyerce antillensis*
	Ulvophyceae	?	?

However, species of *Costasiella* feed on *Avrainvillea* except for *C. pallida* and *C. coronata*, that feed on *Vaucheria* (Jensen 1986; Swennen 2007). The only other sacoglossan feeding on *Avrainvillea* is *Elysia tuca*, but results were obtained by artificial lab experiments and were never observed in nature (Clark & Busacca 1978). The specific quality of chloroplast retention in *Costasiella* may differ from that of chloroplast retention in Plakobranchoidea. Therefore, it cannot be ruled out that properties of the chloroplasts of *Avrainvillea* differ from those of chloroplasts in the cells of plakobranchoidean sacoglossans and play a different role in this endosymbiosis.

Mechanisms that enable the slug to prolong the life of its sequestered chloroplasts has not been understood until today. Several works proclaimed a horizontal gene transfer and protein synthesis of algal nucleus encoded proteins in *Elysia chlorotica* (Pierce et al. 2007; Rumpho et al. 2008, 2009; Schwartz et al. 2010). However, a horizontal gene transfer did not take place in *Plakobranchus ocellatus* nor in *Elysia timida* (Wägele et al. 2010). Even if there were a horizontal gene transfer, this fact would not explain how proteins encoded by the slug's nucleus are delivered to the retained chloroplasts.

Searching for sacoglossans can be quite time-consuming and a mystery to people who are not short-sighted, whether on Guam, USA (above) or on Lizard Island, Australia (below).

5 Summary

The results of this study revealed new data on aspects of the evolution of Sacoglossa under special consideration of their food algae (Ulvophyceae) and the incorporation of chloroplasts.

A phylogeny based on molecular markers, i.e. 28S rDNA, 16S rDNA and *coxI*, was performed. Two major lineages, the shelled Oxynoacea including *Cylindrobulla* and the shell-less Plakobranchacea, were found monophyletic. While Limapontioidea cannot be considered to be mono- or paraphyletic due to missing resolution, Plakobranchoidea are monophyletic with the two families Boselliidae and Plakobranchidae. The group of the monophyletic *Plakobranchus* and *Thuridilla* is sistertaxon to *Elysia*. In Limapontioidea the families Limapontiidae, Costasiellidae and Hermaeidae are monophyletic. However, Polybranchiidae does not appear monophyletic, since the genus *Cyerce* is separated from the remaining genera included in this analysis, but cannot be rejected since phylogenetic position of *Cyerce* remains unresolved.

Photosynthetic activity of chloroplasts in the cells of sacoglossans were measured with the help of a Pulse Amplitude Modulated Fluorometer (PAM). There are three different classes of chloroplast retention: a) no functional retention, b) functional short-term retention of not more than two weeks and c) functional long-term retention over several weeks up to months. A functional retention was found in Plakobranchoidea and in the monogeneric family Costasiellidae. Results obtained from *Costasiella* are not comparable to results obtained from any other sacoglossan and are considered to be preliminary. A functional long-term retention was observed in four plakobranchidean slugs, i.e. the Indo-Pacific *Plakobranchus ocellatus*, the Atlantic *Elysia chlorotica*, *Elysia crispata* and the Mediterranean *Elysia timida*. These species do not form a monophyletic clade and long-term retention evolved independently four times.

The protein encoding chloroplast gene *tufA* has been established as barcode for food of sacoglossans. For the first time, food of several species has been identified: *Cylindrobulla* sp. fed on *Halimeda*, *Plakobranchus ocellatus* on *Halimeda*, *Caulerpa*, ?*Udotea* and several other species of Ulvophyceae that have not been identified up to now. Food of five species of *Thuridilla* was barcoded but for some of them no reference sequences of Ulvophyceae are available yet. Food was confirmed for several species of *Volvatella*, *Costasiella* and *Elysia*. A new food source was found for *Ercolania viridis*: *Ulva*.

Neither short- nor long-term retention is correlated to the food organisms, i.e. the plastid donators.

