Evolution of chloroplast sequestration in Sacoglossa (Mollusca, Gastropoda)

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Bonn, den 23.01.2014

CONTENT

Preface

For almost four years I am studying functional kleptoplasty in Sacoglossa and I am still getting more fascinated by this research field day by day. In the last months, parts of the work generated some media interest showing that this topic is made of "more than just slimy slugs".

I want specifically thank my supervisor Prof. Dr. Heike Wägele for giving me the chance to work on Sacoglossa and all the trust and help she gave and still gives me. The whole project would never have been realized without her ideas and particularly helpful discussions about experiments.

In this line I want to thank Prof. William F. Martin and Dr. Sven B. Gould of the University of Düsseldorf for their immense help with certain projects and an extraordinary cooperation that improved the project considerably. Many thanks also to Jan de Vries from the University of Düsseldorf, with whom I spent a successful and funny field trip on the Florida Keys.

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I want to thank my family and especially my mother and my sister for believing in me and for being there for me.

Ultimately, I want to thank my dearly beloved wife Sarah for giving me patience, strength and love. There are no words that cover her meaning for me.

Summary

Sacoglossa (Gastropoda, Panpulmonata) is a small taxon of marine gastropods with about 300 described species. It consists of two major lineages: the shelled Oxynoacea and the shell-less Plakobranchacea. The latter is sub-divided into the cerata-bearing Limapontioidea and the Plakobranchoidea characterized by so-called parapodia. Despite recent efforts phylogenetic relationships in Sacoglossa are not clarified thoroughly. Sacoglossa are mainly famous for their ability to incorporate functional kleptoplasts of their algae prey and to endure prolonged starvation periods with the help of the still photosynthesizing plastids, but the role of the food source to establish this peculiar relationship and how the slugs benefit from the sequestered plastids is uncertain.

In this study the phylogenetic relationships of families in Sacoglossa, based on four genes (*28S*, *H3*, *COI*, *16S*), was analyzed. The monophyletic Oxynoacea is sister to the Plakobranchacea. In Plakobranchacea the Platyhedylidae is the first offshoot and the Limapontiodea as well as the Plakobranchoidea appear paraphyletic, although morphological data support their monophyly. Limapontiidae, Costasiellidae and Hermaeidae are monophyletic, unlike the paraphyletic Polybranchiidae, where *Cyerce* is separated from the remaining taxa. Sister to the Plakobranchoidea is the Polybranchiidae without *Cyerce*. The monophyletic Boselliidae is sister to *Plakobranchus* and *Thuridilla* and together form the sister-clade to *Elysia*.

The ability to incorporate functional plastids also occurs in the genus *Costasiella*, the only Limapontiodea known for it so far. This affects the estimation of the evolution of functional kleptoplasty considerably. Currently two independent origins are the most likely scenario. A total of six species are identified to endure starvation periods for more than 20 days while keeping the plastids photosynthetically active, though probably there are more so-called long-term-retention forms that still need further investigation.

The molecular analysis of food sources was shown to be particularly important to clearly identify plastid origin. It provided evidence that plastids from certain algae are needed to establish functional kleptoplasty. However, our results also show that unknown abilities of

the slug are necessary as well, since non-retention forms also feed upon particular algae identified in long-term retention forms.

Contrary to previous analyses, the slugs are not photoautotrophic, though harboring a photosynthetically active organelle. Starving experiments under non-photosynthetic conditions indicate that the reason of the survival of starvation periods may be that the plastids rather serve as food storage than as organelles for solar energy.

The results presented here were in some way surprising and therefore a change of the focus on the work of Sacoglossa with regard to functional kleptoplasty has become necessary. Plastid and slug physiology, as well as differences between non-retention and long-term-retention forms need to be investigated. The phenomenon of functional kleptoplasty remains fascinating and becomes partly even more challenging, which is why it deserves further detailed studies.

Chapter 1

General Introduction

Symbiosis, the interaction of different species either in a mutualistic, commensalistic or parasitic relationship, is fairly common in nature and was first introduced by de Bary (1879). The interaction may be obligate: one symbiont is not able to live without the other, as in the probably most prominent symbiotic relationship: the lichens (Honegger, 1998). Often the symbiotic relationship is established with at least one photosynthetic partner and is widespread among poriferans, cnidarians and molluscs, for example (Trench, 1979; Smith & Douglas, 1987; Venn, Loram & Douglas, 2008; Wägele et al., 2010a). An exceptional relationship between animals and their food sources is found amongst a certain group of gastropods: the Sacoglossa (Mollusca, Panpulmonata). Here, not the whole algae is the symbiont, but only the plastids from the food algae, sequestered within cells of the digestive glands and still photosynthesizing even after weeks of starvation of the slugs (Trench, 1969; Hinde & Smith, 1974; Trench, 1975; Rumpho et al., 2001; Händeler et al., 2009). However, the underlying system is not an interaction of different organisms. That is why the term "symbiosis" does not seem to be appropriate, so that a new term for this particular relationship was established: functional kleptoplasty (Waugh & Clark, 1986; Clark, Jensen & Stirts, 1990; Rumpho et al., 2006). Functional kleptoplasty is also found elsewhere among heterotrophic organisms: in Foraminifera (Lee, 2006), Ciliophora (McManus, 2012) and Dinoflagellata (Gast et al., 2007). However, the Sacoglossa is the only metazoan taxon known for this phenomenon to date. The thesis presented here tries to contribute in our understanding, what is so special about the Sacoglossa, what is functional kleptoplasty in particular and how the slugs benefit from these stolen organelles.

TAXONOMY AND PHYLOGENY

Sacoglossa is a worldwide distributed taxon comprising of small marine gastropods (size between 0.2 and 6 cm) occurring in the intertidal flat to depths of several meters in tropical and temperate waters (Jensen, 2007). The name of the order Sacoglossa was derived by von Ihering (1879) because of an autapomorphic structure called 'ascus' or 'saccus'. Within this particular structure, previous used teeth of the highly specialized radula are stored for their whole lifetime and, contrary to other Gastropoda taxa, not re-mineralized (Bergh,

1878; Jensen, 1997). The radula is uniserate, with single teeth formed in the so-called upper-limb region that is ascending dorsal, followed by the forward-facing leading tooth and then the lower-limb region that is descending ventrally. The lower limb is dominated by used teeth and ends up in the ascus [Gascoigne, Sartory & Lemche, 1974; Jensen, 1996; 1997), Fig. 1A]. With the leading tooth the Sacoglossa feed on their algae prey by using it like a saw that cuts a slit in the cell wall of the algae. This procedure is done quickly and subsequently the slugs are sucking out the cell content of the algae with the help of the muscles of the buccal apparatus (Jensen, 1981). The nutrients and organelles are then phagocytized in cells of the digestive gland [see review of (Rumpho et al., 2011)]. Normally, three different radula types are distinguished: blade-shaped teeth, teeth with triangular, denticulate cusps and sabot-shaped teeth [(Jensen, 1997); Fig. 1 B-D]. Evidences that the tooth shape is somehow correlated to the food sources (Jensen, 1993) could so far not be confirmed (Händeler & Wägele, 2007).

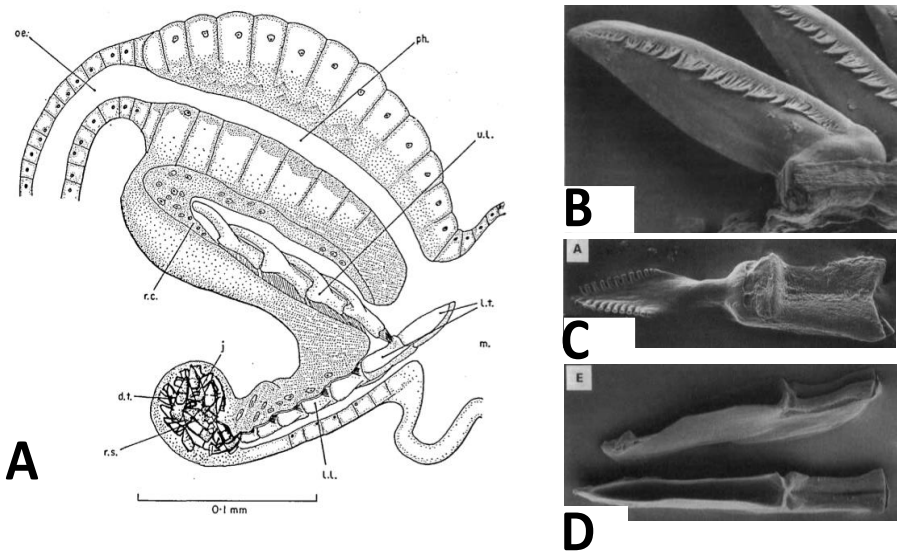

Figure 1 Anatomy of the buccal apparatus and overview of radula forms. **A** Median longitudinal section of the buccal apparatus of *Limapontia depressa* taken from (Gascoigne et al., 1974). d.t. discarded teeth, j. juvenile tooth, l.t. leading tooth, m. mouth, o.e. oesophagus, p.h. pharynx, r.c. radular caecum, r.s. radular sac (ascus), u.l. upper limb **B** Blade shaped teeth of *Oxynoe antillarum* **C** Triangular tooth with lateral denticles of *Cyerce antillensis* **D** Sabot-shaped teeth of *Ercolania* [Pictures B-D after (Jensen, 1993)].

To date about 400 sacoglossan species are known, but only 300 are considered valid [(Jensen, 2007); Tab. 1]. Three major taxa are distinguished: the shell-bearing Oxynoacea, the Limapontioidea, that posses dorsal appendices (cerata) and the Plakobranchoidea, with lateral modifications of the food (parapodia) [(Jensen, 1997; Kohnert et al., 2013); Fig. 2-4, Tab. 1].

Table 1 List of sacoglossan genera according to (Jensen, 2007; Händeler et al., 2009; Krug et al., 2013). # indicates the number of valid taxa.

Order		Family	Genus	#
Oxynoacea		Cylindrobullidae	*Cylindrobulla*	5
		Volvatellidea	*Volvatella*	16
			Ascobulla	7
		Juliidae	*Julia*	6
			Tamanovalva	3
			Berthelinia	
		Oxynoidae	*Lobiger*	5
			Lophopleurella	1
			Oxynoe	8
			Roburnella	1
Plakobranchacea	Plakobranchoidea	Boselliidae	*Bosellia*	5
		Plakobranchidae	*Elysia*	85
			Elysiobranchus	2
			Plattyclaya	2
			Plakobranchus	5-10
			Thuridilla	24
		Platyhedylidae	*Gascoignella*	3
			Platyhedyle	1
	Limapontioidea	Polybranchiidae	*Caliphylla*	1
			Cyerce	12
			Mourgona	3
			Polybranchia	8
			Sogenia	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

From a morphological point of view, the shell bearing Oxynoacea are the most basal Sacoglossa with currently 10 genera recognised and presenting about 20% of the described sacoglossan species [(Jensen, 1997; Händeler et al., 2009), Tab. 1]. In former phylogenetic analyses the affinity of the genus *Cylindrobulla* (as a member of the Oxynoacea or as sister taxon to the Sacoglossa) was handled differently and, in the latter case, often used as outgroup [(Clark, 1994; Jensen, 1996; Händeler & Wägele, 2007; Händeler et al., 2009), Fig. 2A, 3]. This uncertainty was mainly based on the unknown relationship of the Sacoglossa within Heterobranchia, so that an adequate outgroup was hard to define (Wägele & Klussmann-Kolb, 2005; Klussmann-Kolb et al., 2008; Dinapoli & Klussmann-Kolb, 2010). Only recently, the so-called Opisthobranchia, to which the Sacoglossa were assigned in former times, proofed to be paraphyletic (Schrödl, Jörger & Wilson, 2011). Newer phylogenetic studies now clearly demonstrate that the Sacoglossa belong to the Panpulmonata with *Siphonaria* as sister taxon (Jörger et al., 2010; Neusser et al., 2011; Kocot, Halanych & Krug, 2013). Morphological analyses then showed that species of *Cylindrobulla* have an ascus (Lätz et al. 2013, unpublished) and molecular phylogenetic studies also clearly show that *Cylindrobulla* is a member of the Oxynoacea (Jörger et al., 2010; Maeda et al., 2010; Neusser et al., 2011). For these reasons *Cylindrobulla* is treated in this thesis as a member of the Oxynoacea.

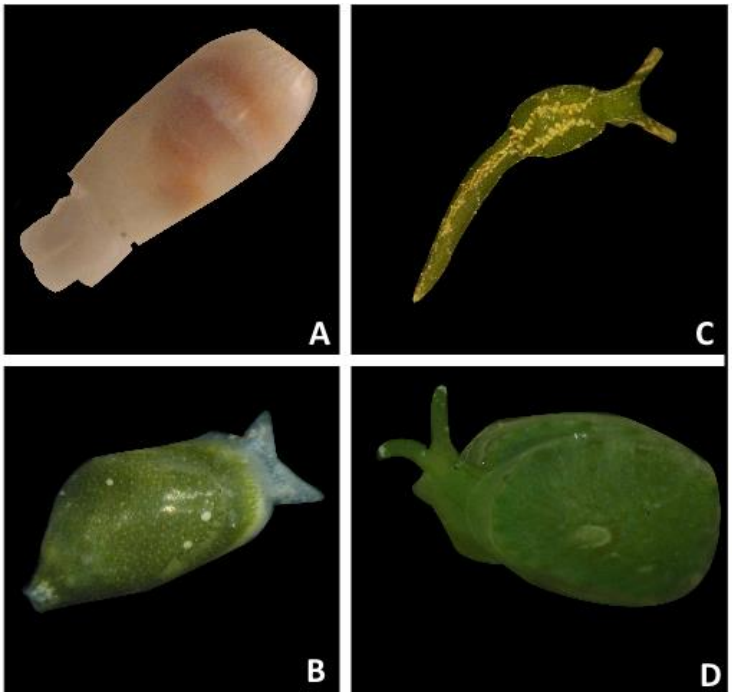

Figure 2 Representative species of Oxynoacea. **A** *Cylindrobulla* "schuppi", **B** *Volvatella viridis,* **C** *Oxynoe antillarum* **D** *Berthelinina limax*

In *Cylindrobulla* and *Ascobulla* specimens are able to retract completely into the cylindrical shell. Both, the shell and the body plan of these two genera, are the most basal found within the Sacoglossa (Jensen, 1983; 1997). It is therefore surprising that molecular analysis do not group them together within the Oxynoacea as the most basal taxa [(Jörger et al., 2010; Neusser et al., 2011; Maeda et al., 2012) Fig. 3B]. In contrast, members of the Volvatellidae and the Oxynoidae bear a reduced shell and are not able to retract into it. The shell of the latter is additional covered by parapodia, whereas in *Lobiger* four parapodia surround the shell. Members of the Juliidae are characterised by a bivalved shell, a feature unique within gastropods. Its ontogenesis is not understood to date, but it is suggested that the formation differs strongly from that of Bivalvia. Intriguingly, in molecular analyses the Juliidae appear paraphyletic, although monophyletic in morphological analyses [(Jensen, 1996; Händeler et al., 2009; Maeda et al., 2012) Fig. 3].

The sister taxon to the Oxynoacea is the Plakobranchacea. The latter consists of the Limapontioidea (Fig. 4) and the Plakobranchoidea (Fig. 5). The Limapontiodea is the genus-richest taxon within the Sacoglossa (about 17 genera described, Tab. 1), and is

mainly characterized by their dorsal appendices in which the digestive glands sometimes branches (Fig. 4).

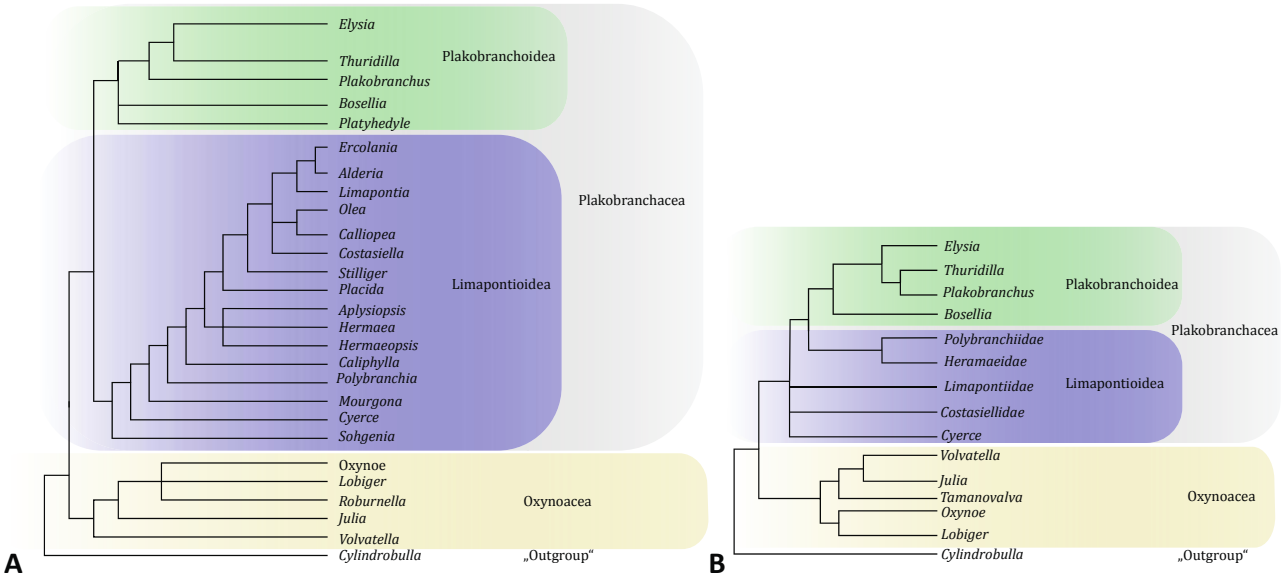

Figure 3 Phylogeny of Sacoglossa. **A** Morphological phylogenetic reconstruction after (Jensen, 1996) **B** Molecular phylogenetic reconstruction after (Händeler, 2011). In both phylogenies *Cylindrobulla* was used as outgroup. Based on newer findings *Cylindrobulla* is however a member of the Oxynoacea. In (Händeler, 2011) the Platyhedylidea were not included.

Regardless of the monophyly of the Limapontioidea based on morphology (Jensen, 1996), molecular phylogenetic analysis always revealed a paraphyletic relationship (Händeler et al., 2009; Maeda et al., 2010). Furthermore the relationship of Limapontiodea and Plakobranchoidea is not satisfactorily resolved [(Jörger et al., 2010; Händeler, 2011; Neusser et al., 2011; Maeda et al., 2012), Fig. 3]. The Platyhedylidea, composed of worm-like mesopsammic species *Gascoignella* and *Platyhedyle,* appear as sister taxon to all the other Plakobranchacea in molecular analysis (Jörger et al., 2010; Neusser et al., 2011) although morphological analysis placed them within the Plakobranchoidea [(Jensen, 1996), Fig. 3A]. Further analyses are needed to clarify the position of this group, which probably exhibit a highly derived morphology. Their basal position within Plakobranchacea in phylogenetic analysis (Jörger et al., 2010; Neusser et al., 2011) may be caused by long-branch effects based on highly derived genes (Kück et al., 2012).

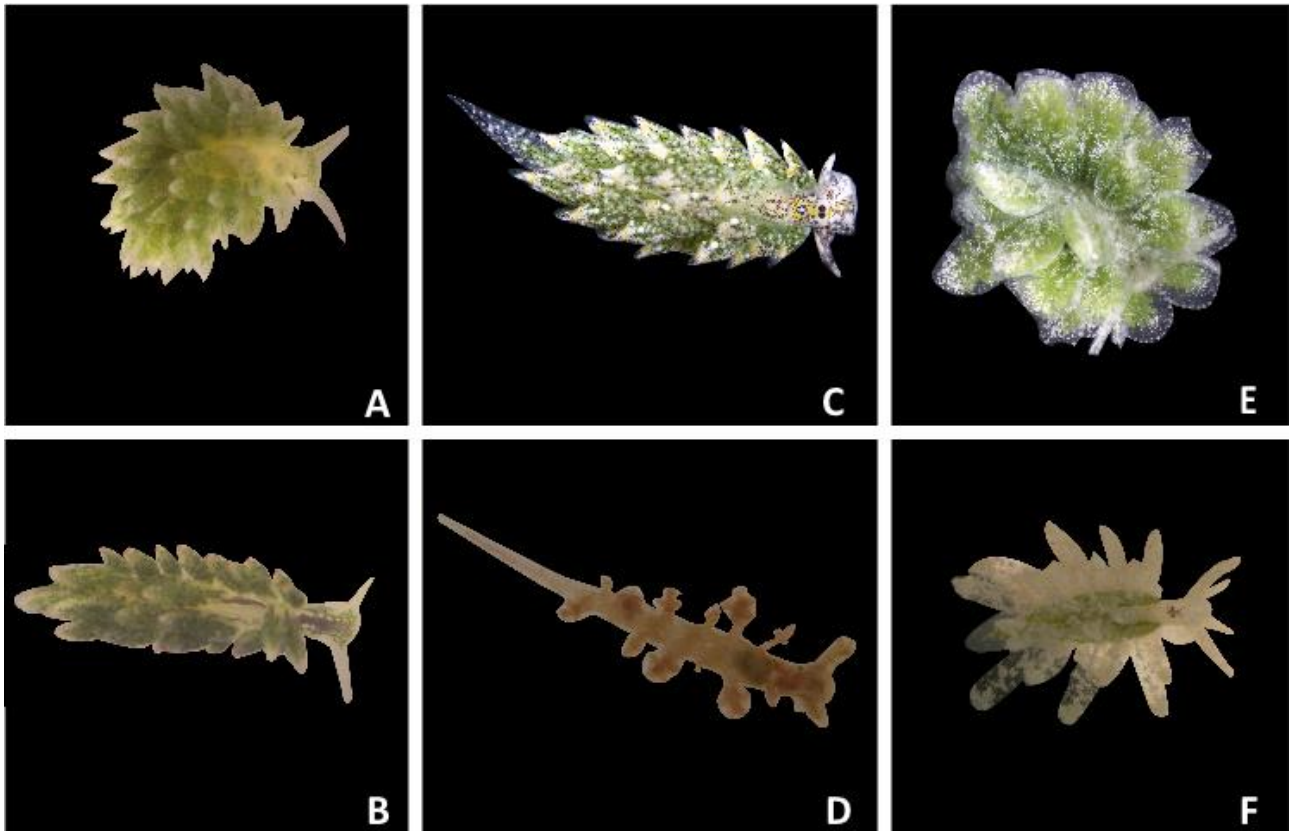

Figure 4 Representative species of Limapontioidea. **A** *Placida* sp., **B** *Ercolania* sp., **C** *Costasiella ocellifera*, **D** *Hermaea bifida*, **E** *Mourgona germaineae*, **F** *Cyerce antillensis*.

The Plakobranchoidea are the most derived taxon and consists of eight genera, but includes with the genus *Elysia* the species richest taxon of all Sacoglossa (about 85 species known) [(Jensen, 1996; 1997; Händeler et al., 2009), Tab. 1, Fig. 5]. In general, Plakobranchoidea are characterised by parapodia, although in *Bosellia* these parapodia are highly reduced or completely absent (Fig. 5A). *Bosellia* is placed at the base of the Plakobranchoidea as sister taxon to the remaining plakobranchoida in molecular analyses, but in morphological analysis its position was not resolved so far [(Jensen, 1996; Händeler et al., 2009); Fig. 3]. In molecular analyses the genera *Thuridilla* and *Plakobranchus* form a monophyletic sister clade to *Elysia* (Händeler et al., 2009), however morphological examinations placed *Thuridilla* as sister taxon to *Elysia* (Jensen, 1996). Above all, the Plakobranchoidea are famous for their ability to incorporate functional plastids of their algae food sources.

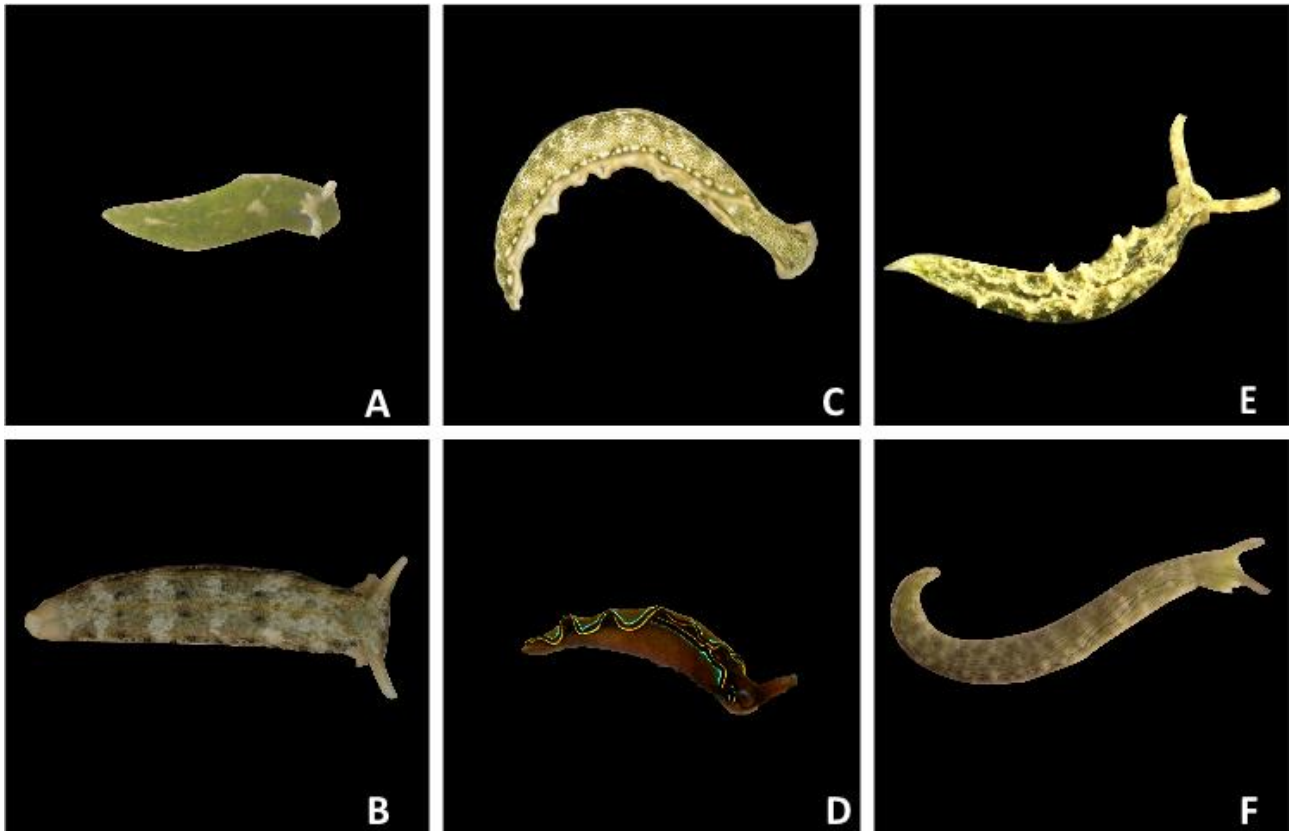

Figure 5 Representative species of Plakobranchoidea. **A** *Bosellia marcusi*, **B** *Plakobranchus ocellatus*, **C** *Thuridilla kathae*, **D** *Thuridilla livida*, **E** *Elysia patina*, **F** *Elysia pratensis*.

FUNCTIONAL KLEPTOPLASTY WITHIN SACOGLOSSA

From "Of leaves that crawl" (Trench, 1975) till "solar-powered" (Rumpho, Summer & Manhart, 2000; Wägele et al., 2010a): Sacoglossa fascinated scientists over decades because of their ability to incorporate plastids and keep them functional. The first report on this phenomenon mentioned green pigments in sea slugs (de Negri & de Negri, 1876) which were later thought to be zooxanthellae (Yonge, 1940). It was Kawaguti (1965) who first identified these as algal plastids by performing electron microscopical studies (Kawaguti & Yamasu, 1965). Subsequent studies examined the capabilities of plastids within the slugs and their importance in surviving starvation periods (Trench, 1969; Trench, Greene & Bystrom, 1969; Greene, 1970; Greene & Muscatine, 1972; Hinde & Smith, 1972; Trench, trench & Muscatine, 1972; Muscatine & Greene, 1973; Trench & Gooday, 1973; Hinde & Smith, 1974; Trench, Boyle & Smith, 1974; Hinde & Smith, 1975; Trench, 1975; Hinde, 1978; Clark et al., 1981; Evertsen et al., 2007; Giménez-Casalduero & Muniain, 2008; Evertsen & Johnsen, 2009; Klochkova et al., 2012; Yamamoto et al., 2012).