6 Appendix

Table 6.1 Survey on the sequences used for the phylogenetic analysis in Händeler et al. 2009. Sequences starting with GQ were produced by myself; those starting with GU and coloured grey were produced by P. Krug. Sequences in grey boxes were taken from GenBank, those starting with EU were produced by myself, published in Händeler & Wägele 2007 and mainly indicate that the different sequences were amplified from the very same individual.

Species	28S rDNA	16S rDNA	*coxI*
Oxynoacea			
Cylindrobulla beauii	EF489371	EU140897	GQ996665
Volvatella viridis	GQ996604	EU140890	GQ996664
Julia exquisita	GQ996653	EU140895	GQ996661
Tamanovalva limax	GQ996605	EU140896	GQ996666
Lobiger viridis	GQ996654	EU140892	–
Lobiger viridis	GQ996655	EU140894	–
Oxynoe antillarum	GQ996606	DQ480217	GU191071
Oxynoe viridis	GQ996607	EU140891	GQ996656
Plakobranchacea			
PLAKOBRANCHOIDEA			
Bosellia mimetica	GQ996608	EU140872	GQ996657
Bosellia sp.	GU191031	GU191040	GU191066
Elysia amakusana	GQ996621	EU140851	GQ996686
Elysia bennettae	GQ996637	EU140868	GQ996675
Elysia chlorotica	GU191035	GU191054	GU191073
Elysia cornigera	GQ996624	GU191055	GU191068
Elysia crispata	GQ996634	DQ480199	DQ471225
Elysia crispata	GQ996635	DQ781367	DQ471224
Elysia macnaei	GQ996628	EU140854	GQ996689
Elysia marcusi	GQ996641	GU191056	GU191067
Elysia obtusa	GQ996627	EU140860	GQ996685
Elysia ornata	GQ996622	EU140848	GQ996688
Elysia ornata	GQ996623	EU140850	GQ996687
Elysia papillosa	GU191033	GU191049	GU191070
Elysia patina	GQ996642	GU191048	GU191069

Elysia pratensis	GQ996631	DQ480162	DQ471258
Elysia pusilla	GQ996639	EU140864	GQ996674
Elysia serca	GQ996640	DQ480192	DQ471244
Elysia stylifera	GU191032	GU191046	GU191057
Elysia subornata	GQ996632	DQ480165	DQ781351
Elysia timida	GQ996625	EU140859	GQ996672
Elysia timida	GQ996626	EU140858	GQ996673
Elysia tomentosa	GQ996633	EU140853	GQ996692
Elysia tuca	GQ996638	DQ480186	DQ471251
Elysia viridis	GQ996636	EU140863	GQ996668
Elysia viridis	AY427462	EU140861	GQ996669
Elysia zuleicae	GQ996643	GU191047	GU191075
Elysia sp. 1 (Wägele et al. 2006)	GQ996629	EU140856	GQ996690
Elysia sp. 5	GQ996630	EU140855	GQ996691
Plakobranchus ocellatus	GQ996619	EU140876	GQ996679
Plakobranchus ocellatus	AY427459	EU140875	GQ996680
Thuridilla albopustulosa	GQ996652	EU140889	–
Thuridilla bayeri	GQ996609	EU140886	GQ996683
Thuridilla bayeri	AY427461	DQ480207	DQ471278
Thuridilla carlsoni	GQ996614	EU140877	GQ996681
Thuridilla gracilis	GQ996610	EU140883	GQ996684
Thuridilla hoffae	GQ996618	EU140880	GQ996670
Thuridilla hopei	GQ996612	EU140882	GQ996677
Thuridilla hopei	GQ996613	EU140881	GQ996678
Thuridilla kathae	GQ996615	EU140879	GQ996676
Thuridilla lineolata	GQ996611	EU140887	GQ996682
LIMAPONTIOIDEA			
Caliphylla mediterranea	GU191020	GU191041	–
Cyerce antillensis	GQ996648	EU140841	GU191072
Cyerce nigricans	GQ996644	EU140843	GQ996658
Cyerce sp. 2 (Wägele et al. 2006)	GQ996645	EU140844	GQ996659
Mourgona osumi	GQ996646	EU140847	GQ996667
Polybranchia cf. *orientalis*	GQ996647	EU140846	GQ996671
Polybranchia viridis	GU191027	GU191052	–
Aplysiopsis enteromorphae	GU191034	GU191053	GU191059
Hermaea bifida	GU191026	GU191043	-
Hermaea cruciata	GU191025	GU191042	GU191058
Costasiella cf. *kuroshimae*	GQ996650	DQ480215	DQ471252
Costasiella nonatoi	GQ996649	GU191037	GU191065
Costasiella ocellifera	GQ996651	DQ480216	DQ471253
Alderia modesta	GU191030	DQ364417	DQ364309

Alderia willowi	GU191036	DQ364419	GU191074
Ercolania boodleae	GU191021	GU191050	–
Ercolania felina	GU191022	GU191038	GU191060
Ercolania fuscata	GU191023	GU191039	GU191061
Ercolania kencolesi	GQ996620	EU140840	GQ996660
Ercolania sp.	GU191024	GU191051	GU191062
Placida dendritica	GQ996616	EU140871	GQ996663
Placida dendritica	GQ996617	EU140870	GQ996662
Placida kingstoni	GU191028	GU191044	GU191063
Placida verticillata	GU191029	GU191045	GU191064

Table 6.2 Survey of species and number of specimens used for PAM measurements in Händeler et al. 2009. #(investigated specimens) gives the number of specimens in total.

Species	#(investigated specimens)	#(specimens investigated by myself)
Oxynoacea		
Oxynoe antillarum	10	0
Oxynoe cf. *viridis*	1	0
Lobiger viridis	1	1
Julia exquisita	1	0
Julia sp.	1	0
Plakobranchacea		
PLAKOBRANCHOIDEA		
Bosellia mimetica	12	10
Elysia bennettae	2	0
Elysia cornigera	10	0
Elysia crispata	4	0
Elysia ornata	9	0
Elysia pusilla	14	3
Elysia subornata	7	0
Elysia timida	10	0
Elysia tomentosa	3	1
Elysia viridis	6	0
Elysia zuleicae	6	0
Elysia sp.1	1	0
Plakobranchus ocellatus	18	14
Thuridilla carlsoni	5	2
Thuridilla gracilis	16	3
Thuridilla hopei	17	10
Thuridilla kathae	2	1
LIMAPONTIOIDEA		
Costasiella cf. *kuroshimae*	6	6
Costasiella nonatoi	2	0
Cyerce nigricans	1	0
Ercolania kencolesi	7	0
Placida dendritica	4	0
Polybranchia orientalis	2	2

Table 6.3 Survey of the molecular markers that were used for phylogenetic analyses: partial 28S rDNA, partial 16S rDNA and 1st and 2nd position of partial *coxI* of Sacoglossa. Compositions of different data sets is indicated: all sequences are used in a data set to analyse phylogeny of Sacoglossa except for sequences in a grey box (see figure 3.1); sequences in bolde type were used in Händeler et al. 2009; sequences in italics were used in a data set comprising only Plakobranchoidea (see figure 3.2); Sequences of the family Limapontiidae and of the genus *Elysia* respectively were analysed separately. LI stands for Lizard Island, Australia.