Since more than 90% of proteins needed for photosynthesis are nuclear encoded (Timmis et al. 2004), the hypothesis of a lateral gene transfer (LGT) from the algal nucleus to the slugs' genome was accepted to support plastid longevity. For almost 20 years this hypothesis was followed, although evidence for a LGT was low and mainly based on a handful of algal genes "identified" by single gene PCR approaches (Mujer et al., 1996; Pierce, Biron & Rumpho, 1996; Hanten & Pierce, 2001; Rumpho et al., 2001; Pierce et al., 2003; Rumpho et al., 2008; Pierce, Curtis & Schwartz, 2010). Rumpho et al. (2008), for example, found sequences of *PsbO* via PCR amplification within *Elysia chlorotica*, which were identical with sequences obtained from *Vaucheria litorea*, its heterokontophyte food source and considered this as proof for a LGT. Fundamentals, such as protein targeting were not addressed but in particular these processes are crucial for providing the plastid with the appropriate proteins to maintain photsynhtesis [see (Wägele et al., 2011)]. However, recent transcriptomic and genomic studies clearly demonstrate that no genes were transferred from the algal nucleus into the slugs' genome (Pelletreau et al., 2011; Wägele et al., 2011; Bhattacharya et al., 2013). What actually enables plastid longevity and herein functional kleptoplasty is still not known.

The identification of functional kleptoplasty in Sacoglossa is to date mainly based on CO_2 fixation experiments and on measurements of the chlorophyll a fluorescence by a Pulse amplitude modulated fluorometer (PAM) (Greene & Muscatine, 1972; Hinde & Smith, 1972; 1974; Kremer & Schmitz, 1976; Clark et al., 1981; 1990; Giménez-Casalduero & Muniain, 2006; Evertsen et al., 2007; Evertsen & Johnsen, 2009; Händeler et al., 2009; Yamamoto et al., 2009; Klochkova et al., 2010; Middlebrooks, Pierce & Bell, 2011; Schmitt & Wägele, 2011; Klochkova et al., 2012; Christa et al., 2014). PAM-measurements provide information of the activity of photosystem II and the use of absorbed light energy, displayed as the maximum quantum yield value (F_v/F_m) [reviewed in (Cruz et al., 2013)]. It has the benefit of allowing the observation of individuals over a certain starvation period with continuous examination of the condition of the photosystem II [(Cruz et al., 2013) Fig. 5]. However, while the PSII is still active, net CO_2 fixation may cease. On the other hand, CO_2 fixation experiments have the drawback that individuals have to be fixed at a distinct point and an examination over a certain starvation period of an individual is not possible. Nevertheless, the results give an excellent impression of how capable the plastids still are

(or not) in regard to phototrophic CO_2 fixation over a certain starvation period (Greene & Muscatine, 1972; Hinde & Smith, 1972; Clark et al., 1981). Thus the ideal situation would be a combination of both methods, but its realisation is limited. This is due to the fact that the amount of individuals necessary for these investigations is hard to collect, since some species are very rare. Therefore the non-invasive technique of PAM-measurements is favoured, though it might be less accurate under certain circumstances.

The classification of functional kleptoplasty used by most authors is based on PAM-measurements and subdivided into three categories (Händeler et al., 2009): (i) no-retention with no F_v/F_m values, (ii) short-term-retention with F_v/F_m >0.4 for at least one day during starvation and (iii) long-term retention with F_v/F_m >0.5 for at least two weeks during starvation. Based on this classification all Oxynoacea are non-retention forms and in Limapontioidea only for the genus *Costasiella* evidences exist that functional kleptoplasty occurs (Clark et al., 1981; Händeler et al., 2009). Nearly all so far examined members of the Plakobranchoidea are retention forms, with only a few non-retention forms. Until the beginning of this thesis 45 species were investigated with regard to their functional kleptoplasty retention and only five species are identified as long-term-retention forms (Table 2).

Table 2 Classification of the retention form of Sacoglossa based on chlorophyll a fluorescence measurements by using a PAM. The classification in non-retention (NR), short-term-retention (StR) and long-term-retention (LtR) is based on Händeler et al. (2009). The fifth LtR form, *Elysia chlorotica*, was so far never analysed by PAM measurements and is therefore not included in this table.

Taxon	Species	Retention form	Method	Reference
Oxynoacea	*Cylindrobulla* "schuppi"	**NR**	PAM	(Händeler, 2011)
	Oxynoe viridis	**NR**	PAM	(Händeler et al., 2009)
	Oxynoe antillarum	**NR**	PAM	(Händeler et al., 2009)
	Julia exquisita	**NR**	PAM	(Händeler et al., 2009)
	Lobiger viridis	**NR**	PAM	(Händeler et al., 2009)
	Volvatella viridis	**NR**	PAM	(Händeler et al., 2009)
Limapontioidea	*Costasiella* sp. 863	**NR**	PAM	(Händeler, 2011)
	Costasiella sp. 864	**StR**	PAM	(Händeler, 2011)
	Cyerce nigra	**NR**	PAM	(Händeler, 2011)

	Cyerce nigricans	**NR**	PAM	(Händeler et al., 2009)
	Placida dendritica	**NR**	PAM	(Händeler et al., 2009)
	Placida sp.	**NR**	PAM	(Yamamoto et al., 2009)
	Stilligher berghi	**NR**	PAM	(Klochkova et al., 2010)
	Ercolania annelyleorum	**NR**	PAM	(Wägele et al., 2010b)
	Ercolania boodle	**NR**	PAM	(Klochkova et al., 2010)
	Ercolania kencolesi	**NR**	PAM	(Händeler et al., 2009)
	Ercolania viridis	**NR**	PAM	(Händeler, 2011)
Plakobranchoidea	*Bosellia mimetica*	**StR**	PAM	(Händeler et al., 2009)
	Bosellia sp.	**StR**	PAM	(Yamamoto et al., 2009)
	Elysia artroviridis	**StR**	PAM	(Klochkova et al., 2010)
	Elysia asbecki	**StR**	PAM	(Wägele et al., 2010b)
	Elysia benettae	**StR**	PAM	(Händeler et al., 2009)
	Elysia clarki	**LtR**	PAM	(Middlebrooks et al., 2011)
	Elysia cornigera	**StR**	PAM	(Händeler et al., 2009)
	Elysia crispata	**LtR**	PAM	(Händeler et al., 2009)
	Elysia nicrocapitata	**StR**	PAM	(Klochkova et al., 2012)
	Elysia ornata	**StR**	PAM	(Händeler et al., 2009)
	Elysia pusilla	**StR**	PAM	(Händeler et al., 2009)
	Elysia sp. 841	**StR**	PAM	(Händeler, 2011)
	Elysia sp. 865	**StR**	PAM	(Händeler, 2011)
	Elysia sp. 871	**StR**	PAM	(Händeler, 2011)
	Elysia subornata	**NR**	PAM	(Händeler, 2011)
	Elysia timida	**LtR**	PAM	(Händeler et al., 2009)
	Elysia tomentosa	**StR**	PAM	(Händeler et al., 2009)
	Elysia trisinuata	**StR**	PAM	(Yamamoto et al., 2009)
	Elysia viridis	**StR**	PAM	(Evertsen & Johnsen, 2009)
	Plakobranchus ocellatus	**LtR**	PAM	(Händeler et al., 2009)
	Thuridilla bayeri	**StR**	PAM	(Händeler, 2011)
	Thuridilla carlsoni	**StR**	PAM	(Händeler et al., 2009)
	Thuridilla gracilis	**StR**	PAM	(Händeler et al., 2009)
	Thuridilla hopei	**StR**	PAM	(Händeler et al., 2009)
	Thuridilla kathae	**StR**	PAM	(Händeler et al., 2009)
	Thuridilla lineolata	**StR**	PAM	(Evertsen et al., 2007)
	Thuridilla livida	**StR**	PAM	(Händeler, 2011)
	Thuridilla vatae	**StR**	PAM	(Yamamoto et al., 2009)

The determination of the diversity of functional kleptoplasty within Sacoglossa is an important issue to understand the evolution of functional kleptoplasty: Händeler et al. (2009) discussed that at the stemline of the Plakobranchoidea functional kleptoplasty evolved, whereas Maeda et al. (2010) hypothesized its evolution at the basis of the Plakobranchacea. While the latter included *Costasiella* as functional kleptoplastic genus, this genus was not considered in the analysis of the former. A re-evaluation of the dataset of Händeler et al. (2009) by including *Costasiella* and an enlarged study with higher taxon sampling is compulsory needed to determine an exact origin and the distribution of functional kleptoplasty within Sacoglossa.

Despite the evolution of functional kleptoplasty, the evolutionary benefits for plastid retention are not obvious at all. As Maeda et al. (2012) showed, the long-term-retention form *Plakobranchus ocellatus* acquires continuously fresh plastids from different food sources instead of relying on a single plastid source and starve when its not available. Moreover, animals are not able to maintain their biomass under starvation periods [(Giménez-Casalduero & Muniain, 2008; Klochkova et al., 2012; Yamamoto et al., 2012), see below, Fig. 7], what makes it more difficult to verify this as an evolutionary benefit. It therefore seems more conceivable that a polyphagous feeding strategy would provide a higher benefit than relying on sequestered plastids during starvation.

FOOD SOURCES OF SACOGLOSSA

Sacoglossa mainly feed on algae of the order Ulvophyceae sensu Floyd und O'Kelly (Jensen, 1980; Floyd & O'Kelly, 1990; Jensen, 1997; Williams & Walker, 1999; Curtis et al., 2005; Curtis, Massey & Pierce, 2006; Pierce et al., 2006; Händeler & Wägele, 2007; Yamamoto et al., 2009; Händeler et al., 2010; Klochkova et al., 2010; Maeda et al., 2012). This morphological highly diverse group consists of siphonal organised algae like the Bryopsidales or Dasycladales as well as siphonocladal organised algae like the Cladophorales and cellular organised members (Ulvales) [Van den Hoek, 1995; Vroom, Smith & Keeley, 1998; Verbruggen et al., 2009; Cocquyt et al., 2010; Sauvage et al., 2013), Fig. 6]. The methods to identify these food sources changed considerable within the past years. In former times, food sources were determined by feeding observations, feeding experiments or by electron microscopic approaches (Kawaguti & Yamasu, 1965; Jensen,

1980; 1981; Trowbridge, 1991; Williams & Walker, 1999; Curtis et al., 2005; Hirose, 2005). However, these methods are very time consuming and unreliable since the differentiation between feeding and only sitting on an alga is prone for errors. Furthermore, if species are polyphagous, not every food algae may be covered by such experiments, especially when the food item is not collected during field trips.

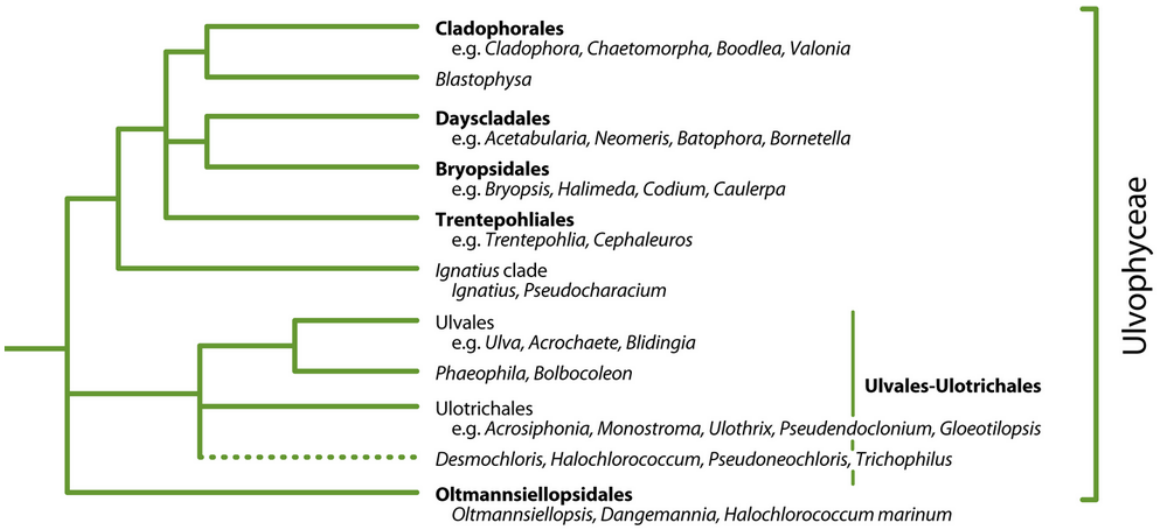

Figure 6 Phylogeny of Ulvophycea taken from (Leliaert et al., 2012).

A very rapid and more precise method is the DNA-barcoding that proofed to be a promising tool in an unambiguous identification of food sources, even without collecting of potential food algae, especially in polyphagous species (Curtis et al., 2006; Pierce et al., 2006; Händeler et al., 2010; Wägele et al., 2011; Maeda et al., 2012). In plant biodiversity research the application of plastid encoded markers *matK*, *tufA* and *rbcL* are state of the art in identifying species by DNA-barcoding (Hollingsworth, Graham & Little, 2011). Unfortunately, contrary to land plants the *matK* gene is not found in the Ulvophyceae plastid genome (Pombert et al., 2006). Yet, *rbcL* and *tufA* are successfully used to identify plastids in algae and in Sacoglossa (Curtis et al., 2006; Pierce et al., 2006; Händeler et al., 2010; Saunders & Kucera, 2010; Wägele et al., 2011; Maeda et al., 2012; Christa et al., 2013).

Food sources and functional klepotplasty

Since the mechanisms for establishing functional kleptoplasty, and especially functional long-term retention, are unknown, the food gets more and more in focus as an important factor (de Vries et al., 2014). While some species like the long-term-retention form *Plakobranchus ocellatus*, *Elysia clarki* and *Elysia crispata* are polyphagous, others are stenophagous, like *Elysia timida* and *Elysia chlorotica* (Händeler & Wägele, 2007). It is therefore important to know if all sequestered plastids of the polyphagous long-term-retention species are kept for the same time or if certain plastids are digested or degrade earlier. Recently, it is suggested that FtsH, plastid encoded in the food source of *E. chlorotica* (*Vaucheria litorea)* and *E. timida* (*Acetabularia acetabulum*), but nuclear encoded in *Bryopsis*, food source of several non-retention forms, might be an important feature of the plastids to establish functional kleptoplasty (de Vries et al., 2014). FtsH removes damaged D1, a core protein of the PSII, and hence enables its replacement by a new protein. Yet, the presence of FtsH in the other food source of the long-term-retention forms needs to be investigated to confirm this hypothesis. Clearly, not only the food source is important to establish functional kleptoplasty, also special abilities of the slugs like control of the digestion are doubtlessly needed. The molecular identification and analysis of the food sources, however, seems to be a smart beginning.

The klepotplasts are still able to fix carbon after the sequestration and some studies suggest that this fixation is lasting for a few weeks, although strongly decreasing with time (Greene & Muscatine, 1972; Hinde & Smith, 1972; Clark et al., 1981). In *Plakobranchus ocellatus*, for example, CO_2 fixation was only a third after 27 days of starvation compared to the beginning (Greene & Muscatine, 1972). However, some studies showed that the slugs lost weight nearly in the same rate, irrespective of exposure to photosynthetic active light or kept in darkness (Giménez-Casalduero & Muniain, 2008; Klochkova et al., 2010; Yamamoto et al., 2012), leading Klochkova et al. (2010) to the assumption that other factors than phototrophic CO_2 are required for the survival of starvation periods in Sacoglossa. A photoautotrophic organism needs photosynthetic fixed carbon to survive and to at least maintain biomass, but in the case of *Elysia nicrocapitata* the slugs lost 99% of their biomass during a five months starvation period (Klochkova et al., 2010). Furthermore, there are only few evidences that the plastids actively provide the slugs with

photosynthates as stated by (Trench & Gooday, 1973) or if photosynthates are simply accessed by digesting plastids.

Figure 7 Size of individuals of *Plakobranchus ocellatus* freshly collected after feeding (upper picture) and after a starvation period of 6 months (lower picture).

Despite the question if photosynthesis is important for the survival of starvation periods, the plastids are still active and transcripts are traceable even after a few months of starvation (Mujer et al., 1996; Green et al., 2000; Rumpho et al., 2009; Pierce et al., 2010). Middlebrooks, Bell & Pierce (2012), for example, showed that in the long-term-retention form *Elysia clarki* chlorophyll a is still synthesized after a period of two weeks, although not for 14 weeks. As a result the slugs lose chlorophyll, like shown for other species (Hinde & Smith, 1972; Clark et al., 1981; Ventura, Calado & Jesus, 2013). This is also most probably the reason for the "bleaching" of starved animals (Fig. 8) rather than the loss of plastids as stated by others (Curtis et al., 2006). So far no report of sacoglossan sea slugs exists that all sequestered plastids are lost before the slugs die (Trench, Greene & Bystrom, 1969).
What the plastids are capable of within the cells of the digestive glands of the Sacoglossa, what triggers their longevity and how the slugs do benefit from the plastid is still mysterious.

Figure 8 Chlorophyll pigmentation of an individual of *Mourgona germaineae* before starvation and after six days of starvation.

AIMS OF THE STUDY

Despite that a large amount of former studies focused on the enigmatic phenomenon of the solar powered sacoglossans, several incongruences and questions still have to be addressed. Functional kleptoplasty was discussed to have evolved at the base of the Plakobranchoidea, although evidence was present that members of the Costasiellidae probably also harbour functional plastids (Clark et al., 1981; Händeler et al., 2009). It is still not known what actually contributes to establish a functional kleptoplastic relationship, since a LGT is excluded to provide genetic support for the plastid. The identification of plastid origin is crucial to find those that are required to establish functional long-term-retention. Furthermore the role of photosynthesis in the survival of the sea slugs revealed conflicting results in former studies, though these were not accordingly addressed.

My study intends to solve some of these issues outlined above and is divided into seven chapters, whereby chapters 2-6 represent individual studies that each contain a detailed introduction, a material & method section, results and discussion. The following topics were addressed:

Is every food source likewise important to establish functional kleptoplasty in the long-term-retention form *Plakobranchus ocellatus* (Chapter 2)?

How is the genus *Costasiella* related to the Plakobranchoidea and is functional kleptoplasty present in this particular genus (Chapter 3)?

Are there particular plastids needed to established functional kleptoplasty or is the food source of minor importance (Chapter 4)?

Are the slugs photoautotroph (Chapter 5)?

What is the most likely scenario of the evolution of functional kleptoplasty in Sacoglossa (Chapter 6)?

Chapter seven eventually provides a general discussion concerning the results of all chapters and a detailed outlook is given.

Chapter 2

What remains after 2 months of starvation? – Analysis of sequestered algae in a photosynthetic slug, *Plakobranchus ocellatus* (Sacoglossa, Opisthobranchia) by barcoding

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ABSTRACT

The sacoglossan sea slug, Plakobranchus ocellatus, is a so-called long-term retention form that incorporates chloroplasts for several months and thus is able to starve while maintaining photosynthetic activity. Little is known regarding the taxonomy and food sources of this sacoglossan, but it is suggested that *P. ocellatus* is a species complex and feeds on a broad variety of Ulvophyceae. In particular, we analysed specimens from the Philippines and starved them under various light conditions (high light, low light and darkness) and identified the species of algal food sources depending on starvation time and light treatment by means of DNA-Barcoding using for the first time the combination of two algal chloroplast markers, *rbcL* and *tufA*. Comparison of available *CO1* and *16S* sequences of specimens from various localities indicate a species complex with likely four distinct clades, but food analyses do not indicate an ecological separation of the investigated clades into differing foraging strategies. The combined results from both algal markers suggest that in general, *P. ocellatus* has a broad food spectrum, including members of the genera *Halimeda*, *Caulerpa*, *Udotea*, *Acetabularia* and further unidentified algae, with an emphasis on *H. macroloba*. Independent of the duration of starvation and light exposure, this algal species and a further unidentified *Halimeda* species seem to be the main food source of *P. ocellatus* from the Philippines. It is shown here that at least 2 (or possibly 3) barcode markers are required to cover the entire food spectrum in future analyses of Sacoglossa.

Keywords: Chlorophyta, DNA-Barcoding, Kleptoplasty, Photosynthesis, Rbcl, TufA

Abbreviations: DT (Dark treatment), LT (Low light intensity treatment), HT (High light intensity treatment), LTR (long term retention of chloroplasts)

INTRODUCTION

The unique ability of sacoglossan sea slugs to incorporate functional chloroplasts over weeks or months during starvation is a fascinating phenomenon that is still not well understood (see latest studies of (Pelletreau et al., 2011; Rumpho et al., 2011; Wägele et al., 2011; Pierce et al., 2012)). Only 3 – 10% of the genetic material required to produce necessary organelle proteins is contained within the plastids (Timmis et al., 2004). Thus, a horizontal gene transfer from the food algal nucleus to the slug genome was claimed for many years (Mujer et al., 1996; Hanten & Pierce, 2001; Pierce et al., 2003; Rumpho et al., 2008). This would have satisfactorily explained the manner in which chloroplasts incorporated from various algal sources remain functional for weeks or even months in the host slug. However, Wägele et al. (2011) rejected this hypothesis on the basis of broad EST analyses on *Plakobranchus ocellatus* (Hasselt, 1824) and *Elysia timida* (Risso, 1818), both known to belong to the chloroplast long term retention forms (LTR), which sustain photosynthetic ability over weeks and months [Evertsen et al., 2007; Händeler et al., 2009; Wägele et al., 2011); unpublished data]. Rumpho et al. (2011) came to the same conclusion for *Elysia chlorotica* (Gould, 1870), the most well-known sacoglossan species with photosynthetic abilities. It is evident that emphasis lie on the known long-term retention forms (LTR), *Elysia chlorotica* (Mujer et al., 1996; Green et al., 2000; Rumpho et al., 2001; 2008) and *Elysia timida* (Marin & Ros, 1992; Giménez-Casalduero & Muniain, 2008; Giménez-Casalduero et al., 2011; Schmitt & Wägele, 2011). Only recently *Plakobranchus ocellatu*s, a widely distributed species in the Pacific and one of the most effective LTR taxa first investigated by (Kawaguti & Yamasu, 1965), has come into focus (Wägele & Johnson, 2001; Hirose, 2005; Evertsen et al., 2007; Händeler et al., 2009; Maeda et al., 2012). Despite the absence of supporting nuclear genes, P. ocellatus shows an exceptional ability to starve over months with a relatively slow loss of photosynthetic activity (see results in [(Hirose, 2005; Händeler et al., 2009); unpublished results]. For this reason, P. ocellatus is of high interest for understanding the mechanism of functional long-term photosynthesis in Sacoglossa. Little is known concerning the biology, larval development, behaviour, and food sources of P. ocellatus. Even the taxonomy is not clarified, and the monotypic situation of this species is under general discussion and is questioned by the present authors (see Rudman 1998, Fig. 1).

One of the most important components to understanding the evolution of photosynthesis in Sacoglossa is determining the algal origin of incorporated chloroplasts. In a few monophagous species, like *Elysia chlorotica* and *E. timida* – feeding on *Vaucheria litorea* (Agardh, 1873) and *Acetabularia acetabulum* (Silva, 1952), respectively – the source of retained chloroplasts is evident. However, this is not the case for *P. ocellatus*, for which data on food items in the natural environment are still rare. Recently the algal food sources of two specimens from Guam (USA) and Lizard Island (Australia) have been identified via DNA-Barcoding as established by Händeler et al. (2010) using the chloroplast marker *tufa* (Wägele et al., 2011). Maeda et al. (2012) analysed specimens from Japan by applying the barcode marker rbcl. Several different incorporated chloroplasts originating from various algal taxa were found, similar to the varied food sources observed in some *Elysia* species (Jensen, 1980; Curtis et al., 2006; Händeler et al., 2009; 2010). This raises the question of which, if not all, chloroplast ingested from various algal species contribute to photosynthetic performance. We therefore investigated specimens of *P. ocellatus* from the Philippines maintained without access to algal food sources and under various light regimes to identify chloroplast source species sustained during the starvation period. *RbcL* (Curtis et al., 2006; Pierce et al., 2006; Maeda et al., 2012) or *tufA* (Händeler et al., 2010; Wägele et al., 2011), as previously used in the few studies in which chloroplasts originating from chlorophytes have been identified, are compared here for the first time. Since the investigated specimens in this experiment originated from different localities than those mentioned in the former studies, we also analysed fragments of the *CO1* and *16S* slug sequences with regard to possible cryptic speciation.

MATERIALS AND METHODS

Experimental set-up

41 healthy specimens of *Plakobranchus ocellatus,* collected in the Republic of the Philippines in November 2010 and transferred to Bonn, Germany, on 30th of November in 2010 by Frank Richter (Meerwasseraquaristik Richter near Chemnitz, Germany), were chosen for the starvation and light regime experiments. 5 specimens set as control group were directly preserved in 96% EtOH and stored at -20°C for DNA extraction (specimens Ploc101 – Ploc 105, Table 1), after measurement of photosynthesis as reported in Händeler et al. (2009). 36 specimens of *P. ocellatus* were divided randomly into three groups, each

containing 12 specimens, and kept in aquariums with artificial aerated sea water at 26°C (Hobby-Marine, Hobby® sea salt, Germany). The aquaria were cleaned regularly and the water changed every second day. One group was kept in complete darkness during the experiment (DT), the other two groups were kept under a Day/Night cycle of 12h:12h with illumination of 40 µmol photons m-2 s-1 (LT) and 200 µmol photons m-2 s-1 (HT), respectively. Illumination was provided by a Daylight Lamp (Androv Medical, Model AND1206-CH), simulating the full spectrum of day light. Downwelling irradiance of this lamp was measured with an Avantes AvaSpec 2048 spectrophotometer and connected to an Avantes CC-UV/VIS cosine. The distance between the light source and the spectrophotometer was about 50 cm. Irradiance was calibrated against an Avantes NIST traceable application standard. Photosynthetic rates of the specimens were monitored on a weekly basis with a PAM (Pulse Amplitude Modulated Fluorometer, Walz, Effeltrich, Germany) after dark acclimatisation (for details see Händeler et al. 2009). For this, two specimens were randomly selected from each treatment. After dark acclimatisation, three separate yield values per animal were documented and the mean value for both specimens calculated. Due to technical problems, measurements from the final period (64 days) are not available (see Fig. 2). Two randomly chosen specimens of each treatment group were fixed in 96% EtOH after 14, 21, and 35 days and after 49 and 64 days only for HT and LT treatment group. Due to the large number of clones to be investigated (12 per slug specimen), the absolute number of slugs in the barcoding experiments was reduced to 4 in the control group, 5 in the high irradiance treatment (HT), 5 from the low irradiance treatment (LT) and 3 from darkness (DT). During the experiments, 4 specimens died and were therefore not included in the phylogenetic analyses. Because of high mortality in the DT-group after 4 weeks, no specimens were available for barcoding after 35 days. 36 specimens of the 41 specimens were included in the phylogenetic analysis.

DNA extraction and amplification

DNA was extracted using the DNeasy ® Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions and stored at -20°C.

For analysis of possible cryptic speciation within *Plakobranchus*, gene fragments of *COI* and *16S* were amplified for all specimens from the Philippines. Sacoglossan-specific primers for *COI and 16S* are specified in Table 2 and were used according to earlier published protocols

(Händeler et al., 2009). Amplification reactions were carried out using 0.5µl of genomic DNA in a 20µl final volume reaction supplied with 5.5µl sterilized water, 2µl Qiagen® Q-Solution, 10µl of double concentrated QIAGEN ® Multiplex PCR Master Mix and 1µl of 10pmo/µl concentrated primer each. Amplification of *COI* was performed by denaturation for 15min at 95°C, followed by 25 standard cycles (94°C for 45 sec, 48°C for 45 sec and 72°C for 90 sec.) and a final extension at 72°C for 10min. Amplification of *16S* was performed by denaturation for 15min at 95°C, followed by 9 touch-down cycles (94°C for 90 sec, 58°C (-1°) for 90 sec, 72°C for 90 sec) followed by 25 standard cycles (94°C for 90 sec, 49°C for 90 sec and 72°C for 90 sec.)