Species	28S rDNA	16S rDNA	*coxI*
Oxynoacea			
Cylindrobullidae			
Cylindrobulla beauii	**EF489371**	**EU140897 (728)**	**GQ996665 (728)**
Cylindrobulla sp.	847	847	847
Volvatellidae			
Ascobulla sp.	DQ927243		DQ974683
Ascobulla fragilis	–	AY345022	AY345022
Volvatella viridis	**GQ996604 (789)**	**EU140890 (789)**	**GQ996664 (789)**
Volvatella cf. *ventricosa*	–	S121	S121
Volvatella sp. (juvenile)	–	858	858
Juliidae			
Julia exquisita	**GQ996653 (783)**	**EU140895 (783)**	**GQ996661 (783)**
	826	826	826
Tamanovalva limax	**GQ996605 (792)**	**EU140896 (792)**	**GQ996666 (792)**
Oxynoidae			
Lobiger viridis	**GQ996654 (785)**	**EU140892 (785)**	–
	GQ996655 (802)	**EU140894 (802)**	–
	–	EU140893 (805)	–
Oxynoe antillarum	**GQ996606 (816)**	816	**GU191071**
	821	821	–
	–	**DQ480217**	–
Oxynoe azuropunctata	828	828	–
Oxynoe viridis	**GQ996607 (786)**	**EU140891 (786)**	**GQ996656 (786)**
	827	827	–
Plakobranchacea			
PLAKOBRANCHOIDEA			
Boselliidae			
Bosellia mimetica	**GQ996608 (744)**	**EU140872 (739)**	**GQ996657** (744)
	HM187620 (745)	*EU140873 (745)*	*HM187642 (740)*
Bosellia sp.	***GU191031***	***GU191040***	***GU191066***

PLAKOBRANCHIDAE			
Elysia amakusana	***GQ996621 (703)***	***EU140851 (703)***	***GQ996686 (703)***
Elysia "asbecki"	***GQ996629 (722)***	***EU140856 (722)***	***GQ996690 (722)***
Elysia bennettae	***GQ996637 (779)***	***EU140868 (779)***	***GQ996675 (779)***
	HM187619 (797)	*HM187586 (797)*	*HM187621 (797)*
Elysia chlorotica	***GU191035***	***GU191054***	***GU191073***
	HM187618 (807)	*DQ480200*	*NC_010567*
Elysia cornigera	***GQ996624 (812)***	*HM187587 (812)*	*HM187622 (812)*
	HM187617 (810)	*HM187588 (810)*	*HM187623 (810)*
	–	**GU191055**	**GU191068**
Elysia crispata	***GQ996634 (837)***	*HM187589 (837)*	*HM187624 (837)*
	GQ996635 (838)	*HM187590 (838)*	***DQ471225***
	–	**DQ480199**	**DQ471224**
	–	**DQ781367**	–
Elysia cf.	–	851	851
grandifolia	–	849	849
Elysia macnaei	***GQ996628 (726)***	***EU140854 (726)***	***GQ996689 (726)***
Elysia marcusi	*HM187616 (835)*	*HM187591 (835)*	*HM187625 (835)*
	GQ996641 (836)	*HM187592 (836)*	*HM187626 (836)*
	–	**GU191056**	**GU191067**
Elysia obtusa	***GQ996627 (778)***	***EU140860 (778)***	***GQ996685 (778)***
Elysia ornata	***GQ996622 (734)***	***EU140848 (734)***	***GQ996688 (734)***
	GQ996623 (798)	***EU140850 (798)***	***GQ996687 (798)***
	–	EU140849 (369)	–
Elysia papillosa	***GU191033***	***GU191049***	***GU191070***
Elysia patina	***GQ996642 (813)***	*HM187593 (813)*	***GU191069***
	HM187615 (825)	*HM187594 (825)*	*HM187627 (825)*
	–	**GU191048**	–
Elysia pratensis	***GQ996631 (811)***	*HM187595 (811)*	*HM187628 (811)*
	HM187614 (824)	*HM187596 (824)*	***DQ471258***
	–	**DQ480162**	–
Elysia pusilla	***GQ996639 (794)***	***EU140864 (794)***	***GQ996674 (794)***
	–	EU140867 (799)	799
	–	EU140866 (796)	796
	–	EU140865 (764)	764
Elysia serca	***GQ996640 (829)***	*HM187597 (829)*	*HM187629 (829)*
	HM187613 (830)	*HM187598 (830)*	***DQ471244***
	–	**DQ480192**	–
Elysia cf. *setoensis*	*HM187612 (803)*	*HM187599 (803)*	–
Elysia stylifera	***GU191032***	***GU191046***	***GU191057***
Elysia subornata	***GQ996632 (832)***	*HM187600 (832)*	*DQ781351*
	HM187610 (833)	*HM187601 (833)*	–
	–	**DQ480165**	–
Elysia timida	***GQ996625 (752)***	***EU140859 (766)***	***GQ996672 (766)***
	GQ996626 (761)	***EU140858 (769)***	***GQ996673 (769)***
	–	EU140857 (750)	750
Elysia tomentosa	***GQ996633 (711)***	***EU140853 (711)***	***GQ996692 (711)***
	HM187611 (710)	*EU140852 (710)*	*HM187630 (710)*
Elysia translucens	–	*HM187602 (845)*	*HM187631 (845)*