For the barcoding of sequestered algal chloroplasts, the genes *rbcL* and *tufA* were analysed. PCR was performed using a touch-down protocol with Ulvophyceaen-specific primers (see Table 2). These primers were tested a priori on a broad spectrum of various ulvophyceaen species with excellent results. 2.5µl of genomic DNA was used as template in a 20µl final volume reaction supplied with 3.5µl sterilized water, 2µl Qiagen® Q-Solution, 10µl of double concentrated QIAGEN ® Multiplex PCR Master Mix and 1µl of 10pmol/µl concentrated primer each. PCR for amplification of *rbcL* was performed by an initial denaturation for 15min at 95°C, followed by 9 touch-down cycles at 94°C for 45sec, 60°C (-1°C per cycle) for 45 sec, 72°C for 90 sec, followed by 25 standard cycles (94°C for 45 sec, 51°C for 45 sec and 72°C for 90 sec) and a final extension at 72°C for 10 min. For *tufA*, amplification was performed as follows: initial denaturation for 15min at 95°C, followed by 9 touch-down cycles at 94°C for 45 sec, 57°C (-1°C per cycle) for 45 sec, 72°C for 90 sec, followed by 25 standard cycles (94°C for 45 sec, 48°C for 45 sec and 72°C for 90 sec) and a final extension at 72°C for 10 min.

DNA purification, cloning and sequencing

PCR products were size-fractionated in a 1.5 % agarose gel for 90 min at 70V. Subsequently bands were extracted from the gel according to desired gene-fragment length (around 950 for *tufA* and 560 for *rbcL*) using Machery-Nagel NucleoSpin® Extract II kit following manufacturer's instructions. Isolated fragments were ligated into pGEM t-easy Vector (Promega, Germany) and cloned into competent *E. coli* XL1-blue cells (Stratagene, Heidelberg, Germany). For each specimen, 12 clones were sequenced by Macrogen Inc, Amsterdam. Sequence identity was verified by BLAST search using the NCBI homepage.

Sequence analysis

Slug analysis: All available *CO1* and *16S* sequences of *Plakobranchus ocellatus* in GenBank were downloaded and added to the sequences obtained in this study. Based on the phylogeny published in Händeler et al. (2009), which shows *Thuridilla* as a sister taxon to *P. ocellatus*, several *Thuridilla* species were selected as outgroups, but none of these species were specifically designated as outgroup (Table 1). Both genes were concatenated and analysed in one dataset with a length of 1030 bp. The best-fit model GTR+I+G was selected by jModeltest with Akaike information criteria [v 0.1.1; (Posada, 2008)] for analysing the data. A maximum likelihood tree was constructed using the RaxML online Server (Stamatakis, Hoover & Rougemont, 2008). Sequence divergence was determined by calculating p-distances using HyPhy (Kosakovsky Pond, Frost & Muse, 2005). *CO1* was separately analysed in addition to the concatenated dataset (Table 3).

TufA and *rbcL* of sequestered algae: Following Händeler et al. (2010), consensus sequences were created when sequence divergence of two sequences of the same individual was less than 1%. Alignments were created for each gene using the Mafft online server (http://mafft.cbrc.jp/alignment/server/) with relevant sequences detected a priori by BLAST search and subsequently obtained from GenBank. Alignment length was 668bp for *tufA* (thus we used shorter sequences than obtained for comparison with already available sequences) and 560bp for *rbcL*. Sequence affinity was determined by Neighbor-Joining trees constructed with Geneious (vers. 5.5.3). No evolutionary model was applied in order to identify absolute genetic similarity with available algal genes.

RESULTS

Sequence analysis of slugs

Comparison of available information of live specimens shows that some minor colour distinctions can be observed in the studied specimens (Fig. 1). Analysis of the concatenated dataset of the gene fragments *CO1* and *16S* showed monophyly of *Plakobranchus ocellatus* with high bootstrap support (Fig. 3) within the *Thuridilla* clade. All specimens from the Philippines, including specimens from Japan and Guam cluster into one clade (Taxon A)

with a sequence divergence between 0.2 to 1.5% in the concatenated data set and 0.16 to 2.58% in the *CO1* analysis. Clade A is the sister group to a single specimen (Clade B) from Lizard Island (Liz2) (sequence divergence about 9% and 12% in concatenated and CO1 dataset, respectively). Two specimens from Guam (PlocGm1 and PlocGm4), exhibiting a sequence divergence of 0.3% and 0.48%, cluster together and are herein called Clade C. These two are the sister group to one specimen from Lizard Island (Liz1) (Clade D) with a sequence divergence each to the latter of 5.59% and 5.78% (concatenated dataset). The divergence between the two Lizard Island specimens is 9.1% and 11.15% (concatenated dataset and *CO1*, respectively). The highest sequence divergence in the concatenated dataset as well as in the *CO1* analysis was found between a specimen from Guam (PlocGm4) and the specimen PlocLiz2 from Lizard Island (9.93% and 12.28%, respectively). These come close to those distances observed within the few *Thuridilla* species included in our study (about 11 to 14% and 14 to 17%, respectively). All four clades herein defined as A to D are therefore clearly genetically distinct from each other with an average percentage of about 8% in the concatenated dataset and 10% in the *CO1* analysis. The separate analysis of *CO1* reveals even higher genetic distances between these 4 outlined taxa (see Table 3).

Measurements of functional photosynthesis

Individuals of the three treatments showed nearly the same photosynthetic activity at the beginning of the experiment with a yield value of approximately 800 indicating a healthy, non-starved condition typical for specimens of *Plakobranchus ocellatus* (Fig. 2, see Händeler et al. 2009, (Yamamoto et al., 2009)). DT and LT individuals showed functional photosynthesis with rather constant and high yield values of about 700 lasting over a period of 28 and 42 days, respectively. Actually, values of both treatment groups were quite similar until the DT specimens perished (at approximately 35 days). In contrast, photosynthetic activity of HT individuals decreased considerably after 30 days in high light conditions, thus showing a significant decrease of photosynthetic activity compared to that of the animals kept in low light or dark conditions.

Sequence analysis and barcoding of Ulvophyceae

TufA and *rbcL* were successfully amplified and cloned from all slug specimens used in the special treatment study in order to barcode ulvophycean food items. Of each specimen and each gene, 12 clones were sequenced to compare the reliability and usefulness of the marker as a Barcode-Gene in food analysis. For every specimen, except Ploc101, a minimum of 10 sequences was successfully sequenced for *rbcL*, whereas for *tufA* less than 10 clones were gained for Ploc103, Ploc109, and Ploc124 (see Table 4, unsuccessful sequenced samples showed double stranded pherograms). Bacterial contamination was higher in the analysis of *tufA* but is not further shown here. A prior analysis of all obtained clones with a wide variety of selected algal *tufA* and *rbcL* genes from GenBank allowed a preliminary assignment to certain algal taxon. Subsequently these taxa were more closely selected and more related taxa extracted from GenBank.

With regard to the number of identified haplotypes, *rbcL* revealed 8 that could be assigned to 4 different *Halimeda* species, 2 *Udotea* species, 1 *Acetabularia*, and 1 *Pseudochlorodesmis* species. The number of obtained *tufA* haplotypes were much lower: 2 haplotypes could be assigned to 2 *Halimeda* species and 1 to *Caulerpa* (Table 4 and 5). Thus, *rbcL* recovered 6 species which were not found with *tufA*: *Halimeda* sp. 1 and sp. 2, *Udotea* sp. 1 and sp. 2, *Acetabularia acetabulum*, and *Pseudochlorodesmis sp. TufA* recovered one algal species, *Caulerpa fastigata* that was not detected in the *rbcL* analysis. Only *Halimeda macroloba* and *H. borneensis* were obtained in both gene analyses.

Halimeda macroloba was the most common algal species identified, independent of the applied barcode gene, length of starvation or light treatment (Table 4). The genetic distance of the *rbcL* genes deposited in GenBank for *Halimeda macroloba* (FJ624513) and *Halimeda kanaloana* (FJ624512) is 0.02, therefore a differentiation between those two species based on *rbcL* sequences is not possible at this time. No *tufA* sequences are available for *Halimeda kanaloana.* However, *tufA* sequences obtained in this study match exactly those of *Halimeda macroloba* (HM140244, AM049960). Therefore we assigned all sequences from the *rbcL* analysis with identicalness to the *H. macroloba* GenBank sequence to *Halimeda macroloba*.

The second most prominent haplotype obtained is an unidentified species of *Halimeda*, herein called *Halimeda* sp. 2. This chloroplast type was only identified using *rbcL* in nearly every starvation period, and treatment group (Table 4).

Halimeda borneensis was found in both gene analyses, although the specimens (except Ploc103) were not the same. This indicates that analysis of one and the same slug does not yield same results for both genes. Overall, *H. borneensis* chloroplasts were recovered in six different specimens kept in high or low light, but not in darkness (Table 4).

Further algae identified by analysing *rbcL* include another unidentified *Halimeda* species (*Halimeda* sp. 1), two unidentified species of *Udotea* (*Udotea* sp. 1, *Udotea* sp. 2*), Acetabularia acetabulum*, and an unidentified species of *Pseudochlorodesmis* (Table 4). These algae were not revealed in the *tufA* analyses, although the primers used here worked before when applying on these algae in direct sequencing.

In addition to the previously mentioned *H. macroloba* and *H. borneensis*, the analyses of the *tufA* gene revealed *Caulerpa fastigiata* (not present in the *rbcL* analysis).

There is a trend that haplotypes of *Halimeda macroloba*, *H. borneensis* and *Halimeda* sp. 2 are kept longer during starvation than haplotypes of all other algae. These three chloroplast types were also found multiple times in one and the same specimen irrespective of treatment. All other haplotypes were identified only by single sequences in the various specimens and treatments (Table 4). Chloroplasts of *Acetabularia acetabulum*, *Pseudochlorodesmis* sp., and *Halimeda* sp. 1 were only detected before starvation (*rbcL* analysis).

The lowest number of chloroplast types (3) was detected in DT specimens (see Table 4) compared to HT and LT (4 and 5, respectively), but it should be emphasized that only 3 DT specimens were investigated, instead of 5. Comparing the number of obtained haplotypes in the starvation period of 64 days, LT specimens still show 4 different algal chloroplast types, whereas in the HT group only *Halimeda macroloba* was found.

Food algae and locality of Plakobranchus ocellatus

Alongside the results obtained from the starvation studies with slugs from the Philippines, Table 5 shows all available *tufA* and *rbcL* data from *Plakobranchus ocellatus* originating from two other localities (Lizard Island, Great Barrier Reef, Australia and Guam, Marianen Islands, USA) in order to identify possible ecological and locality variations. The two specimens from Lizard Island revealed chloroplasts of *Halimeda macroloba* in the *rbcL* analysis and of an unknown ulvophycean species with the highest similarity to *Pseudocodium floridanum* (AM909697) in the *tufA* analysis. Thus, the food spectrum of *P. ocellatus* at the Australian locality seems to be less diverse, but only two specimens were investigated from that region. In contrast, the two specimens investigated from Guam exhibited nearly the same broad range of food items as the specimens from the Philippines (Table 5). Only *Acetabularia acetabulum* was not detected.

DISCUSSION

In recent literature *Plakobranchus ocellatus* is considered to be monotypic with a broad distribution from Japan, the Great Barrier Reef, the Philippine Islands, Vietnam, Malaysia, Hawaii and Bali (Jensen, 1992; 1996; 1997; Hirose, 2005; Evertsen et al., 2007; Händeler et al., 2009; Maeda et al., 2010; Trowbridge, Hirano & Hirano, 2011; Yamamoto et al., 2012). Although a variation in morphology and colour patterns is obvious between specimens from different origins (Fig. 1), no further species have been described thus far, and the synonymisation of *P. ianthobaptus* Gould, 1852 was never re-evaluated. Usually, only one specimen assigned to *P. ocellatus* is included in phylogenetic analyses (Jensen, 1992; 1996; 1997; Maeda et al., 2010). Nevertheless, Jensen (1997) estimated the number of *Plakobranchus* species to be between 1 and 14. In Händeler & Wägele (2007) and Händeler et al. (2009) a concatenated dataset of the genetic markers *28S*, *16S*, and *CO1* revealed that two specimens of *P. ocellatus* from different localities grouped as sister taxa. *CO1* is generally accepted for species discrimination within many phyla (with few exceptions) [barcodeoflife.org; (Hebert et al., 2003; 2004)]. In this gene, a distance greater than 10% is considered a fair value for characterizing distinct species and a distance less than 3% distance is considered to represent intraspecific variability (Hebert et al., 2003; 2004; Hajibabaei et al., 2006). *CO1* has already been used successfully in several instances as a barcode in gastropods (e.g., (Weigand et al., 2010; Wägele et al., 2010b; Huelsken et al.,

2011). In our analysis, the highest distance values of *CO1* are between 7 and 12%, thus indicating cryptic speciation. Even in the analysis of the concatenated dataset of *CO1* and *16S*, the highest sequence divergences lie between 5 and nearly 10%. A comparison of one available 16S sequence of the Japanese specimens studied by Maeda et al. (2012) with our material did not show a clear affiliation of the Japanese population to one of the groups identified here. Overall, we consider our results to show sufficient evidence that several distinct *Plakobranchus* clades are present, thus confirming the assumptions of Jensen (1997) and P. Krug (California State University, Larval Ecology and Evolution, Los Angeles, CA, USA, pers. comm.). At least four different cryptic clades appear to be present in the Pacific and Indopacific Ocean.

The results of our food source analyses do not reflect the genetic and geographic variation. Foraging primarily on *Halimeda* species with the option to consume other ulvophycean species is typical for all slugs irrespective of locality and clade investigated here. In contrast, *Plakobranchus* specimens from Japan mainly fed on *Caulerpella* species, although other related species within Halimedinae (*Poropsis*, *Rhipidosiphon*, Rhipiliaceae spp.) including *Halimeda*, were also detected (see Maeda et al. 2012).

Although a vast body of literature exists concerning photosynthetic slugs (see comprehensive review of Wägele & Martin, 2013), no study to date has investigated which algal chloroplast type contributes to long term photosynthesis under starvation conditions nor considered the influence of irradiance on the survival of chloroplast type. Here we analysed the nutrition of *Plakobranchus ocellatus* using DNA-Barcoding before and during starvation (for more than two months) and under different light conditions (high light, low light and darkness) to compare chloroplast sources and survival of chloroplasts under these conditions. Rapid decrease of photosynthesis in our specimens kept in high irradiances indicates higher photodamage of the RCII protein D1 than in lower irradiances or even darkness, as was already reported by Vieira et al. (2009) for *Elysia viridis* and for isolated *Vaucheria* chloroplasts (Green, Fox & Rumpho, 2005). However, it must be considered that specimens living in their natural environment can be exposed to much higher irradiances in tropical areas. We measured more than 1000µmol m-2 sec-1 during midday in the few centimeter depths where specimens were collected on Lizard Island. Usually the animals burrow in the sand to avoid high irradiances (pers. observations). The

higher death rate in animals kept in darkness might be attributed to the lack of light-dependent synthesis of essential nutritive substances, but we know no reports. Most recently Yamamoto et al. (2012) detected a higher death rate in *P. ocellatus* and *Elysia trisinuata* when animals were kept in darkness, but they also discussed problems in cultivating the animals in small containers with fouling water. Additionally, the short time series (20 days) and extremely low light regime, (28µmol m^{-2} sec^{-1}) in their so-called high light treatments all differed considerably from our settings. Our LT with 40µmol m^{-2} sec^{-1} even exceeded their HT. These authors provided *Rhipidosiphon javensis* (*Udotea javensis*) as a food source for *Plakobranchus ocellatus*, but verification of feeding was not possible.

Comparing the number of algal haplotypes during the various light treatments and starvation days, the highest survival is observed in specimens kept in low light conditions. Out of the 5 identified chloroplast types, 4 are still present after 64 days of starvation. Only *Caulerpa fastigiata* is missing. In contrast, only *Halimeda macroloba* was recovered after 64 days in HT specimens. Since the number of clones containing *H. macroloba* per investigated slug specimen was high (10 and 12) we believe our results indicate the long survival abilities of this algal chloroplasts and that these chloroplast are probably the only species contributing to the photosynthesis measured after 64 days of starvation. We are not able to satisfactorily explain here the higher presence of *H. macroloba* chloroplasts and the loss of all other haplotypes during long-term starvation and high irradiances, but the following hypotheses seem likely. First, chloroplasts of *H. macroloba* are more robust than all others, therefore disintegration/digestion is delayed. They are less influenced by photodamage due to better adaptations to high irradiances, which might also delay disintegration. Second, perhaps the slugs mainly fed on *H. macroloba*, therefore the likelihood of incorporating their chloroplasts is much higher than incorporating other chloroplast types. It cannot be excluded that both arguments are true, although the independent studies of Maeda et al. (2012) indicate a general broad diet, and not particular food items. We do not know the algal composition from the habitat where the Philippine specimens were collected. However, the habitats of the Guam and Lizard Island species exhibited a diverse algal composition, with a high dominance of *Halimeda*, *Udotea* and *Caulerpa*, but which also included species that were not detected as food items (e.g., Avrainvillea).

It is also difficult to state the contribution of *Halimeda macroloba* chloroplasts to overall photosynthesis of the slug. Whereas yield values dropped in HT animals, which eventually seem to be sustained only *H. macroloba* chloroplasts, yield values in the specimens of the LT dropped only minimally and still comprise 4 different chloroplast types. Also, in animals kept in darkness and exhibiting still 2 chloroplast types, the yield values were high. Yield values do not directly allow for quantification of chloroplasts; they merely give an indication of the quality of the chloroplasts. Hence we cannot exclude that several types of chloroplasts can survive in the slug, which raises a statistical question of how many chloroplasts from which algal species were incorporated, and of these, which chloroplasts species we are able to detect. Furthermore, the irradiance might damage all of the chloroplast types in a similar way, so that ultimately we could only recover those that were consumed in a higher percentage before starvation.

Our results here confirm that *Plakobranchus* specimens from tropical areas are opportunistic algal feeders, which can survive on various algae, but with a preference for *Halimeda*. Otherwise sequences of the different incorporated algae specimens would be present in equal numbers. In contrast, the specimens investigated by Maeda et al (2012) from Japan showed a high seasonality of kleptoplast composition. Astonishingly, *Halimeda*, was not the dominant food item, it was actually absent in several specimens collected in 2005 and completely absent in most animals collected in 2007. The authors showed that *Caulerpella* was one of the most important chloroplast providers. These differences might be due to algal availability and therefore might also change geographically, or are clade specific within the Plakobranchus species complex. Whether other chloroplast sources, including *Caulerpella*, are also as valuable as the *Halimeda* chloroplasts for maintaining functional photosynthesis over a longer period remains to be studied.

We cannot answer why haplotypes obtained in the *rbcL* analysis are not recovered in the *tufA* analysis and vice versa, but this is also observed in other studies on sacoglossans (unpublished data of GC). The problem may lie in the statistics again, degradation, or other unknown factors. Using two barcode markers that worked on many algal species certainly increases the chances of detecting a broader array of kleptoplasts in the slugs. There is yet another unsolved pitfall not addressed so far in the recently published studies, which actually demands a third marker: the so far impossible amplification of members of the

genus *Cladophora* Ulvophyceae (see Saunders & Kucera, 2010). For land plants the chloroplast markers *matK* and *rbcL* are used for DNA-Barcoding with high resolution to species level. Nevertheless, they are generally combined with nuclear markers because of higher reliability (CBOL Plant Working Group, 2009). *MatK* is not present in the chloroplast genome of Ulvophyceae (Pombert et al., 2005; 2006) and therefore cannot be used here. Bhattacharya, Friedl & Damberger (1996) and Haugen, Simon & Bhattacharya (2005) stated that *rbcL* is a problematic barcode for identification of marine algae due to a high number of introns. Furthermore, it lacks enough specificity to discriminate *Halimeda* species properly. Unfortunately this is an important algal genus on which several sacoglossan species feed, and exact species discrimination is desirable. Verbruggen, De Clerck & Kooistra (2005) analysed the rps3 region for a special clade within *Halimeda*, but re-analyses using more taxa showed that a discrete species identification based on this gene is also not possible (Händeler et al. 2010; unpublished data). *TufA* does not contain introns and was therefore advised as a good cp marker gene for DNA-Barcoding (Famà et al., 2002; O'kelly, Bellows & Wysor, 2004; Verbruggen et al., 2009; Händeler et al., 2010; Saunders & Kucera, 2010). It also discriminates well within the genus *Halimeda* (see Händeler et al. 2010).

We suggest here for future studies on chloroplasts in photosynthetic slugs to analyze more than one gene (hopefully finding one that also is applicable for Cladophora), and to consider seasonality and food source availability (see Maeda et al. 2012). Since a horizontal gene transfer from the algal nuclear genome into the genome of Plakobranchus ocellatus has been rejected, the origin and the properties of the specific chloroplasts might provide clues to understanding the long-term retention of kleptoplasts in these enigmatic sacoglossan slugs.

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TABLES

Table 1 List of *Plakobranchus ocellatus* specimens used for phylogenetic analyses (columns for *CO1* and *16S*) and for DNA-Barcoding of food (last two columns *rbcL* and *tufA*) with accession numbers. First column designates the specimens with internal numbers and the treatment used in the experiments. (C) refers to control group; (HT), (LT) and (DT) refers to treatments in high light, low light and darkness, respectively. Specimens designated with an (pa) were not used in the experiments, but only for the phylogenetic analysis. (–) indicates that genes were not analysed

Internal numbers (treatment)	**Locality**	**GenBank accession numbers**			
		Co1	*16S*	*rbcL*	*tufA*
Ploc101 (C)	Philippines	JX272685	JX272648	JX272725	JX272772
				JX272726	
				JX272727	
				JX272728	
				JX272729	
Ploc102 (C)	Philippines	-	-	JX272730	JX272773
				JX272731	
				JX272732	
Ploc103 (C)	Philippines	JX272686	JX272649	JX272733	JX272774
				JX272735	JX272775
				JX272735	
Ploc104(C)	Philippines	JX272687	JX272650	JX272736	JX272776
Ploc105 (C)	Philippines	JX272688	JX272651	JX272737	JX272777
Ploc106 (pa)	Philippines	JX272689	JX272652	-	-
Ploc107 (LT)	Philippines	JX272690	JX272653	-	-
Ploc108 (LT)	Philippines	JX272691	JX272654	JX272738	JX272778
				JX272739	
				JX272740	
				JX272741	
Ploc109 (HT)	Philippines	JX272692	JX272655	JX272742	JX272779
				JX272743	JX272780
				JX272744	
Ploc110 (HT)	Philippines	JX272693	JX272656	-	-
Ploc111 (DT)	Philippines	JX272694	JX272657	-	-
Ploc112 (DT)	Philippines	JX272695	JX272658	JX272745	JX306774
Ploc113 (DT)	Philippines	JX272696	JX272659	-	-
Ploc114 (DT)	Philippines	JX272697	JX272660	-	-

Ploc115 (LT)	Philippines	JX272698	JX272661	-	-
Ploc116 (DT)	Philippines	JX272699	JX272662	-	-
Ploc117 (HT)	Philippines	JX272700	JX272663	-	-
Ploc118 (HT)	Philippines	JX272701	JX272664	JX272746	JX272782
				JX272747	
Ploc119 (HT)	Philippines	JX272702	JX272665	-	-
Ploc120 (LT)	Philippines	JX272703	JX272666	JX272748	JX272783
				JX272749	JX272784
				JX272757	
Ploc121 (DT)	Philippines	JX272704	JX272667	-	-
Ploc122 (DT)	Philippines	JX272705	JX272668	-	-
Ploc123 (DT)	Philippines	JX272706	JX272669	JX272750	JX272785
				JX272751	
Ploc124 (HT)	Philippines	JX272707	JX272670	JX272752	JX272786
				JX272753	JX272787
				JX306775	
Ploc125 (HT)	Philippines	JX272708	JX272671	-	-
Ploc126 (LT)	Philippines	JX272709	JX272672	-	-
Ploc127 (LT)	Philippines	JX272710	JX272673	JX272754	JX272788
Ploc128 (DT)	Philippines	JX272711	JX272674	JX272755	JX272789
				JX272756	
Ploc129 (DT)	Philippines	JX272712	JX272675	-	-
Ploc130 (HT)	Philippines	JX272713	JX272676	JX272758	JX272790
				JX272759	
Ploc131 (HT)	Philippines	JX272714	JX272677	-	-
Ploc133 (LT)	Philippines	JX272715	JX272678	JX272760	JX272791
				JX272761	
Ploc135 (HT)	Philippines	JX272716	JX272679	JX272762	JX272792
Ploc137 (LT)	Philippines	JX272717	JX272680	JX272763	JX272793
				JX272764	JX272794
				JX272765	
Ploc138 (pa)	Philippines	JX272718	JX272681	-	-
Ploc147 (pa)	Philippines	JX272719	JX272682	-	-
Ploc148 (HT)	Philippines	JX272720	JX272683	-	-
PlocGm1 (850)	GUAM	HM187633	HM187604	-	-
PlocGm2 (852)	GUAM	HM187634	HM187605	JX272721	HM140235
					HM140245
					HM140234

					HM140225
					HM140228
PlocGm3 (854)	GUAM	HM187638	HM187606	JX272722	HM140238
				JX272723	HM140236
				JX272724	HM140231
PlocGm4 GUAM (856)	GUAM	HM187635	JX272684	-	-
PlocLiz1 (705)	Lizard Island, Australia	GQ996679	EU140875	JX272766	HM140210
				JX272767	HM140189
				JX272768	HM140187
				JX272769	HM140191
					HM140188
PlocLiz2 (706)	Lizard Island, Australia	GQ996680	EU140876	JX272770	HM140193
				JX272771	HM140212
PlocJap Jap	Japan		AB501307	-	-
Thuridilla kathae	Lizard Island, Australia	GQ996676	EU140879	-	-
Thuridilla hoffae	Samoa: Savaii Island, Vaisala lagoon	GQ996670	EU140880	-	-
Thuridilla carlsoni	Lizard Island, Australia	GQ996681	EU140878	-	-
Thuridilla gracilis	Lizard Island, Australia	GQ996684	EU140883	-	-
Thuridilla lineolata	Indonesia: Sulawesi	GQ996682	EU140887	-	-

Table 2 Primers used in this study for slugs and algal analyses

Plakobranchus ocellatus		
CO1 forward: coxFS	TTTCAACAAACCATAARGATATTGG	(Händeler et al. 2009)
CO1 reverse: coxRS	TAYACTTCWGGGTGW CCA AAA AAYCA	(Händeler et al. 2009)
16S forward: 16S_1	GGAGCTCCG GTTTGAACTCAGATC	(Händeler et al. 2009)
16S reverse: 16S_2	CGGCCGCCTGTTTATCAAAAACAT	(Händeler et al. 2009)
Algae		
rbcL: rbcLF forward	AAAGCNGGKGTWAAAGAYTA	(Pierce et al. 2006)
rbcL: rbcLR reverse	CCAWCGCATARANGGTTGHGA	(Pierce et al. 2006)
tufA: tufAF forward	TGAAACAGAAMAWCGTCA TTA TGC	(Famá et al. 2002)
tufA: tufAR reverse	CCT TCN CGA ATM GCR AAW CGC	(Famá et al. 2002)

Table 3 Genetic p-distances [%] (maximum values) between selected *Plakobranchus* specimens found on Lizard Island (Liz), Guam (Gm), Philippines (Ph) and Japan (Jap). Upper part designates CO1 distances, lower part the concatenated dataset. Bold values are the highest distances found in the analyses

		CO1								
	Clade	D	B	C	A	A	C	A	A	A
	Specimens	PlocLiz1	PlocLiz2	PlocGm 1	PlocGm 2	PlocGm 3	PlocGm 4	Ploc131 (Ph)	Ploc13 7 (Ph)	PlocJap
CO1 + 16S	PlocLiz1	-	11.15	7.43	11.31	11.47	7.75	11.3	11.79	11.63
	PlocLiz2	9.10	-	12.12	11.79	11.63	**12.28**	11.79	11.63	11.47
	PlocGm1	5.59	9.30	-	10.82	10.66	0.48	10.82	11.31	11.47
	PlocGm2	9.00	9.12	8.12	-	0.48	10.82	0.16	2.10	0.65
	PlocGm3	9.20	9.05	8.12	0.39	-	10.66	0.65	2.26	1.13
	PlocGm4	5.78	**9.39**	0.29	8.12	8.12	-	10.82	11.31	11.47
	Ploc131 (Ph)	9.00	9.05	8.12	0.10	0.49	8.12	-	2.26	0.81
	Ploc137 (Ph)	9.42	9.07	8.54	1.36	1.56	8.54	1.46	-	2.75
	PlocJap	9.20	8.85	8.51	0.39	0.78	8.51	0.49	1.75	-

Table 4 Algal species obtained by *tufA* and *rbcL* analyses from Philippine specimens during light and starvation treatments. First column indicates the treatment, second and third column the internal number (#) and number of days of treatment (Days). Columns 4 and 5 give total numbers of haplotypes obtained from the respective specimens. Columns 6 and 7 indicate the algal species and the number of algal clones in brackets found in the respective slug specimen

Light Treatment	**#**	**Days**	**Haplotypes per specimen**		**Algal species and number of clones per slug specimen**	
			tufA	*rbcL*	*tufA (nr. clones)*	*rbcL (nr. clones)*
None	Ploc101	0	1	5	*Halimeda macroloba* (10)	*Halimeda macroloba* (5)
						Acetabularia acetabulum (1)
						Halimeda sp 1 (1)
						Halimeda sp 2 (1)
						Halimeda borneensis (1)
	Ploc102	0	1	3	*Halimeda macroloba* (11)	*Halimeda macroloba* (9)
						Pseudochlorodemsis sp (1)
						Udotea sp1 (1)
	Ploc103	0	2	3	*Halimeda macroloba* (5)	*Halimeda macroloba (5)*
					Halimeda borneensis (2)	*Halimeda* sp 2 (3)
						Halimeda borneensis (3)
	Ploc105	0	1	2	*Halimeda macroloba* (11)	*Halimeda macroloba (11)*
						Halimeda sp 1 (1)
HT	Ploc109	14	2	3	*Halimeda macroloba* (6)	*Halimeda macroloba (8)*
					Halimeda borneensis (1)	*Halimeda* sp 2 (1)
						Udotea sp 1(1)
	Ploc118	21	1	2	*Halimeda macroloba* (10)	*Halimeda macroloba* (9)
						Halimeda sp 2 (1)
	Ploc124	35	2	3	*Halimeda macroloba* (6)	*Halimeda macroloba* (9)
					Halimeda borneensis (1)	*Halimeda* sp 2 (1)
						Udotea sp 2 (1)
	Ploc130	49	1	2	*Halimeda macroloba* (11)	*Halimeda macroloba* (11)
						Halimeda sp 2 (1)
	Ploc135	64	1	1	*Halimeda macroloba* (10)	*Halimeda macroloba* (12)
LT	Ploc108	14	1	4	*Halimeda macroloba* (10)	*Halimeda macroloba* (9)
						Halimeda borneensis (1)
						Udotea sp 1 (1)
						Udotea sp 2 (1)
	Ploc120	21	2	3	*Halimeda macroloba* (10)	*Halimeda macroloba* (10)

					Caulerpa fastigiata (1)	*Halimeda* sp 2 (1) *Udotea* sp 1 (1)
	Ploc127	35	1	1	*Halimeda macroloba* (11)	*Halimeda macroloba* (12)
	Ploc133	49	1	2	*Halimeda macroloba* (11)	*Halimeda macroloba* (8) *Halimeda sp 2 (4)*
	Ploc137	64	2	3	*Halimeda macroloba* (11) *Halimeda borneensis* (1)	*Halimeda macroloba* (5) *Halimeda* sp 2 (4) *Udotea* sp 1 (1)
DT	Ploc112	14	2	1	*Halimeda macroloba* (8) *Caulerpa fastigiata* (1)	*Halimeda macroloba* (10)
	Ploc123	21	1	2	*Halimeda macroloba* (11)	*Halimeda macroloba* (10) *Halimeda* sp 2 (4)
	Ploc128	35	1	3	*Halimeda macroloba* (10)	*Halimeda macroloba* (11) *Halimeda* sp 2 (1)

Table 5 Chloroplast haplotypes identified by DNA-Barcoding in various specimens (numbers given in brackets) of *Plakobranchus ocellatus* from different localities using *tufA* and *rbcL*. Numbers indicate number of found haplotype of a certain algal genus

	Halimeda spp		Caulerpa spp		Udotea spp		Acetabularia acetabulum		Unidentified algae (Pseudochlorodesmis, Pseudocodium)	
Specimens' origin	tufA	rbcL	tufA	rbcL	tufA	rbcL	tufA	rbcL	tufA	rbcL
Philippines (17)	3	4	2	0	0	2	0	1	0	1
Guam (2)	2	2	1	0	1	2	0	0	2	1
Lizard Island (2)	0	2	0	0	0	1	0	0	5	2

FIGURES

Figure 1 External differences in specimens of *Plakobranchus ocellatus* used in this study and collected in various localities. Specimens come from Philippine Islands (**a**), Guam (**b**, **c**), Lizard Island (Australia, **d**). Note the colour difference in the smaller specimen (**b**, about 1 cm) and the less spotted animal (**c**) both from Guam. Animals depicted in **a**, **c** and **d** exhibited a length around 1.5 to 2 cm.

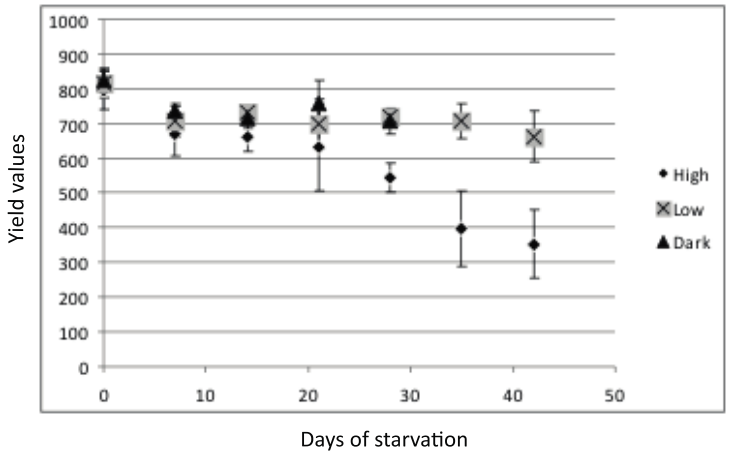

Figure 2 Graphical depiction of the average weekly PAM-readings from two randomly picked individuals from each treatment group (see Materials and methods). Low intensity treatment group (low) received 40 µmol photons m^{-2} s^{-1}, high intensity group (high) received 200 µmol photons m^{-2} s^{-1}, 12 h/day. Measurements for the group kept in complete darkness (dark) could not be conducted after 30 days because all specimens had perished. Note the similar curve of low light and dark treated specimens.

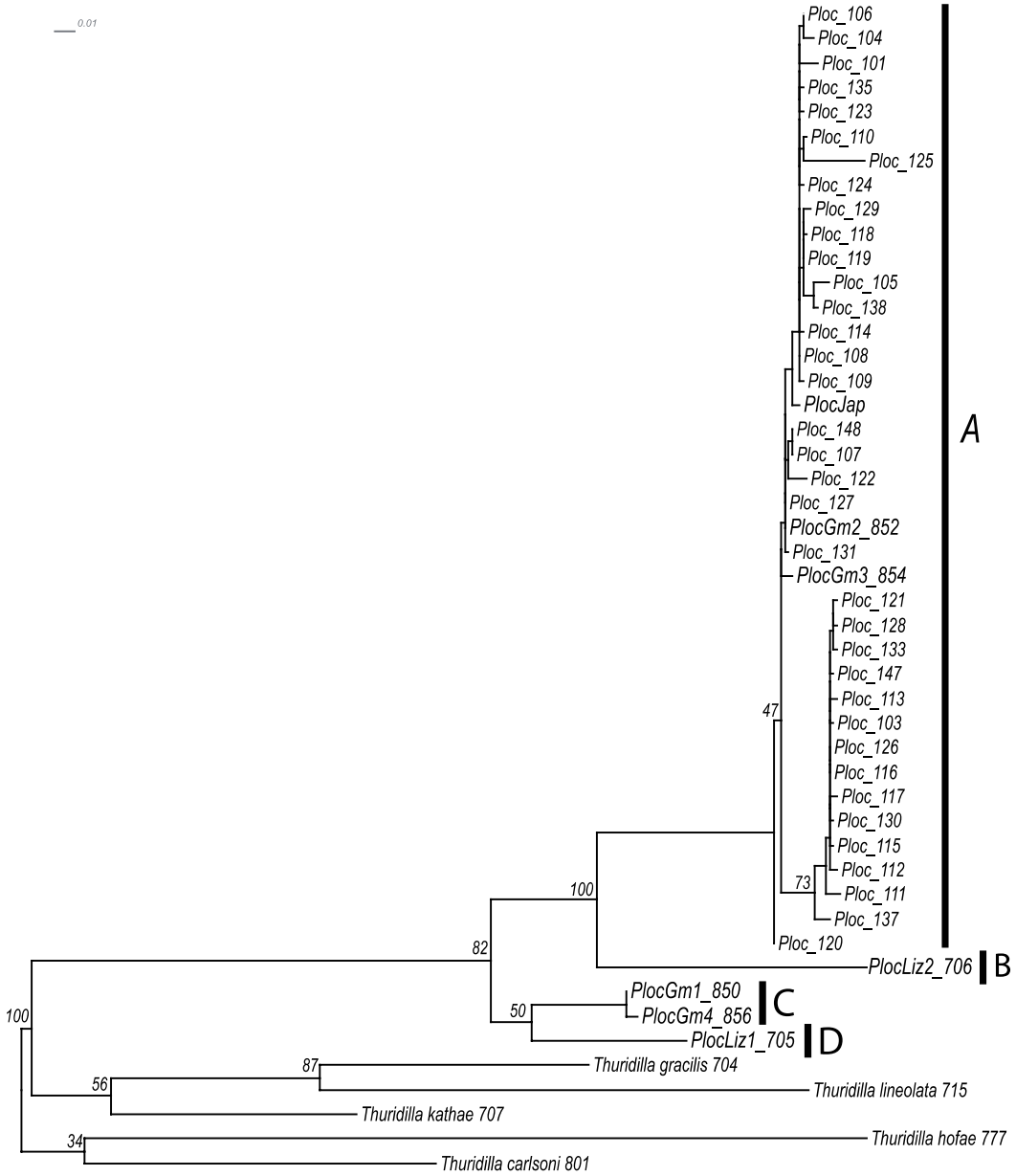

Figure 3 Maximum Likelihood tree (GTR+I+G) of *CO1* and *16S* gene fragments (concatenated dataset) using haplotypes of *Plakobranchus ocellatus* found on the Philippine Islands (smaller letters), Guam (Gm), Japan (Jap) and Lizard Island (Liz). As outgroups several *Thuridilla* species were included.

Chapter 3

Functional kleptoplasty in a limapontioid genus: Phylogeny, food preferences and photosynthesis in *Costasiella* with focus on *C. ocellifera* (Gastropoda, Sacoglossa)

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ABSTRACT

The evolution and origin of functional kleptoplasty (sequestration and retention of functional plastids) within the Sacoglossa is still controversially discussed. While some suggested it evolved at the base of the parapodia-bearing Plakobranchoidea, other suggested an earlier origin at the base of the Plakobranchacea. The latter is supported by positive results on CO_2 fixation in *Costasiella ocellifera*, a ceras-bearing Limapontioidea, and its maintenance of functional kleptoplasts for several weeks. However, the phylogenetic relationship of *Costasiella*, especially with regard to the Plakobranchoidea, was not clarified satisfactorily before, and the photosynthetic ability and the importance of photosynthesis within the genus hardly studied. In this study, we analyzed the phylogenetic position, photosynthetic activity and importance of photosynthesis for survival during starvation of five *Costasiella* species, focusing on *C. ocellifera*. We demonstrate that *Costasiella* is placed at the base of Limapontioidea; therefore, the requirements that allow functional kleptoplasty must have evolved at the base of the Plakobranchacea. Three *Costasiella* species are able to maintain chloroplasts, with *C. ocellifera* representing a long-term-retention form, and *C. kuroshimae* and *C.* sp. 1 short-term retention forms. All three species form a monophyletic group and feed mainly on *Avrainvillea*, whereas the two non-photosynthetic species, *C. nonatoi* and *C.* sp. 2 represent the sister clade that feed on algae other than *Avrainvillea*. Intriguingly, *C. ocellifera* survived under non-photosynthetic conditions for a minimum of 38 days, demonstrating that photosynthates may not be essential in the first place to survive starvation. These findings support our previous suggestion that kleptoplasts primarily represent a nutrition depot, whose function might benefit from ongoing photosynthesis.

INTRODUCTION

Sacoglossa is a group of sea slugs represented by nearly 300 species. They comprise the shelled Oxynoacea, ceras-bearing Limapontioidea and parapodia-bearing Plakobranchoidea (Jensen, 1997; Händeler et al., 2009). Within Metazoa the plakobranchoidean sea slugs are exceptional as some can survive persistent starvation periods due to plastids they sequestered from their food algae (Trench et al., 1972; Green et al., 2000; Händeler et al., 2009; Rumpho et al., 2009). This ability, termed kleptoplasty (Wägele & Martin, 2013), is also known among members of the Foraminifera (Lee, 2006), Ciliophora (McManus, 2012) and Dinoflagellata (Gast et al., 2007). From an evolutionary perspective kleptoplasty represents a possible key character that enhanced adaptive radiation within the Sacoglossa (Wägele, 2004). According to results of Händeler and colleagues (2009), this feature evolved at the base of the Plakobranchoidea, as a property that most likely delayed digestion. In contrast, Maeda et al. (2010) assumed that functional kleptoplasty evolved at the base of the Plakobranchacea and was lost multiple times within different genera. Two different levels of functional retention, based on maximum chlorophyll a fluorescence quantum yield measurement based on Pulse Amplitude Modulated fluorometer [PAM measurements, see revive of (Maxwell & Johnson, 2000)] introduced by Evertsen et al. (2006), are distinguished: (a) values higher than 0.5 for at least one to two days and decreasing values for up to two weeks are referred to as a short-term retention (StR); (b) values higher than 0.4 for 20 days with retention of plastids for several weeks and months are called long-term-retention (LtR). The latter is known from at least five different Plakobranchoidea (Jensen, 1997; Händeler et al., 2009). In contrast, members of the Oxynoacea and the Limpontioidea were all considered non-retention species (NR), in which maximum quantum yield values are zero, with one exception: *Costasiella ocellifera* (Simroth, 1895). Through CO2 fixation experiments, Clark and colleagues (1981) demonstrated for this limapontioidean species incorporation of functional plastids and a survival of up to 65 days while starving. Photosynthetic capability and efficiency during starvation was however not documented by PAM measurements. Moreover, Händeler et al. (2009) reported for *Costasiella kuroshimae* Ichikawa, 1993 maximum quantum yield values similar to those of the plakobranchoid *Thuridilla hopei* (Vérany, 1853) and stressed that functional kleptoplasty may be more widely distributed within the genus *Costasiella* than previously thought. According to the most recent sacoglossan phylogenies, functional kleptoplasty would then have either evolved once at

the base of the Plakobranchoidea, and once independently within the genus *Costasiella* (Händeler et al., 2009) or at the base of the Plakobranchacea with multiple losses of functionality within different genera (Maeda et al., 2010). However, the position of *Costasiella* is not clearly resolved in both phylogenies.

Besides phylogenetic origin of kleptoplasty, the mechanism that allows plastid longevity is also mysterious. Recent studies rejected a lateral gene transfer (LGT), which for a long time appeared the most attractive explanation: transcriptomic analysis and sequencing of slug egg DNA provided no evidence that algal genes supporting plastid longevity had been transferred to the slug nucleus (Pelletreau et al., 2011; Wägele et al., 2011; Bhattacharya et al., 2013). LGT was likewise refuted in the kleptoplastic Foraminifera (Pillet & Pawlowski, 2013) and most likely in the Dinoflagellata (Wisecaver & Hackett, 2010). Different factors enhancing kleptoplast maintenance (e.g. incorporation of specific plastids, light conditions or dual-targeting) have come into focus recently (Pelletreau et al., 2011; Rumpho et al., 2011; de Vries et al. 2014; Christa et al., 2014). Regardless of the underlying mechanism, photosynthesis is thought to play a key role during starvation (Trench & Gooday, 1973; Trench, Boyle & Smith, 1973a). Yet, there is no direct evidence that photosynthates are actively released by the kleptoplasts, as stated by (Trench & Gooday, 1973). This raises the question of whether photosynthesis itself is mandatory for the slugs to survive starvation periods. Recently, Klochkova et al. (2013) even doubted that CO_2 fixation of kleptoplasts is necessary at all to survive starvation periods within the StR form *Elysia nigrocapitata*. Plastids may function as a food depot and photosynthesis may play a secondary role by increasing this internal food depot without an immediate transport of photosynthates into the cytosol (Christa et al., 2014). This depot would only become available if either the plastids degrade or if the slug may digest them when needed.

Here we examined photosynthetic performance of five Costasiella species: *C. ocellifera* (Fig. 1A), *C. nonatoi* (Marcus & Marcus, 1960) (Fig. 1B), *C. kuroshimae* (Fig. 1C), C. sp. 1 (Fig. 1E) and *C.* sp. 2 (Fig. 1D). We analyzed their food sources and present results on their phylogenetic relationship within Limapontioidea. For *C. ocellifera* we analyzed importance of photosynthesis for the survival during starvation. Our results support the hypothesis that functional kleptoplasty in sea slugs evolved earlier within Sacoglossa and we question the contribution of kleptoplasts to the survival of the slugs during periods of starvation.

MATERIALS AND METHODS

Species collection and starving experiments

Specimens of *Costasiella* were collected at several places (Supplementary Table 1) by snorkeling and either fixed immediately in 70% EtOH for food barcoding or examined on Guam, USA, on the Florida Keys, USA or transferred to Bonn, Germany. On Guam and on the Florida Keys, specimens were kept under natural day-night rhythm with light intensities up to 600µmol quanta/m^2s in individual tanks with ocean water and water change every second day, at 24°C. In Bonn, all *Costasiella* specimens were starved at, 20-22°C in individual tanks with artificial seawater under different light conditions and experimental set-ups (see Table 1), with water changed every second day. We set up laboratory light conditions using a full spectrum day light lamp (Androv Medical, Model AND1206-CH). Monolinuron, a photosynthesis-blocking chemical, was purchased in a pet shop from JBL GmbH & Co KG, Neuhofen, Germany as Algol with a concentration of 4000mg/L Monolinuron. The final concentration of Monolinuron was set to 2µg/ml seawater. Monolinuron inhibits the binding site of Plastoquinone (Q_A) at the D1 protein, thus blocks the electron transport between Q_A and the Photosystem II, which leads to an inhibition of the electron transport chain (Arrhenius et al., 2004). Its effectiveness is seen by rapidly increasing ground fluorescence.

PAM measurements

Maximum quantum yield (Fv/Fm) values were measured for all *Costasiella* species with a Diving-PAM (Walz, Germany) (see Table 1 and Supplementary Table 3). One measurement of every individual was taken on a regular base with 15 min dark adaption prior to measurements (individual measurement values available upon request). We performed the measurements with an optic fiber and placed it prior to measurements on the background of the according petri dish to set ground-fluorescence to zero (F_0). Then we placed the optic fiber 3-5mm above the slug to obtain F_0 between 200-500 measured with a red light of 0.15µmol photons/m^2s emitted by a red LED at 0.6 kHz. The maximum chlorophyll a fluorescence (F_v/F_m) was subsequent measured with a white light emitted by a halogen lamp at an irradiance of 10.000µmol photons/m^2s for 0.8s at 20 kHz. For *C. ocellifera* and *C. nonatoi* we set up additional starvation conditions (Table 1) to determine importance of

photosynthesis during starvation. Experiments were stopped for further investigation at day 38, which represent 76% of the maximum starvation period we determined for *C. ocellifera* under a natural days-light rhythm.

Food Barcoding

For Barcoding of food sources of *C. nonatoi* and *C. ocellifera* we used non-starved animals, directly fixed in 70% EtOH after collection. We amplified the plastid gene *rbcL* with Ulvophyceaen specific primer (forward primer rbcLF: 5' AAA GCN GGK GTW AAA GAY TA 3' and reverse primer rbcLR: 5'-CCA WCG CAT ARA NGG TTG HGA-3'; Pierce et al. (2006)). *tufA*, as suggested in Christa et al. (2013), was not investigated, because no ambiguous food sources were found after analysis of *rbcL* sequences. 1 µl of genomic DNA was used as template in a 10 µl final volume reaction supplied with 1 µl sterilized water, 1 µl Qiagen Q-Solution, 5 µl of double concentrated Qiagen Multiplex PCR Master Mix and 1 µl of 10 pmol/µl concentrated primer each. Amplification of *rbcL* was performed by an initial denaturation for 15 min at 95 °C, followed by 9 touch-down cycles at 94 °C for 45 s, 60 °C (-1 °C per cycle) for 45 s, 72 °C for 90 s, followed by 25 standard cycles (94 °C for 45 s, 51 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. PCR products were size-fractionated in a 1.5 % agarose gel for 90 min at 70 V. Bands were extracted from the gel according to desired gene-fragment length (560 for *rbcL*) using Machery-Nagel Nucleo Spin Extract II kit. Isolated fragments were ligated into pGEM t-easy Vector (Promega) and cloned into competent *E. coli* XL1-blue cells (Stratagene). For 12 clones of each individual the cloned *rbcL* product was again amplified in a, 20 µl final volume reaction supplied with 14 µl sterilized water, 5 µl of double concentrated Larova PCR Master Mix (Berlin, Germany) and 1 µl of 10 pmol/µl concentrated primer each (forward primer T7Promoter 5'TAA TAC GAC TCA CTA TAG GG 3' and reverse primer SP6Promoter 5' ATT TAG GTG ACA CTA TAG 3'). Amplification was performed by an initial denaturation for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. Amplification products were purified and all samples were sequenced by Macrogen Inc, (Amsterdam, The Netherlands) and sequence identity was verified by BLAST search using the NCBI homepage. Consensus sequences were generated when sequence divergence of chloroplast genes was lower than 1 %. All created sequences were verified by BLAST search using the NCBI homepage and combined with a set of

corresponding algae sequences (alignment available upon request) to create a dataset of overall 39 rbcL sequences (561bp in length). A maximum likelihood tree was generated using raxMLHPC (v. 7.2.8, (Stamatakis, 2006) on a local Mac OS X (v. 10.7.5) with substitution model GTR + G + I to identify plastid origin.

Feeding experiments

Specimens of *C. ocellifera* were starved under a natural day/night cycle with light intensities up to 600µmol quanta /m^2s on the Florida Keys in individual tanks with water changed ever second day. Maximum quantum yield (Fv/Fm) values were measured daily and, after a decrease to zero, specimens were fed for seven days with *Avrainvillea* (n=6), *Udotea* (n=5), *Penicillus* (n=3) or *Pseudochlorodesmis* (n=5), respectively. Change of maximum quantum yield (Fv/Fm) values was recorded by daily measurements (individual measurements available upon request). Out of each set-up one specimen with the highest yield value was used for barcoding (see below) to verify whether provided food was consumed or not.

Phylogenetic relationship

To analyze phylogenetic relationship of *Costasiella* we investigated partial genes of *16S*, *COI*, *H3* and *28S* sequences of overall 79 Sacoglossa taxa, representing major groups. *Siphonaria pectinata* Linnaeus, 1758 was used as outgroup based on results from (Jörger et al., 2010) and (Neusser et al., 2011). Sequences were downloaded from GenBank or newly produced (Supplementary Table 1). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions and stored at -20 °C. Amplification reactions were carried out using 1 µl of genomic DNA in a, 20 µl final volume reaction supplied with 5 µl sterilized water, 2 µl Qiagen Q-Solution, 10 µl of double concentrated Qiagen Multiplex PCR Master Mix and 1 µl of 10 pmol/ µl concentrated primer each with Sacoglossa specific primer pairs for *16S, COI*, *H3* and *28S* ((Vonnemann et al., 2005; Bass, 2006), Supplementary Table 2). Amplification of partial *COI* was performed by denaturation for 15 min at 95 °C, followed by 25 standard cycles (94°C for 90 s, 48 °C for 90 s and 72 °C for 90 s) and a final extension at 72°C for 10 min. Amplification of partial *16S* was performed by denaturation for 15 min at 95 °C, followed by 9 touch-down cycles (94 °C for 90 s, 58 °C (-1 °) for 90 s, 72 °C for 90 s) followed by 25 standard cycles (94 °C for 90 s, 49 °C for 90 s and

72 °C for 90s). Amplification of partial *H3* was performed by denaturation for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 90 s, 50 °C for 90 s and 72 °C for 90 s). Amplification of partial *28S* was performed by denaturation for 15 min at 95 °C, followed by 9 touch-down cycles (94 °C for 90 s, 65 °C (-1 °) for 90 s, 72 °C for 90 s) followed by 25 standard cycles (94 °C for 90 s, 56 °C for 90 s and 72 °C for 90 s). Sequences of each gene were aligned individually using Mafft (Katoh et al., 2002) and subsequently concatenated (Alignment available upon request). For *COI* only 1st and 2nd positions were used according to the analysis of Händeler et al. (2009). Analysis of the concatenated dataset with Gblocks (Castresana, 2000) did not reveal the necessity for alignment masking. A Maximum likelihood consensus tree was obtained by applying the RaxML algorithms as implemented in raxMLHPC (v. 7.2.8, (Stamatakis, 2006) on a local Mac OS X (v. 10.7.5) with substitution model GTR + G + I and1000 replicates for bootstrapping analysis. Bayesian analysis were performed using MrBayes (v. 3.2, (Ronquist et al., 2012) on a local Mac OS X (v. 10.7.5) with the GTR model and two random starting trees. For each tree three heated and one cold Markov chain were used and run for 4.000.000 generations with sampling each 1000th generation. After 3.148.000 generations the run was stopped, because average standard deviation of split frequencies was lower than 0.005 and log likelihood values of the cold chain did not further increase. The first 1000 trees of both runs were discarded as "burn-in" and a majority rule consensus tree, of the remaining 4296 trees (2148 of both runs), calculated. Posterior Probabilities (PP) were calculated to determine nodal support.

RESULTS

PAM measurements

We analyzed the photosynthetic performance of five *Costasiella* species (Fig. 1A-E) in a natural day/night cycle during starvation by in situ PAM measurements (Fig. 2). *C. ocellifera* survived 52 days in our hands, the longest starvation period of all *Costasiella* species investigated so far (Supplementary Table 3). Maximum quantum yield values exceeded 0.5 for 21 days and 0.2 for the following 28 days before declining to zero. These results are in line with other long-term-retention forms, for example *Elysia clarki* Pierce, Curtis, Massey, Bass, Karl & Finney, 2006 (Middlebrooks et al., 2011). Maximum quantum yield values in *C.* sp. 1 were higher than 0.4 during the eight days of starvation experiment (Fig. 2) similar to results observed for plakobranchoid short-term-retention forms, such as

E. *viridis* ((Montagu, 1804)) or *E*. *nigrocapitata* Baba, 1957 (Klochkova et al., 2010). In contrast, *C*. *kuroshimae* had lower maximum quantum yields, not exceeding 0.4 over a starvation period of 10 days (Fig. 2), similar as in *Thuridilla hopei* (Händeler et al., 2009), a species addressed as a short-term-retention form. Individuals of *C*. sp. 1 and *C*. *kuroshimae* died before maximum quantum yields declined to zero. Neither *C*. *nonatoi* nor *C*. sp. 2 showed any ground fluorescence at all (Supplementary Table 3).