Elysia tuca	***GQ996638 (823)***	*HM187603 (823)*	*HM187639 (823)*
	–	**DQ480186**	**DQ471251**
	–	819	–
Elysia viridis	***GQ996636 (738)***	***EU140863 (738)***	***GQ996668 (738)***
	AY427462	***EU140861 (727)***	***GQ996669 (727)***
	–	EU140862 (737)	737
Elysia zuleicae	***GQ996643 (817)***	***GU191047***	*HM187632 (817)*
	–	–	**GU191075**
Elysia sp. 2	781	–	–
Elysia sp. 5	***GQ996630 (806)***	***EU140855 (806)***	***GQ996691 (806)***
Elysia sp. 1GUAM	–	871	871
	–	868	–
Elysia sp. 4GUAM	–	867	867
Elysia sp. 5GUAM	–	865	865
Elysia sp. 11	–	S178	S178
(seaslugforum.net)	–	S200	S200
Elysia sp. 15	–	S197	–
(Gosliner et al. 2008)	–	869	–
Elysia sp.	–	841	841
BANYULS	–	S143	–
Plakobranchus	***GQ996619 (706)***	***EU140876 (706)***	***GQ996680 (706)***
ocellatus LI	***AY427459***	***EU140875 (705)***	***GQ996679 (705)***
Plakobranchus	–	*HM187604 (850)*	*HM187633 (850)*
ocellatus GUAM	–	*HM187605 (852)*	*HM187634 (852)*
	–	*HM187606 (854)*	*HM187638 (854)*
	–	856	HM187635 (856)
Thuridilla albopustulosa	***GQ996652 (782)***	***EU140889 (782)***	–
Thuridilla bayeri	***GQ996609 (725)***	***EU140886 (725)***	***GQ996683 (725)***
	AY427461	**DQ480207**	**DQ471278**
Thuridilla carlsoni	***GQ996614 (801)***	*EU140878 (801)*	***GQ996681 (801)***
	–	***EU140877 (714)***	*HM187640 (714)*
Thuridilla gracilis	**GQ996610 (704)**	**EU140883 (704)**	**GQ996684 (704)**
	HM187608 (712)	*EU140884 (712)*	*HM187641 (712)*
	–	EU140885 (713)	713
Thuridilla hoffae	***GQ996618 (777)***	***EU140880 (777)***	***GQ996670 (777)***
Thuridilla hopei	***GQ996612 (808)***	***EU140882 (746)***	***GQ996677 (746)***
	GQ996613 (809)	***EU140881 (120)***	***GQ996678 (736)***
	839	–	–
Thuridilla kathae	***GQ996615 (707)***	***EU140879 (707)***	***GQ996676 (707)***
Thuridilla lineolata	***GQ996611 (715)***	***EU140887 (715)***	***GQ996682 (715)***
Thuridilla livida	–	*HM187607 (846)*	*HM187636 (846)*
	–	*DQ480211*	*DQ471276*
Thuridilla vatae	*HM187609 (780)*	*EU140888 (780)*	*HM187637 (780)*

LIMAPONTIOIDEA			
POLYBRANCHIIDAE			
Caliphylla mediterranea	**GU191020**	**GU191041**	–
Cyerce antillensis	**GQ996648 (155)**	**EU140841 (155)**	155
	815	815	815
	–	–	**GU191072**
Cyerce elegans	–	724	724
Cyerce nigra	–	853	853
	–	860	860
Cyerce nigricans	***GQ996644 (790)***	***EU140843 (790)***	***GQ996658 (790)***
	732		701
Cyerce sp. 1 (Wägele et al. 2006)	–	EU140845 (723)	723
Cyerce sp. 2 (Wägele et al. 2006)	**GQ996645 (721)**	**EU140844 (721)**	**GQ996659 (721)**
Cyerce sp. 3	–	848	848
(Gosliner et al. 2008)	–	859	859
Mourgona osumi	***GQ996646 (791)***	***EU140847 (791)***	***GQ996667 (791)***
Polybranchia orientalis	**GQ996647 (709)**	**EU140846 (709)**	**GQ996671 (709)**
	772	772	772
	–	S113	–
Polybranchia viridis	**GU191027**	**GU191052**	–
HERMAEIDAE			
Aplysiopsis enteromorphae	**GU191034**	**GU191053**	**GU191059**
Hermaea bifida	**GU191026**	**GU191043**	–
Hermaea cruciata	**GU191025**	**GU191042**	**GU191058**
COSTASIELLIDAE			
Costasiella kuroshimae	–	**DQ480215**	**DQ471252**
Costasiella cf.	**GQ996650 (804)**	804	–
kuroshimae LI	–	S105	S105
	–	S101	–
	–	S102	–
Costasiella nonatoi	**GQ996649 (814)**	**GU191037**	814
	818	818	818
	–	–	**GU191065**
Costasiella ocellifera	**GQ996651 (822)**	822	822
	–	820	820
	–	**DQ480216**	**DQ471253**
Costasiella sp. 1	–	864	864
(seaslugforum.net)	–	S196	–
Costasiella sp.	–	870	870
GUAM (black)	–	863	863

Limapontiidae			
Alderia modesta	**GU191030**	**DQ364417**	**DQ364309**
Alderia willowi	**GU191036**	**DQ364419**	**GU191074**
Calliopea bellula	AY427464	–	–
Ercolania "annelyleorum"	HQ380188 (787)	EU140839 (787)	HQ380195 (787)
Ercolania sp.	–	855	HQ380198 (855)
Ercolania boodleae	**GU191021**	**GU191050**	–
Ercolania felina	**GU191022**	**GU191038**	**GU191060**
Ercolania fuscata	**GU191023**	**GU191039**	**GU191061**
Ercolania kencolesi	***GQ996620 (788)***	***EU140840 (788)***	***GQ996660 (788)***
	GU191024 as sp.	**GU191051** as sp.	**GU191062** as sp.
Ercolania viridis	HQ380189 (857)	HQ380182 (857)	HQ380199 (857)
	S150	S150	S150
	–	844	–
Limapontia nigra	AY427465	–	–
Limapontia senestra	HQ380190 (834)	HQ380183 (834)	HQ380200 (834)
Placida cremoniana	HQ380191 (862)	HQ380184 (862)	HQ380201 (862)
Placida dendritica	***GQ996616 (768)***	***EU140871 (768)***	***GQ996663 (768)***
	GQ996617 (795)	**EU140870 (795)**	**GQ996662 (795)**
	–	EU140869 (793)	793
Placida kingstoni	**GU191028**	**GU191044**	**GU191063**
Placida verticillata	**GU191029**	**GU191045**	**GU191064**
	HQ380192 (831)	HQ380185 (831)	HQ380202 (831)
Stiliger ornatus	–	HQ380186 (843)	HQ380203 (843)
	–	HQ380187 (840)	HQ380204 (840)
	–	842	842
CEPHALASPIDEA (used as outgroup in the analysis of phylogeny of Sacoglossa)			
HAMINOEOIDEA			
Haminoea hydatis	AY427468	EF489323	DQ238004
DIAPHANOIDEA			
Diaphana sp.	EF489373	EF489325	EF489394
PHILINOIDEA			
Scaphander lignarius	EF489372	DQ974663	EF489324