Starving experiment of C. ocellifera during blocked photosynthesis

Starving experiments with *C. ocellifera* were carried out for a maximum of 38 days (Table 1), representing 76% of the maximum starvation time observed during our preceding experiments for *C. ocellifera*. Shortening the starving period ensured higher healthiness at the moment of tissue fixation, a necessity for further experiments not addressed here. Four animals were kept under a 12h/12h light/dark cycle at 25µmol m^{-2}s (LL) in Bonn, eight animals were kept in complete darkness on the Florida Keys, USA. Maximum quantum yield values of both set-ups were nearly identical and continuously higher than those measured in natural conditions (Fig. 3A). In darkness and low light treatments F_v/F_m never declined below 0.4, whereas in natural conditions after day 33 values were always lower than 0.4 (Fig. 3A). Maximum quantum yield values dropped immediately to less than 0.4 throughout the entire experiment when specimens were simultaneously treated with Monolinuron (Fig. 3B). During the 38 days F_v/F_m values declined in specimens of the LL plus Monolinuron treatments (with a similar slope as the animals starved only under LL), which we did not observe in the animals kept in darkness and with Monolinuron (Fig. 3B).

Nearly all investigated specimens survived the targeted 38 days regardless of experimental set-up. The premature death of seven individuals (two in the control group, two with Monolinuron, one in darkness, tow in darkness with Monolinuron) is most likely attributable to individual fitness, rather than experimental set-up. To test if there is a general ability in *Costasiella* to survive starvation, we conducted starving experiments under LL, LL plus Monolinuron and in complete darkness for the NR form *C. nonatoi*, the sympatric congener of *C*. *ocellifera* (Supplementary Table 3). Since *C. nonatoi* did not exhibit any fluorescence in the former experiments (see above), PAM measurements were not performed. Six of the nine specimens used in the experiments survived 20 days without performing any photosynthesis at all, with a maximum starvation period of 28 days. Three specimens died whilst attempting to leave the petri dishes.

Food-barcoding

In recent works the molecular identification of food sources using *rbcL* proofed to be a rapid and precise tool (Curtis et al., 2006; Pierce et al., 2006; Händeler et al., 2010; Maeda et al., 2012; Christa et al., 2013). This technique revealed more information on food sources than feeding experiments conducted previously (Christa et al., 2013). We applied barcoding to unambiguously identify the food sources of the examined *Costasiella* species, to verify literature data based on feeding observations and to check for a possible correlation of food sources and retention ability. According to our results, all retention forms feed at least on one species of *Avrainvillea*, with *C. ocellifera* exclusively feeding on *Avrainvillea mazei*, *C.* sp. 1 and *C. kuroshimae* on an unidentified species of *Avrainvillea* and *C.* sp. 1 additional on an unidentified *Rhipilia* and *Pseudochlorodesmis* species (Supplementary Fig. 1). In non-retention forms *Avrainvillea* as a chloroplast source is completely missing, although described as food elsewhere (Jensen, 1993): *C. nonatoi* feeds upon unknown taxa related to *Bryopsis* and *Pseudochlorodesmis* sp., *C.* sp. 2 on *Tydemania expeditionis* and the same unknown *Rhipilia* species as *C.* sp. 1 (Supplementary Fig. 1).

Feeding experiments

Specimens of *C. ocellifera* are often not associated with their host algae *Avrainvillea.* Through feeding experiments using various algae found in the surrounding environment, we analyzed whether other food sources may also be consumed and potentially used by *C. ocellifera* to establish functional kleptoplasty (Supplementary Table 4). Only consumption of *Avrainvillea mazei* resulted in a constant increase of F_v/F_m without any specimens dying (Fig. 4, Supplementary Table 4). Providing *Udotea* sp. as food source resulted in a short increase of F_v/F_m, but three out of the five investigated animals did not survive the seven days of experiment. Neither *Pseudochlorodesmis* sp., nor *Penicillus dumestosus* resulted in an increased F_v/F_m (Fig. 4) and some animals died during the feeding period (Fig. 4, Supplementary Table 4). Barcoding revealed that *Udotea*, *Penicillus* and *Pseudochlorodesmis* chloroplasts were not incorporated during these feeding experiments with *C. ocellifera* (Supplementary Table 4). Single sequences of *Bryopsis* (specimen CoocPe1) were identified after starvation and subsequent feeding experiments although this alga was not provided as food source (Supplementary Table 4).

Phylogenetic relationship

In our analysis the monophyletic genus *Costasiella* is a rather basal group within paraphyletic Limapontioidea, and non-retention species of *Costasiella* form a monophyletic sister taxon to the functional-retention-forms (Fig. 1F). The Platyhedylidae is sister taxon to the Limapontioidea and the Plakobranchoidea, thus placed at the base of the Plakobranchacea (Fig. 1F). Some limapontioid genera and families are well supported: the Limapontiidae form a monophyletic group, as well as the Hermaeidae. Monophyletic *Cyerce* is the sister taxon to *Mourgona* and *Polybranchia* resulting in a paraphyletic *Polybranchiidae* (Fig. 1F). Plakobranchoidea group with the limapontioid family Hermaeidae, but support values are very low and the clade not resolved.

DISCUSSION

Functional kleptoplasty and phylogenetic relationship

Our PAM measurements confirm the earlier hypothesis that functional kleptoplasty is not exclusively limited to Plakobranchoidea (Clark et al., 1981; Händeler et al. *20*09), but can also be observed in the limapontioidean genus *Costasiella*. *C. ocellifera* incorporates functional plastids that survive for several weeks, similar as in the long-term retention species of the Plakobranchoidea (e.g., *Plakobranchus ocellatus*, *Elysia chlorotica*, *E. crispata* and *E. timida*). We could further demonstrate that two other *Costasiella* species, *C. kuroshimae* and *C.* sp. 1, are short-term retention forms, similar to *Thuridilla hopei* (Händeler et al., 2009). The remaining two investigated *Costasiella* species *C. nonatoi* and *C.* sp. 2 appear to be non-retention forms that digest the plastids rapidly upon incorporation.

Results on functional kleptoplasty based on carbon fixation rates in other limapontioid genera, e.g. *Mourgona* (Evertsen et al*.,* 2009), have until now not been confirmed in situ through PAM measurements. PAM measurements, a commonly used method for studying photosynthetic ability of Sacoglossa (reviewed in Cruz et al., 2013), exist for only about 40 sacoglossan species, representing roughly 10% of known species (Händeler et al., 2009; Yamamoto et al., 2009; Klochkova et al., 2010; 2012). Of these, only 11 limapontioidean species are examined regarding their ability to incorporate functional

plastids. Further investigations, especially of the latter, are needed to finally conclude whether further sacoglossans- besides some members of the genus *Costasiella* - can perform photosynthesis.

Confirming previous published molecular phylogenetic analyses on Sacoglossa (Händeler et al., 2009; Maeda et al., 2010), our phylogenetic analysis places *Costasiella* at the base of Plakobranchacea, and not close to the Plakobranchoidea — the group known to exhibit functional kleptoplasty in nearly all members for at least a few days. It is interesting that *Costasiella* species with functional kleptoplasts form a sister clade to some *Costasiella* species that do not retain plastids. A differentiation in a photosynthetic clade might be a result of evolutionary pressure caused through oxidative stress in retention-forms or an ecological separation based on food sources. However, these assumptions do not explain the monophyletic non-retention clade and they are also not supported by results on the plakobranchoid genus *Elysia*, where photosynthetic and non-photosynthetic forms are not separated into two distinct clades (Händeler et al., 2009; Maeda et al., 2010; Wägele et al., 2011). According to the phylogenetic relationship presented here and former results (Händeler et al., 2009; Maeda et al., 2010; Wägele et al., 2011), two scenarios are conceivable: (1) functional kleptoplasty evolved at the base of the Plakobranchaceae and was lost secondarily within probably most limapontioidean lineages, Platyhedylidae and within *Costasiella* (Maeda et al., 2010), or (2) functional kleptoplasty evolved independently within the lineage of *Costasiella* and the Plakobranchoidea (Händeler et al., 2009). Until more limapontioidean taxa are investigated with regard to a possible functionally kleptoplasty, none of these hypotheses can currently be favored.

Starving experiment of C. ocellifera with blocked photosynthesis

Starvation periods of 28 days of the non-retention form *C. nonatoi* shows that in Sacoglossa a general ability to survive food depletion - independent on retention abilities – is possible. The long-term retention form *C. ocellifera* survived 38 days of starvation, irrespective whether photosynthesis was either blocked by keeping the animals in darkness or by adding the photosynthesis blocker Monolinuron. We have not compared ultimate survival of *C. ocellifera* specimens under conditions with blocked and non-blocked photosynthesis to non-retention forms. However, we would assume that blocking photosynthesis also reduces the life span. Inhibited photosynthesis by Monolinuron did probably not block every PSII reaction center of the plastids, referring to our results. Some plastids may still

able to fix carbon – although to a much lower degree - and thus contribute in building up energetic polymers that finally can become available for the slugs (Christa et al., 2014). Hence these slugs would probably survive longer than those that are not able to photosynthesize at all, but should die quicker due to the lower amount of energy made available to the slugs. Similar to our results on *Costasiella*, Christa et al. (2014) and Klochkova et al. (2013) observed a high survival of slugs without performing photosynthesis. Earlier results on survival in darkness partly contradict these recent studies, but are very often not discussed (Trench, 1969; Hinde & Smith, 1972; 1975; Hawes, 1979; Giménez-Casalduero & Muniain, 2008; Pelletreau et al., 2011; Wägele et al., 2011; Maeda et al., 2012; Yamamoto et al., 2012; Bhattacharya et al., 2013). Klochkova and co-workers observed that the StR form E. nigrocapitata might survive up to 5 months without photosynthesis during starvation. They stated that additional factors to photoautotrophic CO_2 fixation are important for surviving starvation periods. Evertsen & Johnson (2009) provided a key observation, by examining starch grains in the plastids of starved *E. viridis*. In these specimens starch grains were larger than in fresh collected animals. Vettermann (1973) has shown for the chlorophyte *Acetabularia* that thylakoid membranes of the plastids are transformed into starch when kept in darkness, non-photosynthetic light, or when enucleated. We do not know whether this also happens in all chlorophyte plastids, but since plastids are incorporated into the slugs without the algal nuclei and are probably more shaded than in the algae, a transformation of thylakoid membranes into starch might also occur here. We assume that this starch provides a better energy source for the slug in the long term during starvation. By not digesting plastids at once, the slugs may rely on the prolonged life of the plastids, especially when kept in darkness and under reduced photo-damaging conditions. These plastids with various life spans finally contain a high starch content, which then becomes available by degradation to the slugs.

Food barcoding and feeding experiments

Costasiella species that show functional retention feed upon *Avrainvillea, Rhipilia* and *Pseudochlorodesmis.* This is in contrast to the plakobranchoidean long-term retention forms that mainly feed on *Halimeda*, *Acetabularia*, *Caulerpa* or *Vaucheria* (Christa et al., 2013, unpubl. data). Feeding experiments with algae found in the natural environment did not reveal any additional food sources, but supported our barcoding results with regard to

Avrainvillea as the main source of the plastids. Interesting, all non-retention *Costasiella* species consumed other algal species; *Avrainvillea* was never detected via barcoding although described as food source for *C. nonatoi* in literature (Jensen, 1993). This gives evidence that functional kleptoplasty within *Costasiella* relies on plastids of *Avrainvillea* and we have to ask, whether *Avrainvillea* plastids have similar "surviving" properties as those from e.g., *Vaucheria* and *Acetabularia*, that allow long term maintenance in the slugs.

Conclusion

With our detailed results on a rather under-investigated solar-powered clade within the Sacoglossa with short- and long-term retention forms, we have broadened our knowledge on kleptoplasty in these enigmatic sea slugs. Based on the diversity of functional kleptoplasty found in the genus *Costasiella*, we suggest investigating more species within other limapontioidean taxa, since a general deduction for limapontioidean genera based on few observations on selected species is not possible. Our experiments also show that plastids, that are not digested at once, can be blocked in their activity, but still may function as a food depot that help the slugs to survive starvation periods. The chlorophyte *Avrainvillea* has to be included in the list of those algae that have properties allowing a long survival in the slug's digestive system.

AUTHORS' CONTRIBUTION

GC, HW, KH, WFM and SBG planned the experiments. GC, KH and HW collected the material. GC, HW, DK, JF, MV performed the experiments and analyzed the molecular data. GC, HW, WFM and SBG analyzed the data and wrote the paper.

ACKNOWLEDGEMENT

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Commision (FWC) provided collection permission of material on the Florida Keys. Collection of material in Guam was performed on a permit provided to Peter Schupp.

TABLES

Table 1. List of experimental settings for *Costasiella ocellifera* and *C. nonatoi*. A full spectrum day light lamp was used in all light set-ups. All specimens were kept in individual tanks with artificial seawater and starved over a defined period (Days of starvation). Experiment set-ups were either done without additional parameters (control), with 2µg/ml seawater Monolinuron or in complete darkness. Number of taxa differs due to collection success when a particular experiment was performed.

Species	Light condition	Light intensity	Experimental condition	#	Days of starvation	Mean starting F_v/F_m value	Standard deviation	Mean final F_v/F_m value	Standard deviation
Costasiella ocellifera	12h/12h (day/night)	25 µmol photons m-2 s-1	control	4	38	0.71	± 0.02	0.419	-
			Monolinuron	6		0.393	± 0.72	0.092	± 0.11
	Complete Darkness		control	8		0.731	± 0.06	0.442	± 0.15
			Monolinuron	4		0.393	± 0.05	0.362	± 0.01
Costasiella nonatoi	12h/12h (day/night)	25 µmol photons m^{-2} s^{-1}	control	3	28	-		-	
			Monolinuron	3		-		-	
	Complete Darkness		control	3		-		-	

FIGURES

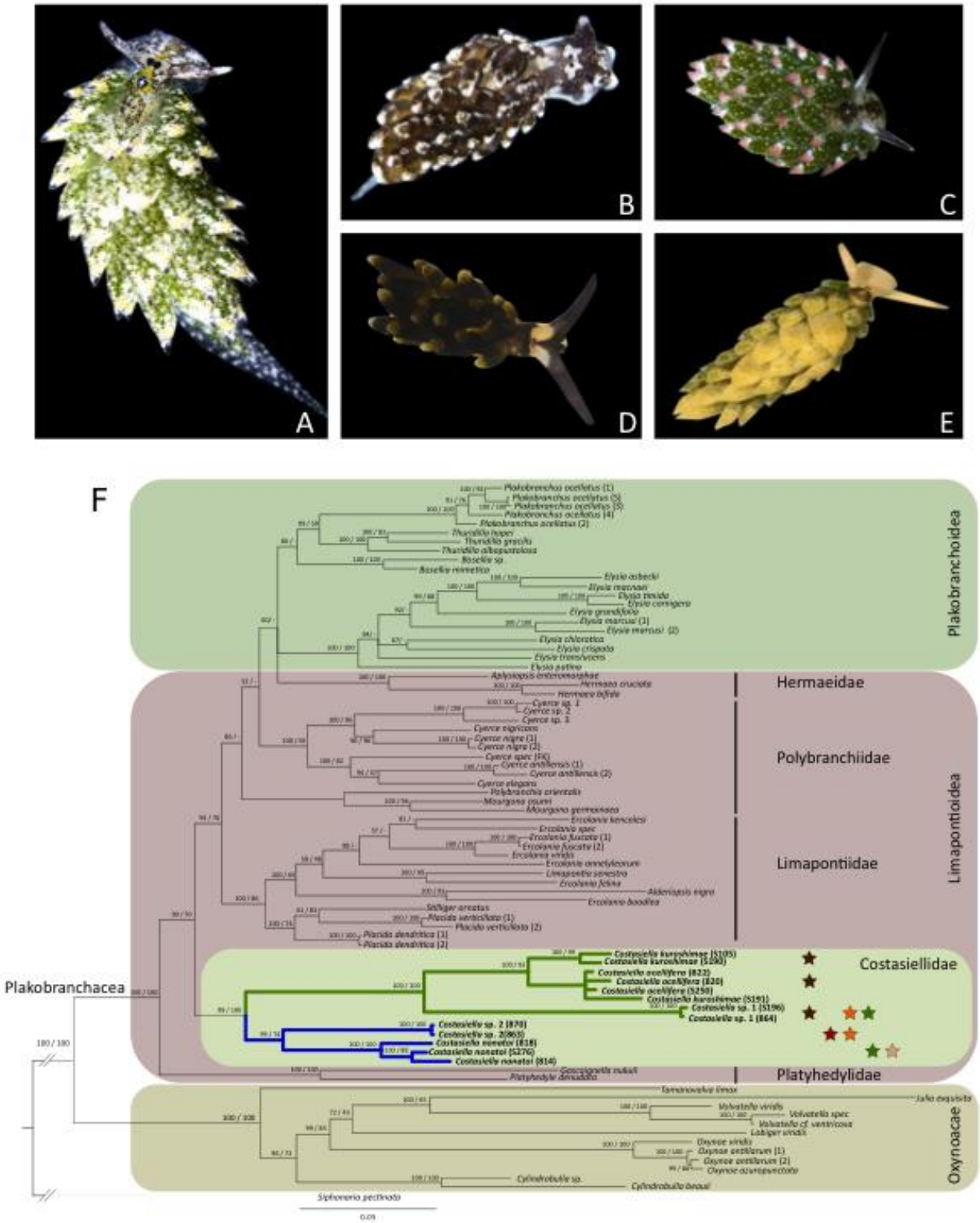

Figure 1. Species of *Costasiella* investigated. **A.** *Costasiella ocellifera* (USA, Florida Keys), **B.** *C. nonatoi* (USA, Florida Keys) **C.** *C. kuroshimae* (USA, Guam) **D.** *C.* sp. 2 (USA, Guam), **E.** *C.* sp. 1 (USA, Guam) **F.** Phylogenetic

relationship of *Costasiella* based on partial sequences of *16S*, 1st and 2nd position of *COI*, *H3* and *28S*. 50% majority rule tree, Bayesian analysis. Numbers at nodes represent Posterior Probabilities (Bayesian analysis) and bootstrap values (ML analysis). *Siphonaria pectinata* was chosen as outgroup. Stars indicate food sources of *Costasiella* species identified by barcoding using *rbcL*: brown = *Avrainvillea*, red = *Tydemania*, orange = *Rhipilia*, green = *Pseudochlorodesmis*, beige = *Bryopsis*.

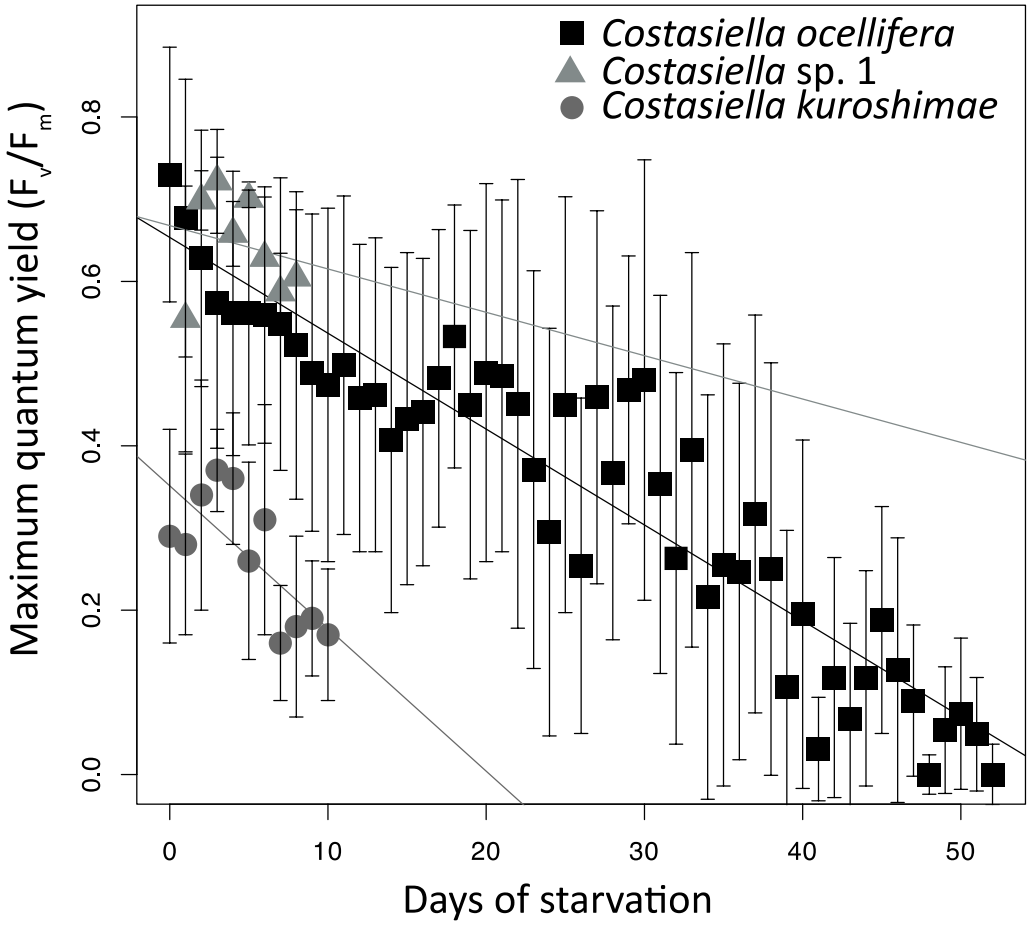

Figure 2. PAM measurements of *Costasiella* species. Maximum quantum yield values (Fv/Fm) of *C. ocellifera* (n=31, squares), *C.* sp. 1 (n=2, triangles) and *C. kuroshimae* (n=6, circles) during starvation periods under natural light conditions. Shown are the mean values of measured specimen and the error bars indicate the standard deviation.

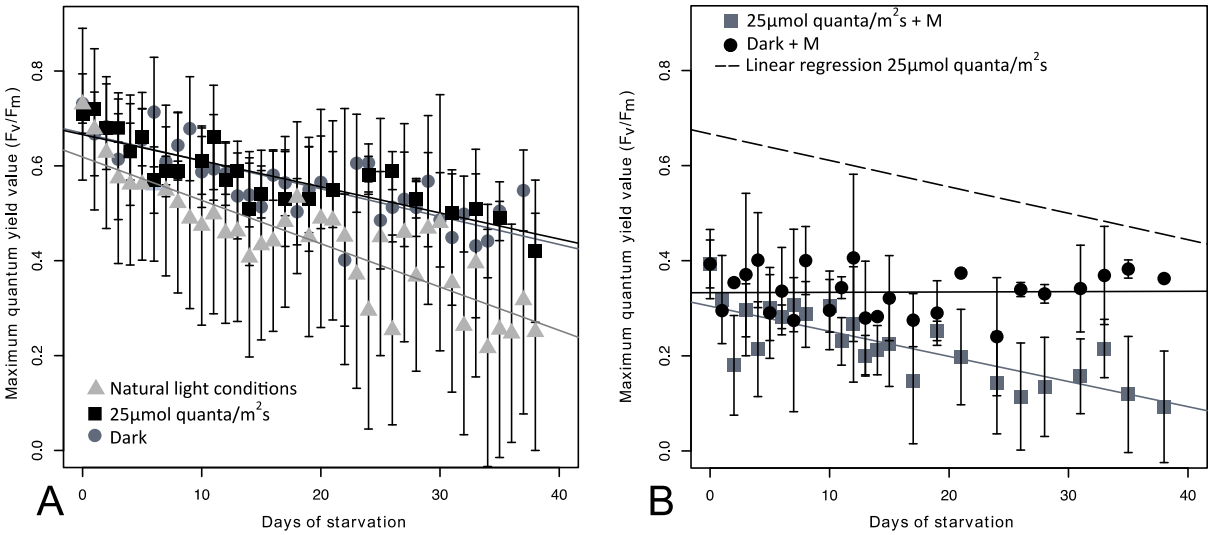

Figure 3. PAM measurements of *Costasiella ocellifera*. Maximum quantum yield values (Fv/Fm) during starvation of 38 days under different light conditions. **A.** Various light conditions; triangles = natural light conditions (=31), squares = 25 µmol quanta/m²s (LL) (n=4), circles = complete darkness (n=8). **B.** Comparison of specimens treated with photosynthesis blocker Monolinuron under various light conditions; squares = 25 µmol quanta/ m²s + Monolinuron (n=6), circles = complete darkness + Monolinuron (n=4). Shown are the mean values of measured specimen and the error bars indicate the standard deviation.

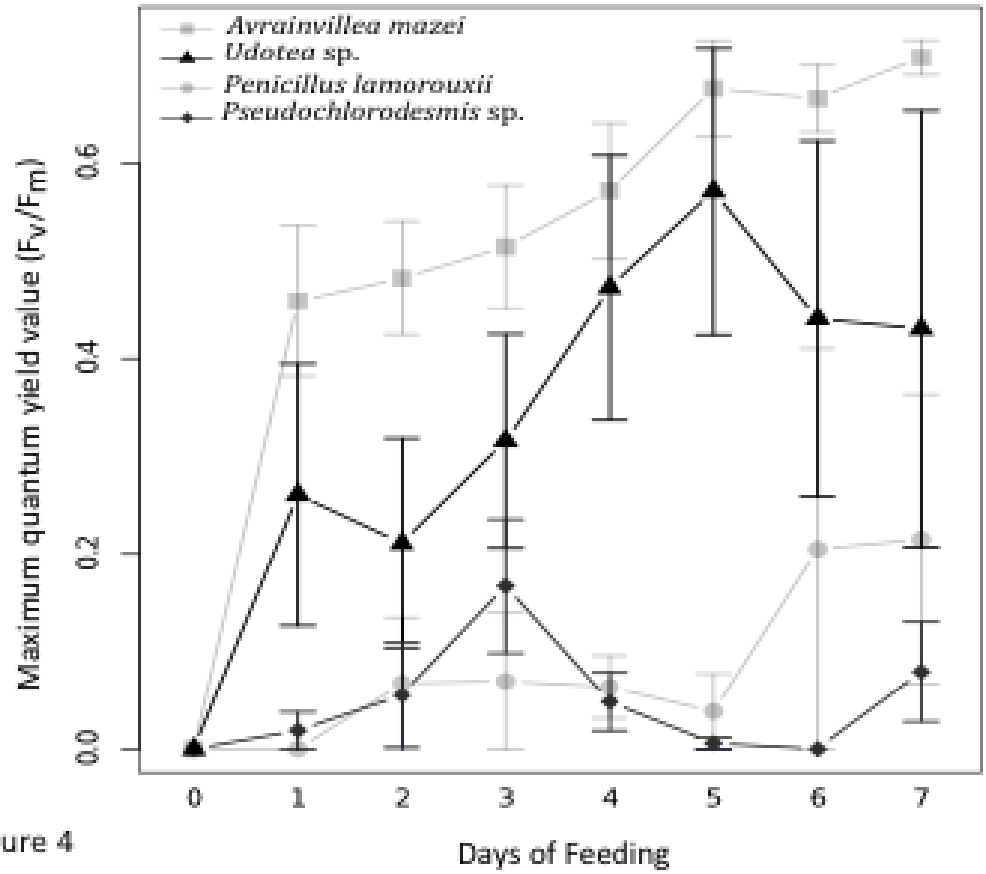

Figure 4

Figure 4. Feeding experiment of *Costasiella ocellifera*. Maximum quantum yield values (Fv/Fm) during 7 days of feeding with different food sources under a natural day-night rhythm with light intensities up to 600µmol quanta $m^{-2}s^{-1}$. Food sources were provided after specimens were starved until maximum quantum yield values declined to zero; squares = *Avrainvillea mazei* (n=6), triangles = *Udotea* sp. (n=5), circles = *Penicillus lamourouxii* (n=3), diamonds = *Pseudochlorodesmis* sp. (n=5). Shown are the mean values of measured specimen and the error bars indicate the standard deviation.

SUPPLEMENTARY DATA

For the Supplementary Data please see the attached CD. There is a folder named "Chapter 3 - Functional kleptopalsty in a limapontioid genus" which contains all additional information.

Supplementary Table 1. Overview of collection places, dates and Accession numbers of examined species in this study. # indicates internal specimens voucher; for better displaying different species in the tree, numbers in brackets are used.

Supplementary Table 2. Primer used for phylogenetic analysis in this study

Supplementary Table 3. Maximum quantum yield values (Fv/Fm) of PAM-measurements of *Costasiella* specimens under natural light conditions. **LtR**= Long-term-retention, **StR**= Short-term-retention, **NR**= No retention. * Food sources revealed by DNA-Barcoding.