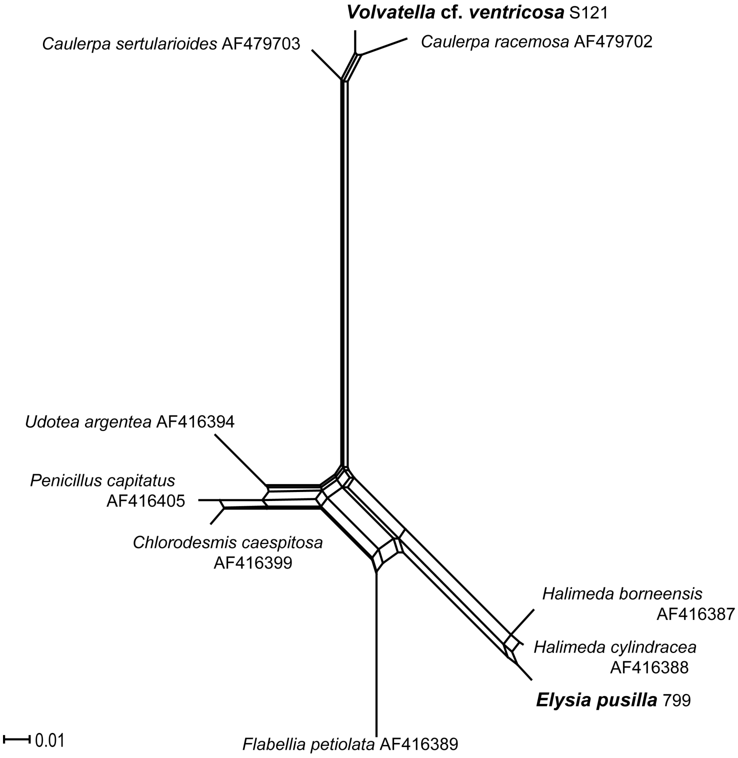


Figure 6.1 Neighbornet analysis of 28S rDNA (489bp) of sequences obtained from DNA extracted from sacoglossans and Ulvophyceae. Sequences obtained from sacoglossans are printed in bold type. Ulvophycean sequences were taken from GenBank; accession no. are given.

Table 6.4 Uncorrected p-distances of 16S rDNA (top right) and *coxI* (bottom left) sequences of Oxynoacea. Distances in a grey box indicate distances within genus level. Distances calculated with PAUP 4.0b.10 lower and equal to 0.01 are written in white. Distances are rounded to two decimal points.

	Cylindrobulla beauii	*Cylindrobulla* sp.	*Ascobulla* sp.	*Ascobulla fragilis*	*Volvatella viris* 1	*Volvatella viris* 2	*V.* cf. *viridis*	*Julia exquisita* 1	*Julia exquisita* 2	*Tamanovalva limax*	*Lobiger viridis* 1	*Lobiger viridis* 2	*Oxynoe antillarum* 1	*Oxynoe antillarum* 2	*O. azuropunctata*	*Oxynoe viridis* 1	*Oxynoe viridis* 2
C. beauii		0.15	0.16	0.18	0.18	0.17	0.18	0.18	0.19	0.21	–	–	–	–	–	0.23	–
Cylindrobulla sp.	0.12		0.16	0.19	0.15	0.18	0.18	0.17	0.17	0.19	–	–	–	–	–	0.20	–
Ascobulla sp.	–	–		0.17	0.18	0.18	0.18	0.17	0.17	0.19	–	–	–	–	–	0.21	–
A. fragilis	0.23	0.22	–		0.17	0.17	0.18	0.17	0.17	0.17	–	–	–	–	–	0.22	–
V. viris 1	0.18	0.18	–	0.19		0.15	0.15	0.17	0.17	0.18	–	–	–	–	–	0.24	–
V. viris 2	0.21	0.17	–	0.19	0.11		0.00	0.19	0.19	0.20	–	–	–	–	–	0.23	–
V. cf. *viridis*	0.21	0.17	–	0.19	0.10	0.00		0.20	0.20	0.20	–	–	–	–	–	0.23	–
J. exquisita 1	0.21	0.15	–	0.19	0.14	0.15	0.15		0.01	0.18	–	–	–	–	–	0.22	–
J. exquisita 2	0.21	0.15	–	0.19	0.14	0.15	0.15	0.00		0.18	–	–	–	–	–	0.22	–
T. limax	0.21	0.20	–	0.24	0.23	0.23	0.23	0.19	0.19		–	–	–	–	–	0.21	–
L. viridis 1	0.20	0.19	–	0.24	0.18	0.19	0.19	0.16	0.16	0.24		–	–	–	–	–	–
L. viridis 2	0.20	0.19	–	0.24	0.18	0.19	0.19	0.16	0.16	0.24	0.01		–	–	–	–	–
O. antillarum 1	0.20	0.17	–	0.24	0.20	0.20	0.19	0.16	0.16	0.24	0.18	0.18		–	–	–	–
O. antillarum 2	0.21	0.18	–	0.24	0.19	0.20	0.20	0.15	0.16	0.24	0.18	0.18	0.01		–	–	–
O. azuropunctata	0.20	0.17	–	0.24	0.19	0.19	0.19	0.16	0.16	0.24	0.18	0.18	0.01	0.01		–	–
O. viridis 1	0.19	0.18	–	0.23	0.21	0.20	0.21	0.18	0.18	0.21	0.19	0.20	0.12	0.12	0.12		–
O. viridis 2	0.19	0.18	–	0.23	0.20	0.20	0.20	0.18	0.18	0.21	0.19	0.20	0.11	0.12	0.12	0.00	

Table 6.5 Food sources of Sacoglossa that do not belong to Ulvophyceae. Exotic food sources can only be found in Plakobranchacea. Data are taken from Händeler & Wägele (2007), Swennen (2007) and Teugels et al. (2008). Xanthophyceae, Phaeophyceae, Bacillariophyta and Eustigmatales are groups of Stramenopiles, which combines algae whose motile cells are typically biflagellate with a heterokont flagellation (Adl et al. 2005).

Species	Food	Higher taxon	Ulvophycean food sources
Plakobranchacea			
PLAKOBRANCHOIDEA			
Plakobranchidae			
Elysia catulus	*Zostera marina*	Angiospermae	–
Elysia chlorotica	*Vaucheria*	Xanthophyceae	*Cladophora* *Chaetomorpha*
Elysia crispata	*Vaucheria*	Xanthophyceae	several (table 4.1)
Elysia diomedea	*Padina*	Phaeophyceae	–
Elysia evelinae	*Biddulphia*	Bacillariophyta	–
Elysia patagonica	veliger: *Nanochloropsis*	Eustigmatales	adults: *Codium* *Bryopsis*
Elysia serca	*Halodule wrightii* *Halophila engelmanni* *Thalassia testudinum* *Syringodium filiforme*	Angiospermae	*Ulva lactuca*
Elysia viridis	*Dasysiphonia* sp.	Rhodophyceae	*Codium* *Bryopsis* *Cladophora* *Chaetomorpha*
LIMAPONTIOIDEA			
Costasiellidae			
Costasiella coronata	*Vaucheria*	Xanthophyceae	–
Costasiella pallida	*Vaucheria*	Xanthophyceae	–
Limapontiidae			
Alderia modesta	*Vaucheria*	Xanthophyceae	*Rhizclonium*
Alderia willowi	*Vaucheria*	Xanthophyceae	–
Calliopea oophaga	opisthobranchiate eggs		–
Calliopea oophaga	opisthobranchiate eggs		–
Limapontia depressa	*Vaucheria* *Conferva*	Xanthophyceae Bacillariophyta	*Rhizoclonium*
Olea hansineensis	opisthobranchiate eggs		–
Stiliger fuscovittatus	*Polysiphonia*	Rhodophyceae	–
Stiliger vesiculosus	eggs of *Favorinus branchialis*		–
Hermaeidae			
Hermaea spp.	*Griffithsia, Delesseria, Heterosiphonia Bornetia, Dasya*	Rhodophyceae	–
Hermaea vancouverensis	*Isthmia*	Bacillariophyta	–

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