Supplementary Table 4. Feeding experiment of *C. ocellifera*. Different food sources were provided and maximum quantum yield values recorded for seven days during feeding. Afterwards one specimen of each feeding set was barcoded (using *rbcL*) to identify if cps of food sources were incorporated or not.

Supplementary Figure 1. ML tree of *rbcL* sequences. Algae sequences with highest BLAST identity to sequences found in *Costasiella* species were included. Highlighted in red are sequences found in photosynthetic inactive species, in blue sequences found in photosynthetic active forms.

Chapter 4

Does it matter, what you eat? Barcoding of sequestered chloroplasts in photosynthetic and non-photosynthetic sacoglossan sea slugs (Mollusca, Gastropoda)

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ABSTRACT

Background

Sacoglossan sea slugs are well known for their unique ability among metazoans to incorporate functional chloroplasts (kleptoplasty) in cells of their digestive gland, enabling the slugs to use these as energy source when starved for weeks and months. However, members assigned to the Oxynoacea (shelled Sacoglossa) and Limapontioidea (often with dorsal processes) are in general not able to keep the incorporated chloroplasts functional during periods of starvation. Since obviously no algal genes are present within three (out of six known) species with chloroplast retention of several months, other factors than horizontal gene transfer that enable functional kleptoplasty have to be considered. Certainly, the origin of the chloroplasts is important, however, food source of most of the about 300 described species, including several photosynthetic active ones, is not known so far. Therefore, a deduction of specific algal food source as a factor to perform functional kleptoplasty was still missing.

Results

We investigated the food sources of 26 sacoglossan species, freshly collected from the field, by applying DNA-Barcoding methods. We used the chloroplast marker genes *tufA* and *rbcL* and subsequently combined and compared them with literature data of species known for their retention capability, either based on PAM-measurements or on CO_2 fixation experiments. For the majority of the investigated species, especially for the genus *Thuridilla*, we were able to identify the precise food source for the first time. Furthermore, published data based on feeding observations were confirmed and enlarged by the molecular methods. We analyzed food sources of sacoglossan taxa in correlation to their chloroplast retention capability and found that certain chloroplasts are most likely essential for establishing functional kleptoplasty.

Conclusions

Applying DNA-Barcoding revealed to be very efficient compared to feeding experiments or ultrastructural analyses and allowed a detailed insight into sacoglossan food sources. We favor *rbcL* over *tufA* for future analyses, because the former revealed more reliable results. *TufA* might be used additionally in ambiguous cases. We narrowed down the algal species that seems to be essential to long-term-functional photosynthesis: *Halimeda, Caulerpa,*

Penicillus, Avrainvillea, Acetabularia and *Vaucheria.* None of these were found in *Thuridilla* gut contents, the only species rich plakobranchoidean genus without long-term retention forms. We also show that the chloroplast type does not solely determine functional kleptoplasty; members of no-retention genera, such as *Cylindrobulla* or *Volvatella,* feed on the same algae as e.g., the long-term-retention forms *Plakobranchus ocellatus* or *Elysia crispata*, respectively. Evolutionary benefits of functional kleptoplasty are still questionable, since a polyphagous life style would be more independent of specific food sources and their abundance.

Keywords: DNA-Barcoding, Food analyses, Kleptoplasty, *rbcL*, Sacoglossa, *tufA*

BACKGROUND

Sacoglossa sea slugs are a relatively small group of heterobranch gastropods with currently about 300 species described (Jensen, 2007), comprising the shelled Oxynoacea and the shell-less Plakobranchacea. The latter is further divided into the ceras-bearing Limapontioidea (although not monophyletic in phylogenetic analyses) and the parapodia bearing Plakobranchoidea (Jensen, 1997; Händeler et al., 2009; Kohnert et al., 2013). Because of their ability to incorporate functional chloroplasts and use them for sustenance during starvation over weeks and months Sacoglossa fascinated scientists over decades. This feature is unique among metazoan life forms (Muscatine & Greene, 1973; Trench, 1975; Rumpho et al., 2000; Wägele & Johnson, 2001; Händeler et al., 2009) and only known elsewhere in members of the Foraminifera (Rhizaria) (Lee, 2006), Ciliophora (Alveolata) (McManus, 2012) and in Dinoflagellata (Alveolata) (Gast et al., 2007). Within Sacoglossa usually three states of kleptoplasty are differentiated, either based on measurements of functional chloroplasts with Pulse Amplitude Modulated (PAM) Fluorometry or on CO_2 fixation experiments (Händeler et al., 2009; Rumpho et al., 2011; Wägele & Martin, 2013). Following the classification of Händeler et al. (2009), specimens that are not able to incorporate chloroplasts functional are considered as no-retention forms (NR); specimens that are able to incorporate functional chloroplasts for up to two weeks of starvation are called short-term-retention forms (StR) and those incorporating functional chloroplasts for over 20 days during starvation are called long-term-retention forms (LtR). The fact that chloroplasts survive for weeks and months in the slug's digestive system and perform

functional photosynthesis, despite the absence of the algal nucleus, has led to the intriguing hypothesis that a horizontal gene transfer must have occurred from the algal organisms into the metazoan life form (Pierce et al., 1996; Rumpho et al., 2000; 2011). Up to now, a few single genes amplified from *Elysia chlorotica*, Gould 1870 (Gould, 1870) were interpreted to encode proteins relevant for photosynthesis, such as *Lhc* (light harvesting complex), *fcp* (fucoxanthin protein), *psbO* (manganese stabilizing protein), and others (Rumpho et al., 2000; 2008; Schwartz, Curtis & Pierce, 2010; Rumpho et al., 2011).

Analyzing transcriptomic data for the first time, Wägele et al. (2011) (Wägele et al., 2011) found no evidence for a horizontal gene transfer in two sacoglossan species known to maintain chloroplasts for several months, *Elysia timida* Risso, 1818 (Risso, 1818) and *Plakobranchus ocellatus* van Hasselt, 1824 (Hasselt, 1824). Similar negative results were obtained later for *Elysia chlorotica* by transcriptomic and genomic data (Pelletreau et al., 2011; Bhattacharya et al., 2013). Conflicting results of (Pierce et al., 2012) that suggested a gene transfer based on few single reads in their transcriptomic data, are most recently been interpreted in a different way (Christa et al., 2014). Thus, in our view the hypothesis that slugs are able to actively support the kleptoplasts via the translational products of transferred algal genes is rejected and the focus for understanding long-term incorporation of kleptoplasts has to include other factors, like properties of chloroplasts and their origin with regard to functional photosynthesis. We clearly demonstrated this recently by investigating *Plakobranchus ocellatus* during various starvation periods (Christa et al., 2013): Despite a broad documented food range in fresh caught animals, only chloroplasts from the ulvophycean *Halimeda macroloba* Decaisne, 1841 (Decaisne, 1842) remained in the digestive gland after a starvation period of two months. Unfortunately, our knowledge on sacoglossan food preferences is still scarce. In general the slugs are considered to be stenophagous and mainly sequester members of the Ulvophyceae sensu Floyd & O'Kelly (1990) (Taylor, 1968; Jensen, 1980; Thompson & Jarman, 1989; Jensen, 1993; Williams & Walker, 1999; Händeler & Wägele, 2007; Händeler et al., 2010). Few species (*Elysia crispata, E. clarki and P. ocellatus*) are known to feed on a high variety of algae (Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2010; Wägele et al., 2011; Maeda et al., 2012; Christa et al., 2013). Only some species are recorded to feed on specific members of Rhodophyta (e.g., *Hermaea bifida*) (Taylor, 1971a; Vogel, 1971), Heterokontophyta (e.g., *Elysia chlorotica*) or sea grasses (*Elysia serca*, Marcus 1955 (Marcus, 1955)) (Jensen, 1980; Krug, 2001; Händeler et al., 2009; 2010). Food sources were usually identified by

observation, feeding experiments (Jensen, 1981; 1993; Williams & Walker, 1999), or by electron microscopically studies of chloroplast types (Kawaguti & Yamasu, 1965; Hirose, 2005). But it is obvious that, especially in potential polyphagous sea slugs, not every food algae may be detected by feeding experiments, especially when not knowing, which ones to offer. On the other hand, slugs may feed on alternative food sources during food limitation rather than having these as host algae. Molecular barcoding has proved to be a high efficient method to identify algal food sources even when using a single barcoding-marker (Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2010; Maeda et al., 2012), instead of two (Christa et al., 2013). This now well-established method opens the opportunity to study sacoglossan food sources and origin of kleptoplasts in a highly reliable mode. To find a pattern in chloroplast origin and functional kleptoplasty, a profound database on sequestered chloroplasts for all sacoglossan groups, and especially those taxa hardly studied at all, e.g., the genus *Thuridilla* (Händeler et al., 2010; Wägele et al., 2011), is needed. Following the methods introduced previously (Curtis et al., 2005; 2006; Händeler et al., 2010) we investigated chloroplast origin in 26 non-starved sacoglossan species, including NR, StR and LtR forms, by DNA-barcoding using the chloroplast markers *tufA* and *rbcL* to enlarge our insight in, and state more precisely, sacoglossan food spectrum. We combined the identified food sources with literature data of species for which retention ability is documented and analyzed in combination with this information if there is a correlation between food sources and retention-form. DNA-barcoding has become an important method in identifying plastid origin in Sacoglossa, but due to varying results and minor pitfalls in the application of *rbcL*, we compared reliability of this gene.

RESULTS

Barcoding of tufA and rbcL

We successfully identified food sources for 30 sacoglossan specimens representing 26 species by applying at least one barcoding marker (Additional file). For 19 of the 26 species included here we were able to identify the food sources for the first time. For the remaining seven species, we could confirm literature data or even enlarged the food range (Additional File 1). Yet, no *rbcL* amplification products were obtained for four specimens and no *tufA* product was obtained for seven specimens (Additional File 1). *TufA* sequences obtained

from *Elysia amakusana* (703) Baba, 1955 (Baba, n.d.), *Elysia* sp. (841), *Cyerce nigra* (860) Bergh, 1970 (Bergh, 1870) and *Costasiella* sp. (863) exclusively represented sequences of bacterial origin.

We were able to identify 14 different algal genera combining both markers: eleven genera can be assigned to Bryopsidophyceae, two to Dasycladophyceae and one to Ulvophyceae. *RbcL* revealed about two times more different haplotypes than *tufA* (30 for *rbcL* and 14 for *tufA*, respectively, Figures 1 and 2), though the number of genera is equal (9 for *rbcL* and 10 for *tufA*, respectively). Nine haplotypes for *rbcL* and three haplotypes for *tufA* could not clearly be assigned to a certain algal genus, because of missing reference sequences in GenBank. We are not able to clarify if some of the unidentified haplotypes of *rbcL* and *tufA* actually represent the same algal species. Especially for *rbcL* almost always a higher number of algal haplotypes for a distinct sacoglossan species was revealed, with the highest number of haplotypes for an unknown sister clade of *Pseudochlorodesmis* (7) and for *Bryopsis* (6) (Additional File 1, Figures 1 and 2).

Food sources and retention form

We combined the information obtained from barcoding and literature data, and analyzed this information with regard to the according retention-form. We found that the food of NR forms covers a broad spectrum of ulvophycean algae and includes items that are not consumed by StR or LtR forms: *Boodlea*, *Chlorodesmis*, *Ulva*, *Urospora*, red algae, angiosperms and eggs of other sacoglossans (Fig 3). In contrast, algal taxa like *Poropsis*, *Rhipidosiphon*, *Rhipocephalus*, *Udotea* and an unidentified member of the Ulvophyceae were not identified so far as food sources for NR-forms.

LtR forms consume various ulvophycean algal species, but prefer *Halimeda, Caulerpa, Penicillus* and *Avrainvillea*, the dasycladalen *Acetabularia* and the heterokontophyte *Vaucheria* (Fig 3). However, some NR and LtR forms belonging to different sacoglossan families [5] sequester the same algal species (e.g. *Halimeda*, *Caulerpa*, *Avrainvillea*, *Vaucheria*); therefore a correlation between food sources and functional kleptoplasty cannot be solely algal based. Interestingly, *Thuridilla* species that only comprise StR forms rarely feed on plastids sequestered by LtR forms of *Elysia* and *Plakobranchus ocellatus* (Figure 3).

Within sacoglossan clades, differences in preferred food items exist. All investigated members of the Oxynoacea are NR forms and stenophagous. This is in contrast to all other (non oxynoacean) sacoglossan genera that include polyphagous species (Figure 3). *Cylindrobulla* is the only member of the Oxynoacea that exclusively feeds on *Halimeda,* whereas the members of the remaining Oxynoacea specialized on *Caulerpa* species, with one exception that the *rbcL* analysis in *Lobiger viridis* revealed additional *Halimeda* as food source (Additional File 1, Figure 3). Within Limapontioidea, several species have a broad food spectrum, like *Costasiella* sp. (864) with at least four and *Cyerce nigra* Bergh, 1871 (Bergh, 1870) with at least five different food sources, respectively. However, *Halimeda* and *Caulerpa*, the major oxynoacean food, is scarcely represented amongst the food items of Limapontioidea and only found in *Cyerce antillensis* and *Polybranchia viridis*, respectively. Interestingly, *Costasiella ocellifera* Simroth, 1895 (Simroth, 1895), the only non-plakobranchoid LtR form [(Clark et al., 1981), unpublished data], feeds exclusively on *Avrainvillea nigricans*.

Four genera of the Plakobranchoidea that all include functional retention forms, are investigated. *Bosellia mimetica* is stenophagous, feeding on *Halimeda*, contrary to the polyphagous *Plakobranchus ocellatus* that feeds on seven different algae (including *Halimeda*, *Caulerpa* and *Udotea)* exhibiting the most diverse food spectrum of all investigated sacoglossans so far; however, these data are obtained from different specimens of different localities (Maeda et al., 2012; Christa et al., 2013). The geographically wide distributed taxon *Plakobranchus* might show geographic differences in the consumed food items. Furthermore, cryptic speciation with already ecological differences in feeding can also not be ruled out (Krug, Händeler & Vendetti, 2011). Based on the molecular phylogeny of Händeler et al. (2009), *Thuridilla* is the sister taxon to *Plakobranchus*, its members principally feeding on a variety of food sources. However, *Thuridilla* species neither consume *Halimeda*, nor *Caulerpa* or *Poropsis* (food sources of *Plakobranchus*), but a bryopsidophycean genus related to *Pseudochlorodesmis* (Figure 2). However, this food item could not be identified more specifically due to lack of reference sequences in GenBank. Members of the fourth investigated genus, *Elysia,* consume food items recorded also for the shelled sacoglossans, *Halimeda* and *Caulerpa.* Additionally, many *Elysia* species feed on the same bryopsidophycean genera similar to those observed in *Thuridilla* (exceptions see above) or *Plakobranchus*. Some species became very

specialized. *Elysia chlorotica* feeds on *Vaucheria* (like the limapontioidean genus *Alderia*), whereas its sister taxon, *Elysia serca*, is reported to feed solely on higher plants (i.e., sea grasses (Jensen, 1980)), a unique feature within Sacoglossa. Several species are confirmed in their specific narrow food spectrum, e.g., *Elysia timida*, which exclusively feeds on the dasycladophycean *Acetabularia acetabulum* P.C. Silva, 1952 (Silva, 1952).

DISCUSSION

Food sources and retention form

We analyzed a wide spectrum of sacoglossan sea slugs with regard to their food preferences and plastid origin by using a combination of two barcoding markers, *rbcL* and *tufA*, instead of one (Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2009; Wägele et al., 2011; Maeda et al., 2012). This method proved to be more rapid and more precise in identification of food sources of a large set of Sacoglossa sea slugs, compared to former feeding experiments or feeding observations. Although not every algal food source is identified on species level, the information we provide here increases our knowledge considerably and can now be used for further ecological or behavioral studies.

We found that the ability to perform photosynthesis for at least several days and weeks is established with certain food sources (members of the genera *Elysia, Bosellia* and *Plakobranchus*, see Figure 3 and (Händeler et al., 2009)). At least one of the following six algae, the bryopsidophycean *Halimeda, Caulerpa, Penicillus* and *Avrainvillea,* the heterokontophyte *Vaucheria*, or the dasycladophycean *Acetabularia,* seem to be essential for establishing long-term-retention. However, non-retention forms also feed on these specific algae and are not able to perform photosynthesis. Both aspects indicate that a special type of chloroplast is not sufficient to establish functional kleptoplasty and intrinsic factors of the slugs also contribute to a functional photosynthesis. Here, we pave the way for further research on these specific algae consumed by LtR forms to better understand which genetically and physiological commonalities these target plastids may have. Pelletreau et al. (2011) recently considered special abilities of chloroplasts from respective host algae as relevant for long-term incorporation, an option already mentioned earlier (Trench, Boyle & Smith, 1973b; Wright & Grant, 1978). A factor now found to possible

enhance plastid longevity is FtsH, a protein important for the repair of Photosystem II. It is plastid encoded in the food algae of the two LtR forms *Elysia chlorotica* and *Plakobranchus ocellatus* (de Vries et al., 2013). With the information of this study we are now able to specifically examine the presence of FtsH in plastids of the identified food sources of other retention forms and correlate plastid origin, photosynthetic capability and plastid longevity. Interestingly, *Thuridilla* species – as close relatives of *Plakobranchus* and *Elysia* – and which exhibit extremely short chloroplast retention (Händeler et al., 2009; Ventura et al., 2013), did not reveal any of these six algal species mentioned above (Additional File 1, Figure 3).

Besides plastid origin, several other factors surely influence survival of chloroplasts and render them nutritional at least in sacoglossans in one way or the other. A horizontal gene transfer as a factor was excluded not only for the sea slugs *Plakobranchus ocellatus*, *Elysia timida* and *E. chlorotica* (Wägele et al., 2011; Bhattacharya et al., 2013), but also for the Foraminifera *Elphidium margaritaceum* and most likely for the dinoflagellate *Dinophysis acuminate* (Wisecaver & Hackett, 2010; Pillet & Pawlowski, 2013). Common to all three different systems is that there is no mechanical grinding before chloroplast uptake. E.g., the sacoglossans pierce the cell wall with their teeth and then suck out the cytoplasm of the algae, which is then processed in the digestive gland. Second, the intracellular digestion of plastids seems to be stopped or regulated in a way yet to be discovered.
Händeler et al. (2009) have shown in their phylogenetic analysis by using ancestral character state reconstruction, that the ancestor of the Plakobranchoidea most likely developed an unknown mechanism, which hinders direct digestion of chloroplasts. However, the genus *Costasiella* was not included in their analysis, which is now verified in comprising both, LtR and StR species [(Clark et al., 1981), (Händeler, 2011), unpublished data]. Thus, at least in *Costasiella,* this mechanism must be developed as well. Evertsen & Johnsen (2009) showed that in *Elysia viridis* starch grains persist throughout degradation of plastids and are not broken up, as was the case in the NR form *Placida dendritica*, (Alder & Hancock, 1843). If the slugs cannot access the photosynthetic produced starch of functional chloroplasts in the first place, the benefit, under starvation conditions, has to arise from another process. In this case the accumulated photosynthates would only become accessible for slug metabolism after the complete degradation of the chloroplast. The nutritional benefit of the chloroplasts would lie in the presence of an additional food

reservoir (starch) that becomes available only after degradation or digestion of the chloroplasts. This is in contrast to former hypotheses that usually assume a continuous supply of photosynthates and therefore a continuous exchange between chloroplast and slug's cytosol (Trench & Gooday, 1973; Gallop, 1974).

General food source of Sacoglossa

Our results in combination with literature data show that sacoglossans generally feed on a high variety of algal species, although the majority prefers bryopsidophycean taxa. Jensen (1997) (Jensen, 1997) assumed *Halimeda* as the ancestral food of Sacoglossa based on her observations on *Cylindrobulla*. We can confirm here by molecular analyses of the gut content that this species exclusively feeds *Halimeda,* whereas all other oxynoacean species feed on *Caulerpa*, though *Lobiger viridis* seems also to at least feed additional on *Halimeda*. The position of *Cylindrobulla* within the Oxynoacea is not resolved yet, thus the ancestral food source of this clade cannot be deduced (Händeler et al., 2009; Jörger et al., 2010; Maeda et al., 2010; Neusser et al., 2011). Future phylogenetic studies are needed to address these questions. All higher sacoglossan taxa switched to other food sources than *Halimeda* (especially members of the Limapontioidea), or broadened their food spectrum (many *Elysia* species).

Why some species feed on several different algal species and others specialized on just one prey species, is difficult to explain. Further information on biology has to be considered, e.g., incorporation of defensive compounds obtained via sequestration of toxic algae, or *de novo* synthesis (rendering the slug independent from algal toxins). We have to emphasize that we were not able to study all species on a broad scale by including specimens from various geographic areas or seasons. Food availability and seasonality, and/or intraspecific or interspecific competition might also force specimens from the same species to switch to other food items and thus influences the finding of certain food items in sacoglossan sea slugs. Further starvation studies on polyphagous species (see e.g. (Christa et al., 2013)) will verify if these species have a preferred food source, as was shown for *Plakobranchus ocellatus* and these investigations will certainly contribute to our understanding of polyphagous strategies (Maeda et al., 2012).

Jensen (1993, 1997) studied morphological differentiation of the radula teeth in connection with algal food. Based on her findings she suggested that certain radula teeth are correlated with the polysaccharide of cell walls of the respective food sources. However, Händeler & Wägele (2007) mapped radula teeth forms on their molecular based cladogram and were not able to confirm this previously suggested correlation. We therefore compared three unusual food switches within the Sacoglossa with regard to a possible correlation of food source and radula tooth shape i) The NR form *Hermaea bifida* (Montagu, 1815) feeds on the red algae *Griffithsia* with cell walls composed of cellulose. However, the radula of *H. bifida* is not different from species feeding on the green alga *Caulerpa* with cell walls composed of xylan (Thompson, 1988). ii) *Elysia serca*, also a NR form (Clark et al., 1990), presents a unique switch to sea grasses with cell walls composed of cellulose (Jensen, 1993; Händeler & Wägele, 2007), but the radula is similar to species feeding on *Halimeda* with cell walls composed of xylan. iii) The NR forms *Alderia modesta* and *A. willowi,* as well as the LtR form *Elysia chlorotica,* feed on the heterokontophyte genus *Vaucheria* composed of cellulose-based cell walls. The radulae of *Alderia* species and *Elysia chlorotica* are similar to species feeding on the green alga *Codium* composed of mannan-based cell walls. Thus, we think that different radula shapes did not force or influence a host switch in these three examples, though it cannot be ruled out for others that are not examined here.

Whether functional kleptoplasty is of higher evolutionary benefit than e.g., feeding on a wide spectrum of algae may be reconsidered. A polyphagous strategy –in contrast to stenophagous strategies – would render the slugs more independent of seasonality or general availability of their host algae. LtR forms like *Costasiella ocellifera*, *Elysia chlorotica* and *Elysia timida* feed on one algal species. *Acetabularia acetabulum*, the only food of *E. timida*, calcifies in fall, and therefore probably forces *Elysia timida* to rely on its incorporated chloroplasts (Marìn & Ros, 1993). Unfortunately we have no similar information on seasonality of *Avrainvillea* and *Vaucheria*, the major food items of *C. ocellifera* and *E. chlorotica*, respectively. *Plakobranchus ocellatus* on the other side has a broad food spectrum. As Maeda et al. (2013) showed in a subtle experiment, the algae show a high seasonality and the slugs combine here functional kleptoplasty and multiple food sources that certainly enhances survival.

Usefulness of barcoding markers

RbcL and *tufA* are now state of the art markers for barcoding algal species (Saunders, 2005) and are therefore intensively used for identification of sequestered chloroplasts in Sacoglossa (Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2010; Maeda et al., 2012; Christa et al., 2013). So far it is not possible to identify the food of every sample we screened, and reasons are unknown. Christa et al. (2013) showed that *rbcL* almost always revealed more haplotypes for one sacoglossan specimen than *tufA*, especially within the genus *Halimeda*. In the present study we see exactly the same pattern. We therefore compared several *rbcL* sequences of randomly chosen *Halimeda* species in order to reveal possible problems by using *rbcL* as barcoding marker for this genus (Figure 4). Our analyses show that for several *Halimeda* species various *rbcL* sequences exist. p-distances between haplotypes of the same species exceeded in some cases those of *rbcL* sequences between different species. For example, the p-distance of haplotypes of *H*. *cuneata* FJ624533 and FJ624532 is 2.5%, the p-distance of *Halimeda gracilis* FJ624494 and *Halimeda lacrimosa* FJ624495 1% (Additional file 5). Assuming that there are no misidentifications (which is important for DNA-Barcoding) with regard to the sequences obtained from GenBank, this problem can lead to an overestimated number of haplotypes in cases where no reference sequences are available. With regard to our study, this might be the case for several algal sequences obtained from *Thuridilla*, which are now assigned as seven different unidentified haplotypes. It is very likely that these problems do not only occur in the taxon *Halimeda*, but also in other ulvophyceaen taxa where detailed studies are still missing. Händeler et al. (2010) demonstrated for *Caulerpa* that identification and annotation using *tufA* on species level could also be problematic due to sequence similarities. Hanyuda et al. (2000) reported introns in *rbcL* sequences from members of the Caulerpaceae, a problem we faced in *Caulerpa* sequences obtained from *Volvatella viridis*. These sequences cannot be aligned with the remaining *rbcL* sequences, and were therefore not included in our analyses here.

An additional problem for identifying food organisms in Sacoglossa is connected to the lack of references for both genes in the databases. Available information does not cover many of the obtained haplotypes; therefore an extension of algal taxon sampling in the future by algal specialists is absolutely necessary. Here we show that the use of two markers for

plastid identification in Sacoglossa only leads to a more detailed identification of ingested plastids in some species. Yet, we suggest that, based on the higher reliability and despite possible multiple haplotypes for some genera, *rbcL* should be used preferentially. *TufA* may be added in cases where an identification of *rbcL* failed or sequence variability of *rbcL* hinders unambiguous identification. Unfortunately both primer pairs are still not applicable at the moment for Cladophoraceae (Hanyuda, Arai & Ueda, 2000; Verbruggen et al., 2009). Constructing new primers for both genes based on chloroplast genomic data probably could solve this problem as soon as a cladophoraceaen plastid genome is available. Until then it has to be kept in mind that barcoding results may cover only a reduced food range. Even with the limitations mentioned above, we consider DNA-Barcoding a more accurate and effective method than feeding observations, especially in polyphagous specimens.

Conclusions

DNA-Barcoding of sequestered chloroplasts in Sacoglossa led to a more specific insight in food sources of sacoglossan sea slugs than by direct observation and is certainly much more efficient. *RbcL* should be used primarily because of higher amplification and sequencing success, while *tufA* should be added in analyses with ambiguous results.

Our results revealed food items for many species not investigated before and additionally confirmed literature data. We could show that food sources are highly variable in some species and morphological features such as radula shape have probably only little impact on food preferences or food switches. There seem to be certain food sources that are essential for functional kleptoplasty. LtR forms preferably consume algal species belonging to the genera *Halimeda, Caulerpa, Avrainvillea, Acetabularia* or *Vaucheria.* Kleptoplasty is only established in slugs feeding on green algae and heterokontophytes, but not in slugs feeding on rhodophytes and sea grasses. NR forms may also feed on algae that are known to be the sole food of LtR forms. Factors like physiology of food items, genetic and physiological properties of the plastids and digestion properties in slugs need to be more investigated in future studies to reveal principles of establishing functional kleptoplasty. The evolutionary benefit of kleptoplasty is still enigmatic, since a polyphagous life style may lead to more independence from specific food source's seasonality and abundance.

METHODS

Sacoglossan specimens used in this study were identified by morphological examination using a taxonomic species file based on original literature (Gosliner, 1995 on *Thuridilla*), as well as identification books (e.g., Gosliner, Behrens & Valdés, 2008) and the sea slug forum (http://www.seaslugforum.net). These identified specimens, covering NR, StR and LtR forms, are listed in Additional File 1 and represent those for which DNA-Barcoding of food source was successful, in order to determine the minimal food items of these species. Specimens were fixed in 96%-EtOH immediately after collection. Slug parts containing digestive glandular tissue were cut off and subsequently DNA extracted using the DNeasy ® Blood & Tissue Kit (Qiagen, Germany) following manufacturer's instructions and stored at -20°C.

DNA amplification

Standard PCR reactions for *rbcL* and *tufA* as reported elsewhere (Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2010; Maeda et al., 2012; Christa et al., 2013) were performed using a touchdown protocol and ulvophycean specific primers. 2.5µl of genomic DNA was used as template in a 20µl final volume reaction supplied with 5.5µl sterilized water, 2µl Qiagen® Q-Solution, 10µl of double concentrated QIAGEN® Multiplex PCR Master Mix and 1µl of 5pmol/µl concentrated primer each. PCR for amplification of *rbcL* was performed with primer pairs rbcL 1 (Pierce et al., 2006) and rbcL R, 5'-CCA WCG CAT ARA NGG TTG HGA-3' (Christa et al., 2013) modifid after (Pierce et al., 2006) by an initially denaturation for 15 min at 95 °C, followed by 9 touch-down cycles at 94 °C for 45 s, 53 °C (-1 °C per cycle) for 45 s, 72 °C for 90 s, followed by 25 standard cycles (94 °C for 45 s, 45 °C for 45 s and 72 °C for 90 s.) and a final extension at 72 °C for 10 min. *tufA* amplification was performed with primer pair tufAF and tufAR (Famà et al., 2002) by an initially denaturation for 15 min at 95 °C, followed by 9 touch-down cycles at 94 °C for 45 s, 57 °C (-1 °C per cycle) for 45 s, 72 °C for 90 s, followed by 25 standard cycles (94 °C for 45 s, 48 °C for 45 s and 72 °C for 90 s.) and a final extension at 72°C for 10 min. PCR products were size-fractionated in a 1.5% agarose gel for 90 min at 70V and bands according to desired gene-fragment length and subsequently gel-extracted using Machery-Nagel NucleoSpin® Extract II (Düren, Germany) kit following manufacturer's instructions. Isolated fragments

were ligated into pGEM T-easy Vector (Promega, Germany) and cloned into competent *E. coli* XL1-blue cells from Stratagene (Heidelberg, Germany). For each specimen 12 clones were sequenced by Macrogen Inc, Amsterdam.

Sequence analysis

Sequence identity of *tufA* and *rbcL* of every clone was verified by BLAST search using Geneious (Biomatters Ltd, New Zealand, v. 6.0.3). Consensus sequences of one slug specimen were created when sequence divergence of chloroplast genes was lower than 1 %, as introduced by (Händeler et al., 2010). All gained sequences were again verified by BLAST search and the first top 5 BLAST results of each sequence were taken to create a dataset of overall 54 sequences for *tufA* (688bp) and 94 sequences for *rbcL* (561bp). Both datasets were aligned with MAFFT plugin as implemented in Geneious [*v6.814b* (Katoh et al., 2002)]. A maximum likelihood tree for each dataset was calculated using PhyML plugin (Guindon & Gascuel, 2003) implemented in Geneious with GTR+I+R as substitution model. For final identifying sequence origin we used a combination of similarity-based and tree-based DNA-barcoding approaches (Little, 2011; Tanabe & Toju, 2013): Sequences were assigned to a certain algal species by a similarity-based approach using BLAST analysis when sequence similarity was 99-100%. We included a 99% match as positive identification of an algal species, thus allowing a small amount of ambiguity nucleotides within our consensus sequences which are not identified as similarity by the BLAST search in NCBI (www.ncbi.nlm.nih.gov). When multiple positive identifications were obtained, sequences were assigned to the corresponding genus based on the position within the ML tree. When not able to define on species level (BLAST similarity <99%), sequences were assigned to higher taxa based on their position within monophyletic algal groups in the ML tree analysis. When sequences obtained in our study did not group within distinct algal taxa, we assigned them to the taxon level Ulvophyceae.

Sequence analysis of rbcL sequences of Halimeda

Randomly chosen *rbcL* sequences of *Halimeda* species were downloaded from Genbank to analyze sequences variability. Overall 72 sequences were used, representing 32 species (Additional file 6). Dataset was aligned with MAFFT plugin as implemented in Geneious [*v6.814b* (Katoh et al., 2002)]. A maximum likelihood tree for the dataset was calculated

using PhyML plugin (Guindon & Gascuel, 2003) implemented in Geneious with GTR+I+R as substitution model. P-distances of sequences were calculated using HyPhy (v 2.1). On the basis of these p-distances the sequence variability of haplotypes of the same algal species were then analyzed (Additional File 5).

Comparison of literature data and new barcoding data

DNA-Barcoding results of the present study were combined with results taken from literature to compare food sources of different retention forms (LtR, StR, NR). Literature data on food sources and retention form were taken from (Jensen, 1997; Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2009; Yamamoto et al., 2009; Händeler et al., 2010; Klochkova et al., 2010; Wägele et al., 2010b; 2011; Klochkova et al., 2012; Maeda et al., 2012; Christa et al., 2013) (Additional File 1 and 6). The retention ability of the majority of the species investigated here was measured by means of a PAM. Therefore, we used the classification scheme of Händeler et al. (2009). Results of Clark et al. (1990) based on ^{14}C methods were re-assigned, when new data were available. For example, *Mourgona germaineae* Marcus, 1970 is classified as StR form by Clark et al. (1990), however PAM measurements revealed no fluorescence at all (unpublished data). We therefore set ^{14}C values obtained by Clark et al. (1990) similar to *M*. *germaineae* as "no retention", e.g. *Caliphylla mediterranea* Costa , 1867 (Costa, 1867), *Hermaea cruciata*, *Placida kingstoni* Thompson, 1977 (Thompson, 1977) and *Elysia serca*.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

HW, KH and GC conceived and designed the experiment. GC, TS and KH performed the experiments. HW, GC and KH analyzed the data. GC, TS, GK and HW wrote the paper. All authors read and approved the final manuscript.

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FIGURES

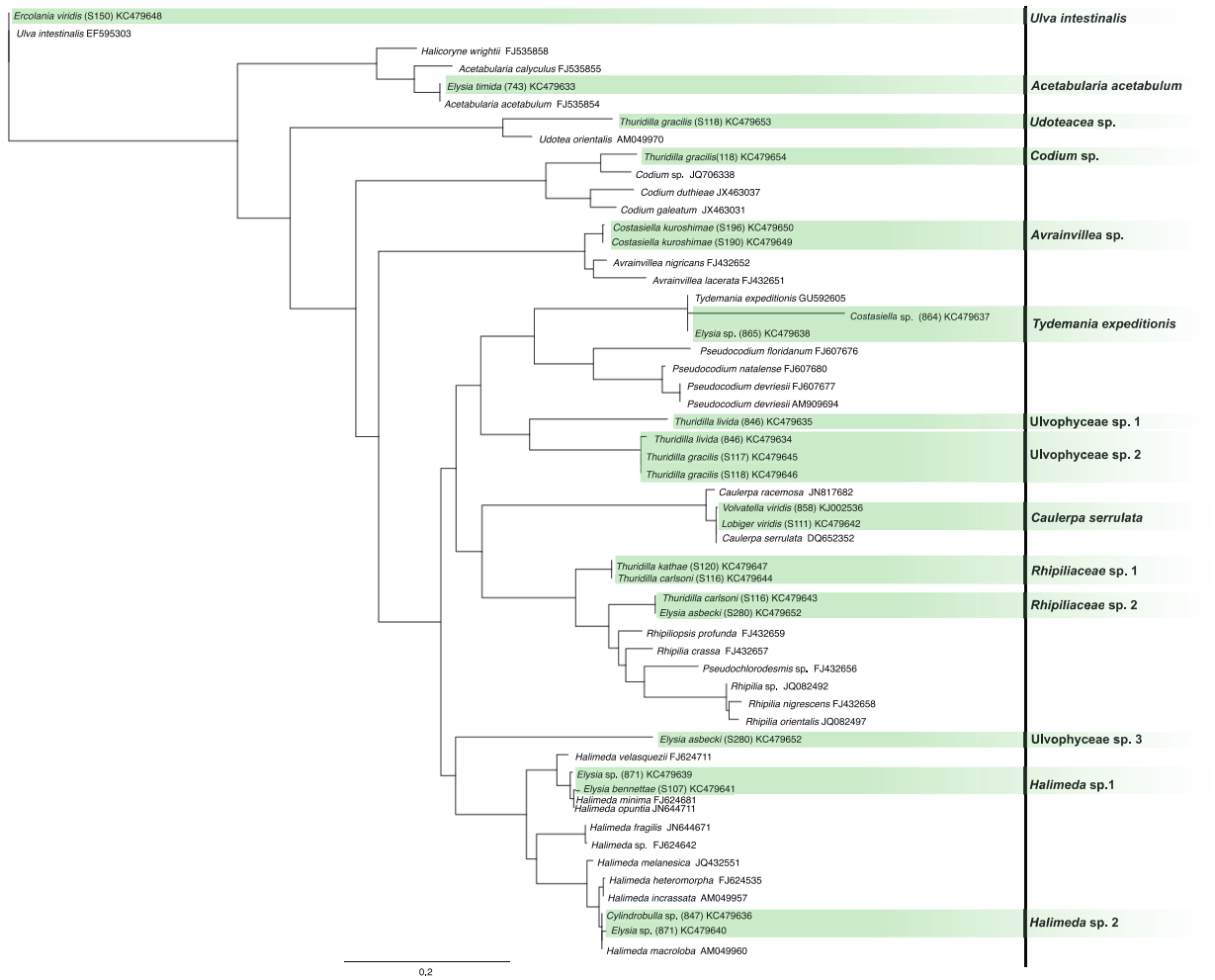

Figure 1 – Food sources identified using *tufA as barcode marker*

ML tree of identified algal haplotypes in Sacoglossa by using *tufA* (highlighted in green). Identified haplotype is noted on the right side. When sequence match was < 99%, higher taxon name of the algae that formed a monophyletic group with the corresponding haplotype was used. Haplotypes with no monophyletic grouping are named "Ulvophyceae spec".

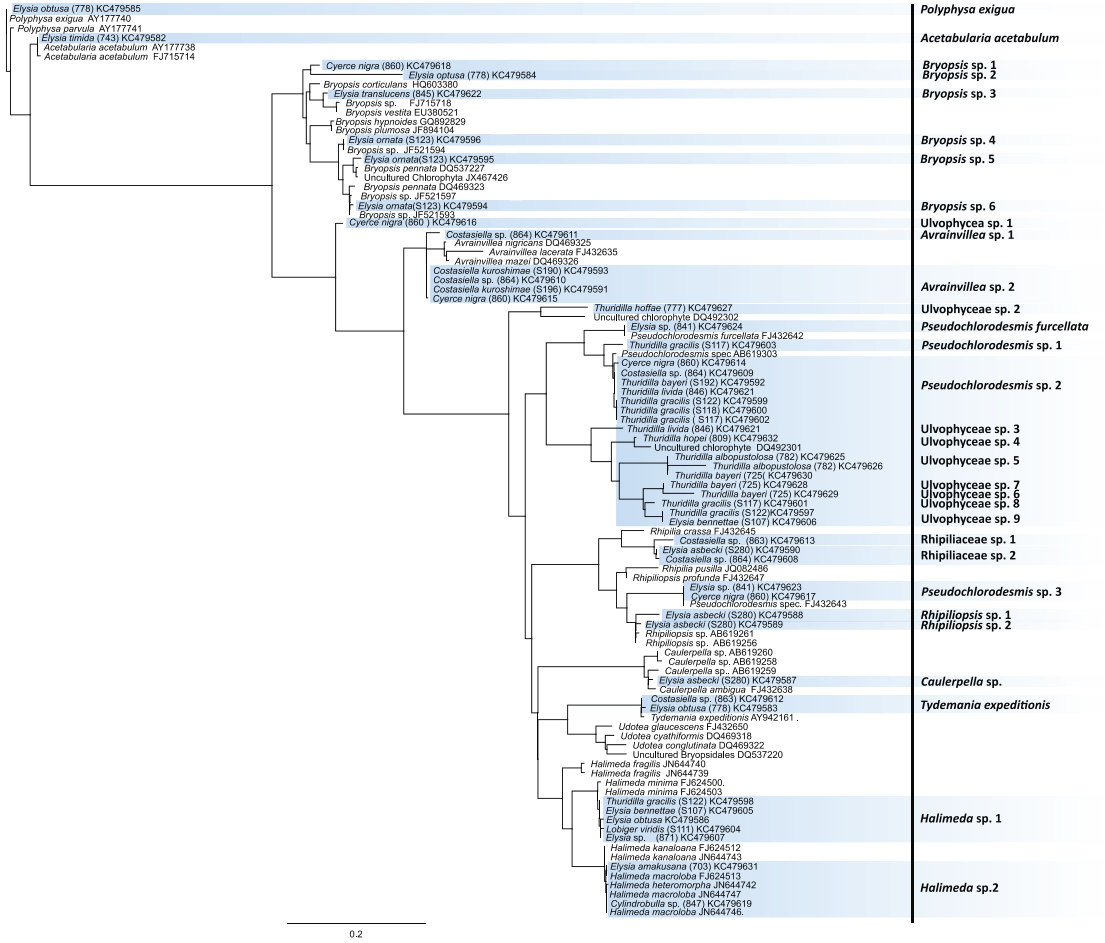

Figure 2 – Food sources identified using *rbcL* as barcode marker

ML tree of identified algal haplotypes in Sacoglossa by using *rbcL* (highlighted in blue). Identified haplotype is noted on the right side. When sequence match was < 99%, higher taxon name of the algae that formed a monophyletic group with the corresponding haplotype was used. Haplotypes with no monophyletic grouping are named "Ulvophyceae spec".

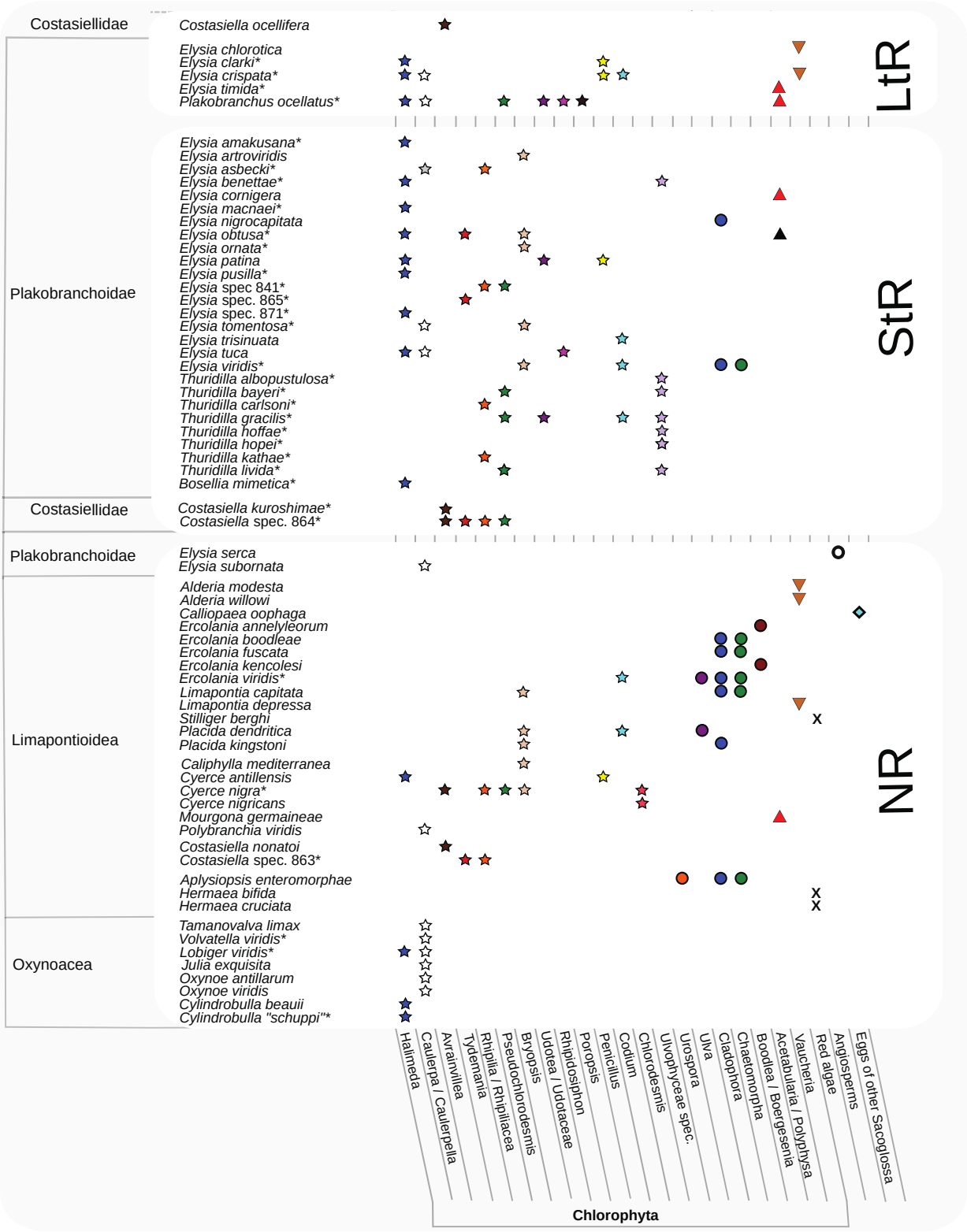

Figure 3 – Food spectrum of Sacoglossa compared to functional retention form

Food sources of 68 species of Sacoglossa are shown. The classification of Sacoglossa is based on works of (Bergh, 1870; Jensen, 1997; Händeler et al., 2009). Asterisk indicates molecular identification of food (overall 33 species). Food sources were either obtained in this study or taken out of literature (Jensen, 1997; Curtis et al., 2005; 2006; Pierce et al., 2006; Händeler et al., 2009; Yamamoto et al., 2009; Händeler et al., 2010; Klochkova et al., 2010; Wägele et al., 2010b; 2011; Klochkova et al., 2012; Maeda et al., 2012; Christa et al.,

2013). Classification of retention ability was done according to literature data (Christa et al. (unpublished data), (Clark et al., 1981; Evertsen et al., 2007; Händeler et al., 2009; Yamamoto et al., 2009; Klochkova et al., 2010; Wägele et al., 2010b; 2011; Klochkova et al., 2012). **LtR** = Long-term-retention, **StR** = short-term-retention, **NR** = no retention. Stars indicate members of the Bryopsidales, filled circles members of the Ulvophyceae other than Bryopsidales; triangles represent Dasycladales, inverted triangles Heterokontophyta, X red algae, circle sea grass and diamond eggs of other Sacoglossa.

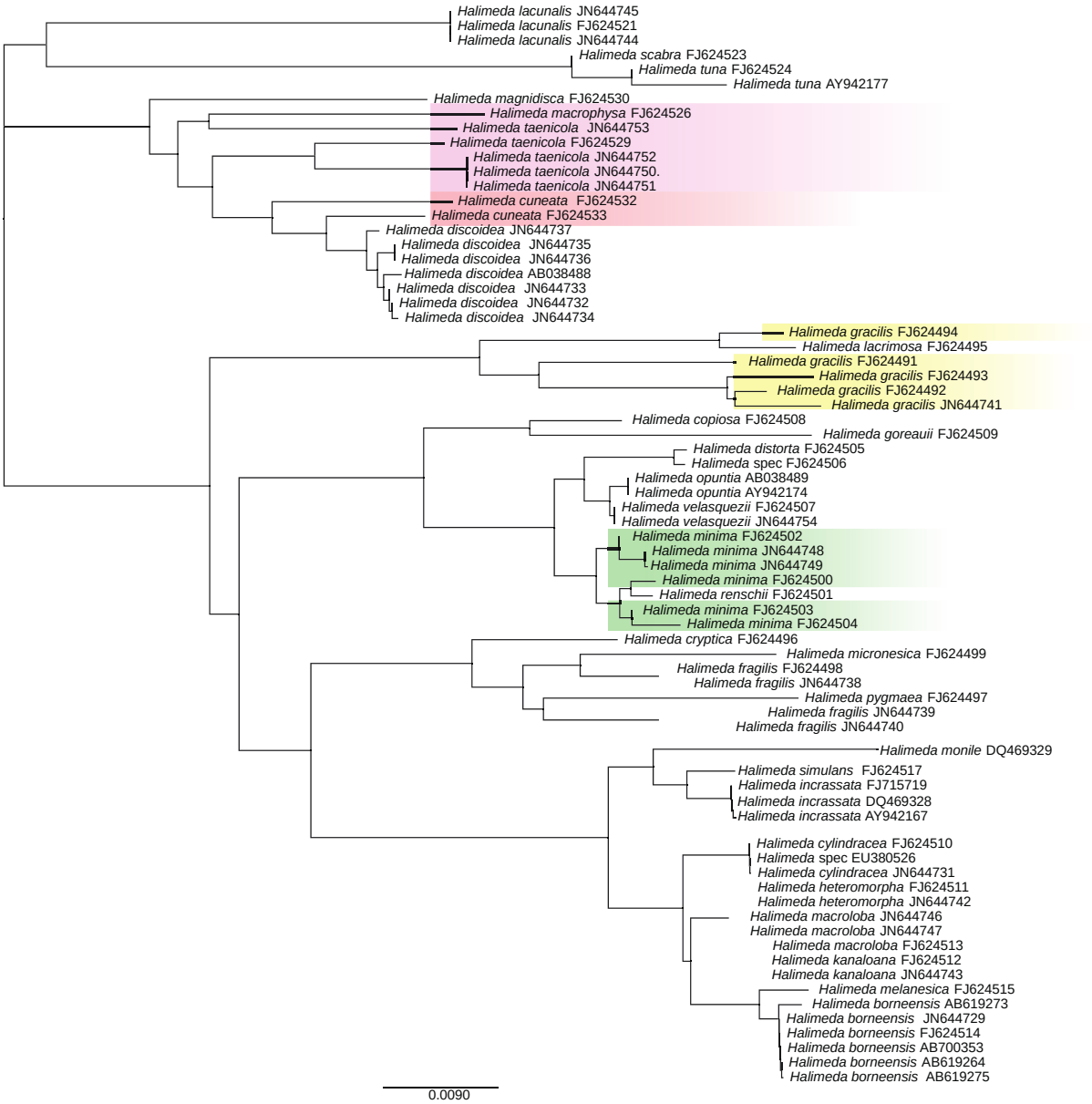

Figure 4 – ML tree of rbcL sequences of *Halimeda* species

ML tree of randomly chosen *rbcL* sequences of *Halimeda*. Multiple haplotypes of a single species are highlighted in colored boxes.

ADDITIONAL FILES

For the Additional Files please see the attached CD. There is a folder named "Chapter 4 - Does it matter what you eat?" which contains all additional information.

Additional File 1 – Origin of specimen and identified food sources with comparison of literature data. In Table 1 we provide a list of Sacoglossa specimens and species analyzed with regard to food items in. Table 2 displays the identified food sources in Sacoglossa specimens by analyzing the chloroplast markers *tufA* and *rbcL*. The number of haplotypes per gene obtained from each slug specimen is listed in the last two columns. * *tufA* sequences of *Thuridilla hopei* (809), Vérany 1853 (Verany, 1853) were published previously (Wägele et al., 2011). In Table 3 a comparison of chloroplast origin by feeding observations (4th column) and identified by DNA-Barcoding (this study and literature data – last column). When not specified specifically, information on retention-form is taken from Händeler et al. (2009) (Händeler et al., 2009). Food sources identified by using *tufA* is indicated with [1], by *rbcL* with [2] in the last column. Literature data on food sources base on the review of Händeler & Wägele (2007) (Händeler & Wägele, 2007) or is indicated otherwise.

Additional File 2 – Sequence affinity based on BLAST search.
Additional File 3 – Alignment of *tufA* sequences used for consensus sequences.
Additional File 4 – Alignment of *rbcL* sequences used for consensus sequences.
Additional File 5 – p-distances of *rbcL* sequences of *Halimeda* species.
Additional File 6 – Classification on retention form in Sacoglossa.

Chapter 5

Plastid-bearing sea slugs fix CO_2 in the light but do not require photosynthesis to survive

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SUMMARY

Several sacoglossan sea slugs (Plakobranchoidea) feed upon plastids of large unicellular algae. Four species — called long-term retention (LtR) species — are known to sequester ingested plastids within specialized cells of the digestive gland. There, the stolen plastids (kleptoplasts) remain photosynthetically active for several months, during which time LtR species can survive without additional food uptake. Kleptoplast longevity has long been puzzling, because the slugs do not sequester algal nuclei that could support photosystem maintenance. It is widely assumed that the slugs survive starvation by means of kleptoplast photosynthesis, yet direct evidence to support that view is lacking. We show that two LtR plakobranchids, *Elysia timida* and *Plakobranchus ocellatus,* incorporate $^{14}CO_2$ into acid-stable products 60 and 64-fold more rapidly in the light than in the dark, respectively. Despite this light-dependent CO_2 fixation ability, light is, surprisingly, not essential for the slugs to survive starvation. LtR animals survived several months of starvation i) in complete darkness and ii) in the light in the presence of the photosynthesis inhibitor Monolinuron, all while not losing weight faster than the control animals. Contrary to current views, sacoglossan kleptoplasts are more likely slowly digested food reserves, than a source of solar power.

INTRODUCTION

Symbioses between animals or heterotrophic protist and algae are fairly common in nature (Venn et al., 2008); prominent examples include the zoochlorellae of *Hydra viridis* (Kovacević et al., 2010; Bosch, 2012) or dinoflagellates (zooxanthellae) of corals (Archibald, 2009) and the many different species of algae found in ciliates (Johnson, 2011). A more curious kind of symbiosis is found among the sacoglossan molluscs (marine slugs) from the Plakobranchoidea. These animals establish a symbiosis with only a part of their algal partner: the plastid. Nearly 150 species of plakobranchoids have been described to date, and like all sacoglossans they feed upon algae by sucking the cytoplasm out of the large, syncytial algal cells upon which they feed. While most sacoglossan species simply digest the plastids, plakobranchoidean species (termed plakobranchids for convenience) exhibit a delayed digestion and four species — *Elysia chlorotica, Elysia timida*, *Elysia crispata* and *Plakobranchus ocellatus* — retain the ingested plastids, the kleptoplasts, in their digestive gland for several months (Trench, 1975; Rumpho et al., 2000; Pierce et al., 2007; Händeler et al., 2009; Wägele et al., 2011). This gland fills most of the animal's body and gives them a distinctive green color (Fig. 1), which is why they are sometimes called "leaves that crawl" (Trench, 1975) or "solar powered slugs" (Rumpho et al., 2000). Because these four species can maintain plastids with functional photosystems for several months, they are designated as long-term retention species (LtR) in contrast to those plakobranchids that are only able to maintain functional plastids for up to two weeks, and which are hence termed short-term retention (StR) species (Händeler et al., 2009). In *E. timida*, the undigested plastids remain ultrastructurally intact and photosynthetically active, as determined by photosystem fluorescence, for more than two months (Händeler et al., 2009; Wägele et al., 2011). Having acquired a load of plastids, the animals can be kept in the laboratory, in the light, for months without additional food (Händeler et al., 2009).

How LtR plakobranchids maintain their kleptoplasts for such long periods of time has been the subject of much speculation and considerable recent research. The predicted proteome of *Arabidopsis* plastids ranges from 1000 to ~3500 proteins (van Wijk & Baginsky, 2011), but plastid genomes only encode for 60 (higher plants) to 200 (red algae) protein-coding genes in the organelle's DNA (Timmis et al., 2004). The remaining plastid proteins (>90%) are encoded in the nucleus, synthesized as precursor proteins on cytosolic ribosomes, and imported from the cytosol through the plastid-specific protein translocon machinery [reviewed in (Gould, Waller & McFadden, 2008; Strittmatter, Soll & Bölter,

2010; Shi & Theg, 2013)]. Because plakobranchids do not sequester algal nuclei, which can however be ingested for a short time during feeding ((Mujer et al., 1996), and because some proteins in higher plant chloroplasts can have turnover rates on the order of 30 to 120 minutes (Greenberg, Gaba & Mattoo, 1987; GODDE, SCHMITZ & WEIDNER, 1991; Sundby, McCaffery & Anderson, 1993), it has been widely assumed that sequestered plastids of plakobranchids also require imported proteins to remain photosynthetically active. The most popular theory for the source of those assumedly essential genes has been lateral gene transfer (LGT) from the algae to the slug, and some PCR-based reports provided evidence in favor of that view, for example involving the gene for the light harvesting protein LHC (Pierce et al., 2007).

The by far most prominent report for putative involvement of LGT in sacoglossans concerns a sequence for the manganese cluster stabilizing protein PsbO of photosystem II in *E. chlorotica* (Rumpho et al., 2008). The PCR amplification products for *PsbO* obtained from *E. chlorotica* were identical in sequence to those from *Vaucheria*, including a canonical bipartite targeting signal (Gruber et al., 2007; Gould, 2008) that directs the PsbO precursor across the four membranes that surround the plastid in *Vaucheria*. However, the *Vaucheria* plastids that are sequestered in *E. chlorotica* are only surrounded by two membranes, the outer two are removed during sequestration (Rumpho et al., 2001). As a consequence, were the *E. chlorotica* PsbO precursor protein (Rumpho et al., 2008) really expressed, the gene product would enter the secretory pathway and thus be excreted from the cell because of its intact and highly conserved signal peptide, rather than being targeted to the remaining inner two membranes of the sequestered *Vaucheria* plastid (Wägele et al., 2011). (Rumpho et al., 2011) noted that this circumstance "…has potential implications for protein targeting, but will not be discussed here", while Pierce et al. (Pierce et al., 2012) in their latest report for LGT in sacoglossans do not address this issue at all.

In order to determine whether or not there is LGT from algae to slugs in LtR plakobranchids, Wägele and colleagues (Wägele et al., 2011) sequenced ESTs from the LtR species *E. timida* and *P. ocellatus* and found no evidence in either species for the expression of any genes of demonstrably green algal nuclear provenance. Similar results for *E. chlorotica* were subsequently obtained, with no transcripts for PsbO or any other *Vaucheria*-derived nuclear genes identified (Rumpho et al., 2011), leading to the conclusion that, contrary to earlier claims, LGT probably does not underpin photosynthetic activity of sequestered plastids in *E. chlorotica* after all. However, (Pierce et al., 2012) reported that

among the 100 million *E. chlorotica* transcripts that they sequenced, about 100 reads might indicate LGT in *E. chlorotica*, although only one pointed to an essential function in photosynthesis (a light harvesting complex protein). But photosynthesis requires the expression of thousands of nuclear genes (Richly et al., 2003; van Wijk & Baginsky, 2011), not 100. Moreover, transcripts for photosynthetic functions are generally abundant, for example, the small subunit of RuBisCO and LHC together constitute ~20% of all transcripts in *Arabidopsis* leaves (Bhalerao et al., 2003). The 100 genes that Pierce et al. (2012) found comprise 0.000001% of the mRNA each, or 0.0001% of the total, so even if those 100 genes are LGTs, they cannot underpin a photosynthetic lifestyle. While Pierce et al. (2012) interpret those 100/100,000,000 reads as evidence for LGT from alga to mollusc, we would interpret that same data to indicate that their sequencing substrate was 99.9999% free of contaminating algal nucleic acids — if there is any LGT in plakobranchid genomes, it is of the one-in-a-million kind.

The very expectation that some sacoglossans have undergone LGT stems from the inference that plastids require many proteins in order to support a photosynthetic lifestyle. Since the genes for the proteins are missing, the next question is: how strong is the evidence that the slugs depend upon photosynthesis to begin with? The main evidence supporting the view that plakobranchids are photosynthetic (in the sense of being photoautotrophic) comes from earlier studies and is of two main types. First, Trench et al. (Trench, 1973) showed that *E. viridis* incorporates ^{14}C from $^{14}CO_2$. A number of other studies also reported the incorporation of ^{14}C from $^{14}CO_2$ in plakobranchids that sequester plastids (Greene & Muscatine, 1972; Kremer & Schmitz, 1976; Hinde, 1978; Ireland & Scheuer, 1979), but animals can also incorporate CO_2 via carboxylation reactions. A second line of evidence for plakobranchids being photosynthetic comes from the observation that once the plastids have been incorporated into the digestive gland, LtR species can survive for months in the absence of additional food (Rumpho et al., 2001; Pierce et al., 2007; Händeler et al., 2009; Middlebrooks et al., 2011; Rumpho et al., 2011; Christa, 2013). Such plakobranchids are said to be “starved” and are typically cultivated in the light (Rumpho et al., 2000; Wägele et al., 2011).

However, a subtlety of such experiments that is not immediately evident to the observer (who is understandably fascinated by the sight of plastid-bearing slugs), but that has been pointed out in earlier work (Hinde & Smith, 1972; 1975; Giménez-Casalduero & Muniain, 2008; Klochkova et al., 2012; Yamamoto et al., 2012), is that starved animals get

smaller as starvation progresses. Starved animals also tend to lose their green color with time, getting pale as starvation progresses (Hinde & Smith, 1972; 1975; Curtis et al., 2006). Here we take a step back in the study of "photosynthetic slugs", as many — including ourselves — have called them in the past, by re-inspecting the role of light. We test the light dependence of $^{14}CO_2$ incorporation into acid-stable compounds in *E. timida* and *P. ocellatus*, the long-term starvation survival of plastid-bearing slugs in light *versus* dark, and the effect of the photosynthesis inhibitor Monolinuron on the ability of *P. ocellatus* to survive starvation in the light. Surprisingly, photosynthesis was not essential for the slugs to survive months of starvation, which explains the lack of gene transfer from alga to animal in these species and, more importantly, calls for a general rethinking of the "photosynthetic slug" story.

RESULTS AND DISCUSSION

The relationship between sacoglossans that perform long-term retention of their sequestered plastids is now widely reported in the literature as an example of acquired photoautotrophy in animals (Rumpho et al., 2000; Weber & Osteryoung, 2010; Rumpho et al., 2011), typically leading to questions of how many and what kinds of genes have been transferred to support this photoautotrophic lifestyle (Rumpho et al., 2008). Critical of that view, we recently tested the gene transfer hypothesis in sacoglossans that perform long-term retention and found no evidence for the expression of any genes of demonstrably green algal nuclear provenance to support plastid longevity in two of the four known LtR species, *Elysia timida* and *Plakobranchus ocellatus* (Wägele et al., 2011). That eyebrow-raising result prompted us to further re-inspect to what degree plakobranchid sacoglossans exhibiting long-term retention depend on photosynthesis in the first place.

E. timida and P. ocellatus display light-dependent CO2 fixation

Previous studies on several plastid-bearing sea slugs have shown that green animals can fix $^{14}CO_2$ (Greene, 1970; Greene & Muscatine, 1972; Trench, 1973; Kremer & Schmitz, 1976; Clark et al., 1981; Marìn & Ros, 1989). However, there are also exchange reactions and carboxylation steps in animal metabolism that would allow $^{14}CO_2$ to be incorporated into

animal tissue in a light-independent manner. For example, propionate is a main primary source of reduced carbon in many animals; it is absorbed from the gut, where it is generated from ingested food by the gut microbial flora. Propionate is channeled into metabolism as propionyl-CoA, that is then carboxylated to methylmalonyl-CoA and rearranged in a vitamin B_{12}-dependent reaction to the citric acid cycle intermediate succinyl-CoA and then succinate, which can be used either for biosynthetic (amino acids, heme, etc.) or for energetic purposes (Marìn & Ros, 1989). Thus, via succinate, $^{14}CO_2$ can be incorporated into animal tissue, but in a light-independent manner. Furthermore, the fixation rates reported so far vary substantially between different species studied (Greene & Muscatine, 1972; Kremer & Schmitz, 1976; Clark et al., 1981; Marìn & Ros, 1989; Rumpho et al., 2001).

We investigated the ability of *E. timida* bearing *Acetabularia* plastids to fix $^{14}CO_2$ in the absence and presence of light. In total we used 24 slugs for three individual experiments. We analyzed the light-dependent incorporation of [^{14}C]-labeled CO_2 after 2 min, 1 h and 2 h. Four slugs were used for every time point and kept either in the light or in the dark. Afterwards, incorporation of labeled carbon was measured. Adults slugs lacking plastids cannot be used as a control here, because individuals of these sacoglossan species do not develop into adults unless they feed, at the larval stage, upon their specific algae, and because plastid-bearing adults die before they can be starved to the stage of lacking plastids altogether. After 2 min incubation with [^{14}C]-labeled CO_2, incorporation in the light was slightly higher than in the dark (0.05 nmol in the light versus 0.04 nmol in the dark). After one hour, slugs in the light showed incorporation 23-fold higher than slugs in the dark (6.73 nmol incorporated $^{14}CO_2$ in the light and 0.30 nmol in the dark). In the light, $^{14}CO_2$ incorporation after two hours was 60 times greater than for slugs kept in the dark (28.1 nmol vs. 0.46 nmol $^{14}CO_2$) (Fig. 2). Thus we can confirm that *Acetabularia* plastids in *E. timida* fix CO_2 in a light-dependent manner, as has been reported for other plakobranchids (Greene, 1970; Greene & Muscatine, 1972; Trench, 1973; Kremer & Schmitz, 1976; Clark et al., 1981; Marìn & Ros, 1989). That CO_2 fixation in the dark is almost abolished demonstrates that light-independent carbon fixation reactions, although they can occur in slug metabolism, are overshadowed by light-dependent CO_2 fixation in sequestered plastids.

For *P. ocellatus* we obtained similar results (Fig. 2), furthermore showing that in comparison to the untreated slugs, in the Monolinuron treated samples the incorporation

of $^{14}CO_2$ was 87% lower after 120 min, indicating that photosynthesis in the slugs is inhibited by the drug. Previous studies reported ^{14}C in a variety of slug metabolites (Greene, 1970; Greene & Muscatine, 1972; Trench, 1973; Kremer & Schmitz, 1976; Clark et al., 1981; Marìn & Ros, 1989), but whether the label stems from photosynthate exported from intact plastids or simply from decomposing plastids is not known. That is, it is possible that sequestered plastids do not export reduced carbon, but are simply digested, a possibility that is supported by microscopic observations suggesting that kleptoplasts accumulate substrate under starvation conditions, rather than secreting it (Cutignano et al., 2009; Maeda et al., 2012). Notwithstanding many studies in the literature addressing the nature of plastid-slug metabolite interactions, it seemed that the more crucial question was whether light-dependent CO_2 fixation was essential for survival of the animals grown without algal food.

Blocking photosynthesis affects neither survival rate nor weight decrease during starvation

Earlier work on the LtR species *E. timida* and *P. ocellatus* delivered conflicting results with respect to the role of light during starvation. Some studies indicated that specimens starved in the dark lost weight faster and had a higher death rate than those starved in the light, from which it was concluded that photosynthesis is important for the survival of these LtR slugs (Hinde & Smith, 1972; 1975; Giménez-Casalduero & Muniain, 2008). However, in those experiments some slugs survived just fine in the dark and *vice versa* some kept in the light died. In the starvation experiments performed on *Elysia timida* (Giménez-Casalduero & Muniain, 2008) the survival rate was only monitored for three, apparently randomly chosen, aquaria (of in total nine), and the exact survival rate across all aquaria was not reported. While Yamamoto and colleagues (Yamamoto et al., 2012) reported a higher death rate for those slugs kept in the dark, too, they noted that the higher dark death rate could be attributable to water fouling, as the survival rate even for those kept in the light was very low. From our experience it is crucial to keep each experimental animal in a separate repository, whilst at the same time regularly monitoring water quality. In all of our experiment, only one animal (one *E. timida* kept at 40µmol quanta $m^{-2}s^{-1}$) died at day 23 of starvation.

Recent results reported for *P. ocellatus* indicate that in the wild, the contribution of photosynthesis by sequestered plastids to the animal's carbon uptake is very minor, and raised the question of whether photosynthesis in kleptoplasts contributes significantly to

nutrition during starvation (Bhattacharya et al., 2013). To readdress the role of light, we blocked photosynthesis in two ways: first by simply culturing slugs in the dark and second by inhibiting photosynthesis through Monolinuron. We kept six specimens of *E. timida* individually in total darkness over a time course of almost three months. Based on PAM fluorescence the maximum quantum yield was better for the dark-kept animals than for those kept in the light (40 µmol quanta $m^{-2}s^{-1}$) (Fig. 3). *P. ocellatus* animals were weighed and their plastid photosynthetic capacity measured through PAM. Using 2 µg/ml of Monolinuron, slugs cultivated at 40 µmol quanta $m^{-2}s^{-1}$ revealed an average inhibition of photosynthesis by 42% as determined by PAM measurements (Fig. 4A). Yet, animals cultivated in the presence of Monolinuron survived just as well as the control set over the 55 days analyzed. Importantly, the control slugs, the Monolinuron-treated slugs, and those kept in the dark, all showed the same degree of weight loss at day 49 when the experiment was ended (Fig. 4B).

In our hands, both LtR plakobranchids studied survived equally well the dark as they did in the light. Furthermore, for Plakobranchus, comparing the individual regressions of weight loss with each other demonstrated that none statistically behaved different from the others. That is, those starved in the dark and those starved in the light and treated with Monolinuron, lost weight in the same way as the control (slugs starved in the light). We did try to determine the weight for *E. timida* as well, but due to the small size of this species, handling was difficult and results unreliable. However, the results clearly indicate that photosynthesis, as a core carbon source cannot be essential for slug survival in these two LtR sacoglossan species. It remains a possibility that the slugs require specific compounds synthesized in plastids for proper development, such as the synthesis of pyrone-containing proprionates (Cutignano et al., 2009), in particular because Ireland and Scheuer (Ireland & Scheuer, 1979) suggested that a significant part of the fixed CO_2 might be dedicated to the biosynthesis of pyrone-containing proprionates. In any case, as far as basic nutrition goes, the slugs are apparently not "solar powered" at all. That readily explains why no algal genes essential for photosynthesis are expressed by slug nuclei: the gene products are not required, and this is why algal nuclear genes are not required to support kleptoplast longevity (Rumpho et al., 2011; Wägele et al., 2011). Accordingly, recent nuclear genome data of egg DNA of *Elysia chlorotica* uncovered no evidence for putatively transferred algal genes (Bhattacharya et al., 2013). Once a nuclear genome becomes available, it will need to

provide evidence that genes of algal origin occur at the same copy number in the assembly as a single copy plakobranchid gene.

The plastids of these LtR species remain capable of photosynthesis, as the PAM and $^{14}CO_2$ incorporation results show (Figs 3, 4 and 2, respectively), but the observation that light has no detectable effect on animal survival or weight loss during starvation indicates that whatever the plastids do, they do not have a life-extending effect on the animals that depends upon photosynthetic activity over the three month time we analyzed. Photosynthesis in sequestered plastids of LtR species might be important for their longevity, but this has yet to be shown. Our experiments measured the survival of the slugs, not the survival of the plastids directly, although the data in Fig. 4A shows that plastids maintained in the dark are, by the measure of PAM fluorescence, just as viable as those maintained under 12h/12h light/dark. The plastids appear to be a source of stored food, and although the similar experiments have yet to be reported for the other two LtR species – *Elysia chlorotica* and *Elysia crispata* – the clear implication from these findings is that light is probably not required for the long-term survival during starvation of those species either.

If plastid fitness had a direct impact on slug fitness, blocking photosynthesis, whether through light deprivation or Monolinuron, should influence the weight and survival rate of the animals. Yet, those *Plakobranchus* specimens kept in the dark or treated with Monolinuron lost weight at the same rate as the starving control specimens (Fig. 4B) and the *E. timida* slugs kept in the dark appeared as healthy as the control set, too. Furthermore, the linear regression of the maximum quantum yield of slugs kept in the dark (Fig. 3) and those treated with Monolinuron (Fig. 4A) runs parallel to those of the control animals, rather than declining more rapidly over time as a proxy of reduced and essential plastid contribution to nutrition. Klochkova and colleagues (Klochkova et al., 2012) recently observed that specimens of the short-term retention (StR) species *Elysia nigrocapitata* survived for five months without performing photosynthesis, during which time the starved animals dramatically lost weight. Notably, *E. nigrocapitata* animals go from 3 cm in length to 3 mm during starvation, but reversibly, if provided with food (Klochkova et al., 2012).

Conclusions

It has been established that LGT is not involved in kleptoplast maintenance following starvation either in slugs (Rumpho et al., 2011; Wägele et al., 2011) or in Foraminifera (Pillet & Pawlowski, 2013). The present findings go a step further by showing that sacoglossan slugs survive for months with the help of kleptoplasts, but without the help of photosynthesis. While the plastids are photosynthetically active, they do not confer an autotrophic lifestyle upon the slugs. It rather appears that the slugs sequester their plastids not directly as a source of photosynthetic capabilities, but as a source of stored food reserves, whose nutritional value does not depend on light subsequent to sequestration. Plastid longevity in LtR sacoglossan slugs remains an interesting phenomenon, but the present results prompt a shift in emphasis from viewing the kleptoplasts as green solar panels towards viewing them as green food reserves.

EXPERIMENTAL PROCEDURES

Elysia timida individuals were collected in Banyuls-sur-Mer (France) between July and September 2012 and transferred to Bonn (Germany). Specimens were kept with food algae in petri dishes with artificial seawater (Tropic Marine®) at 20°C and water changed every two days. For acclimation to lab conditions the slugs were illuminated at 25µmol quanta $m^{-2}s^{-1}$ and a 12h/12h day-night cycle under a "Daylight Lamp" (Androv Medical, Model AND1206-CH) for six days. Then six individuals of *E. timida* were separately starved in petri dishes under 25 and 40µmol quanta $m^{-2}s^{-1}$ under a day/night of 12h/12h and under complete darkness for a maximum of 88 days. Analyses of photosynthetic activity were performed with a Pulse Amplitude Modulated Fluorometer (Diving PAM, Walz, Germany) by measuring the maximum quantum yield of chlorophyll a fluorescence in photosystem II. Specimens kept under light conditions were dark acclimated for 15 min prior to the measurement.

Plakobranchus ocellatus individuals were collected on the Philippines in November 2012 and transferred to Bonn (Germany). Two aquaria were set up with 20L artificial seawater (Tropic Marine®) at 22°C with two specimens of P. ocellatus, respectively, each in individual fishnet. Additionally, two specimens were placed in an aquarium in individual fishnets in 10L artificial seawater at 22°C. One third of the water in every aquarium was

changed weekly and the best water quality established through the use of an internal filter (Eheim, Germany). To one 20L aquarium 2 µg 3-(4-Chlorophenyl)-1-Methoxy-1-Methylurea (Monolinorun) /ml seawater was added. This and the 10L aquarium were illuminated at 25µmol quanta m-2s-1 under a day/night cycle of 12h/12h. The second 20L aquarium was kept in the dark. All specimens were starved for 55 days and afterwards fixed in 4% formaldehyde for further analysis not addressed here. PAM-measurements were taken using a Diving PAM (Walz, Germany). Weight of all six specimens was measured at day 0, 14, 28 and 49 of the experiment by placing the slugs on a spoon, gently removing all remaining water with a paper towel and placing them into pre-set water container onto a scale. Measurements were taken three times and mean values determined. Data of each trial were pooled. For each individual, the weights were scaled to a maximum of 1 and the linear regression calculated. With 20.0059, 'dark' had the lowest slope followed by the monolinuron-treated slugs with 20.0067 and the control set with 20.0114. Using a Tukey test, we tested pairwise whether the slopes of the linear regression lines were equal (H0). The resulting q-values with a significance level of 0.05 showed a significant difference between 'dark' and 'normal' conditions, while the remaining slopes did not significantly differ (p-values: control to monolinuron- treated ≥ 0.0567; monolinuron-treated to darkness ≥ 0.9193; and darkness to control ≥ 0.023).

Elysia timida used for incubations with [14C]-labeled CO2 were collected in the Mediterranean Sea (Banyuls-sur-Mer, France) in October 2012 and transferred to Düsseldorf (Germany). They were maintained for six weeks at 15°C and 33 µmol quanta $m^{-2}s^{-1}$ in 12L-aquaria containing 25 specimens, artificial seawater (37 g/l hw-Marinemix professional (Wiegandt, Germany) and *Acetabularia acetabulum* as food source. They were starved for several days before labeling. To test the light-driven incorporation of CO_2 by *Elysia*, the slugs were incubated in 1.2 ml artificial seawater supplemented with 0.32 mM [^{14}C]-$NaHCO_2$ (18 µCi per incubation, NEN-radiochemicals, MA, USA). For each measurement 4 slugs were incubated together in a transparent plastic 1.5-ml tube. *Plakobranchus ocellatus* individuals used for incubations with [^{14}C]-labeled CO_2 were collected on the Philippines in April 2013 and transferred to Bonn (Germany). To test the light-driven incorporation of CO_2 they were incubated in 5 ml artificial seawater supplemented with 0.16 mM [^{14}C]-$NaHCO_2$ (36 µCi per incubation). Each measurement contained one single organism in an 8 ml glass tube. The measurements for time point 120

were carried out twice and the mean values are shown in Fig. 2. All incubations of both species were performed at room temperature either in the dark or illuminated (72 µmol quanta $m^{-2}s^{-1}$). The incubations lasted 2 minutes, 1 hour or 2 hours, and afterwards the slugs were separated from the radioactive incubation medium, rinsed 5 times with sea water and then homogenized in a small glass-teflon Potter-Elvehjem tissue grinder in 1 ml (*Elysia*) or 3ml H_2O (*Plakobranchus*). The homogenates of *Elysia* were removed and the Potter tube rinsed twice with 1 ml H_2O. These 3 ml, containing the homogenized slugs, were acidified with 150 µl 1M HCl and the open vial was then shaken overnight to remove all the substrate, (labeled) carbon dioxide. Afterwards, incorporation of labeled carbon atoms by the *Elysia* slugs was determined in a scintillation counter after the addition of 12 ml LUMA-Gel scintillation cocktail (LUMAC, the Netherlands). To the homogenates of *Plakobranchus* 3 ml H_2O and 300 µl 1M HCl were added, while the rest of the method to measure acid stable incorporation of carbon dioxide was the same as for *Elysia.*

ACKNOWLEDGEMENT

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FIGURES

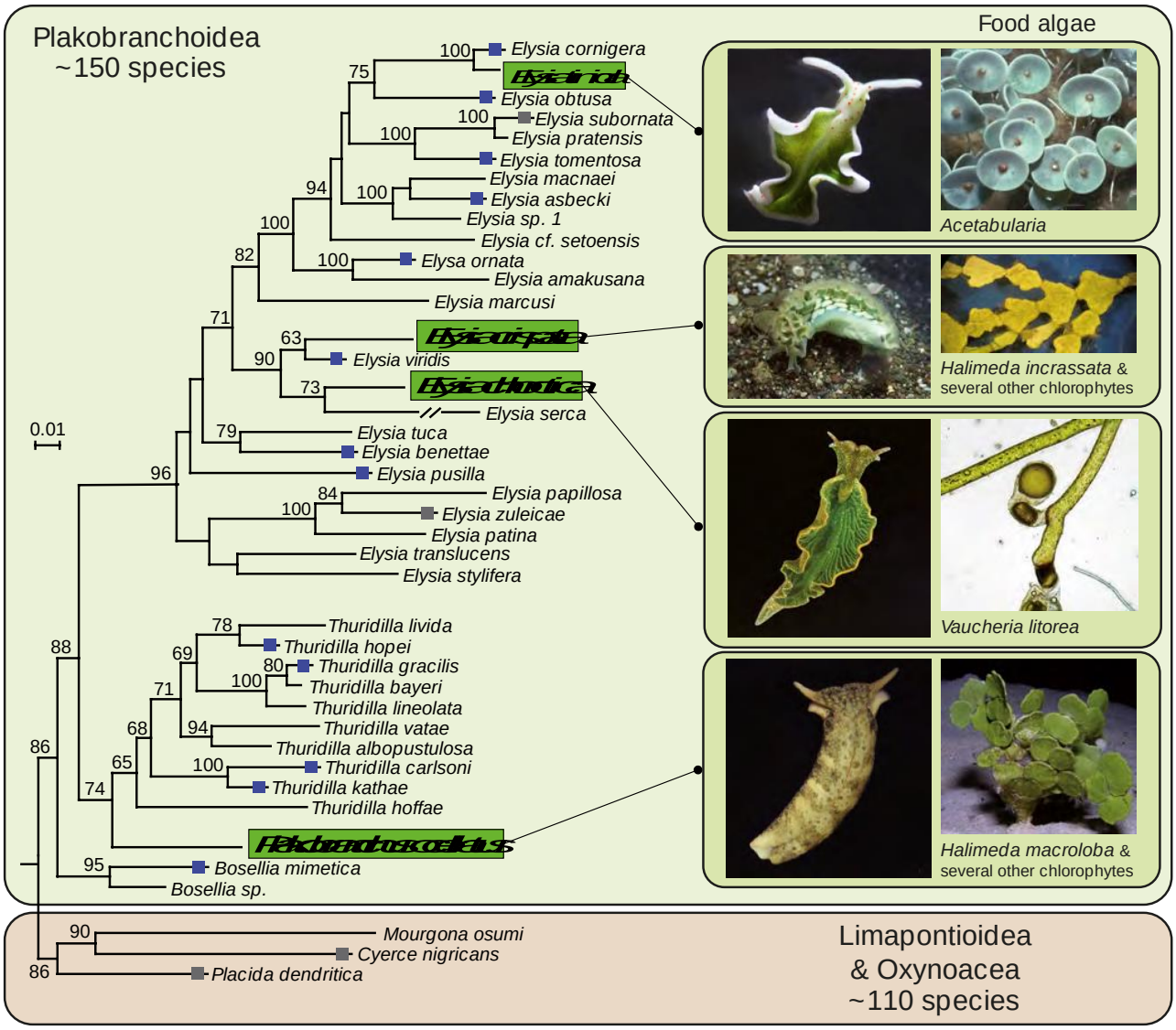

Figure 1 Overview of phylogenetic relationships of Plakobranchoidea (Sacoglossa). Blue rectangles indicate photosynthetic activity over at least 2 weeks of starvation (based on Pulse-Amplitude-Modulation measurements), whereas grey squares indicate species that immediately digest plastids. Slugs showing photosynthetic activities over 2 months are highlighted in green, the respective picture of the species and food alga are provided on the right. Photo of *E. chlorotica* with permission of M. Rumpho, and that of *Vaucheria litorea* with permission of C. F. Carter. Phylogeny based on Wägele et al. (Wägele et al., 2011).

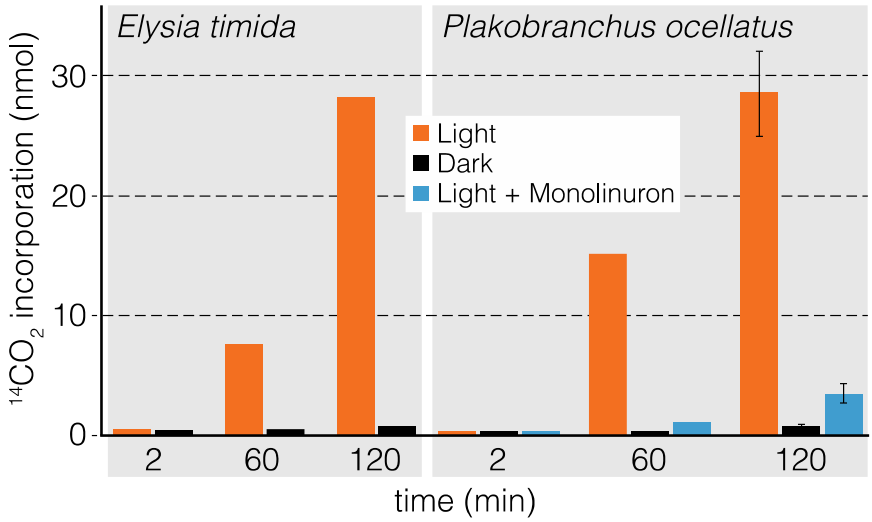

Figure 2 Light-dependent incorporation of $^{14}CO_2$ by *Elysia timida* and *Plakobranchus ocellatus*. CO_2 incorporation in *E. timida* is almost completely blocked when the slugs were kept in the dark. In *P. ocellatus* we additionally blocked photosynthesis using Monolinuron, which led to a 87% decrease of CO_2 incorporation. Due to the size difference, four *E. timida* specimens (always representing an equal amount of weight) and only one *P. ocellatus* were used for each individual time point measured. Only for the 120 min values of *P. ocellatus*, the mean of two individual measurements is shown [values (nmol/inc) of these 120 min incubations of *P. ocellatus* were D: 0.42 and 0.47; L: 24.5 and 32.2; M: 3.14 and 4.35], for all others a single measurement was carried out.

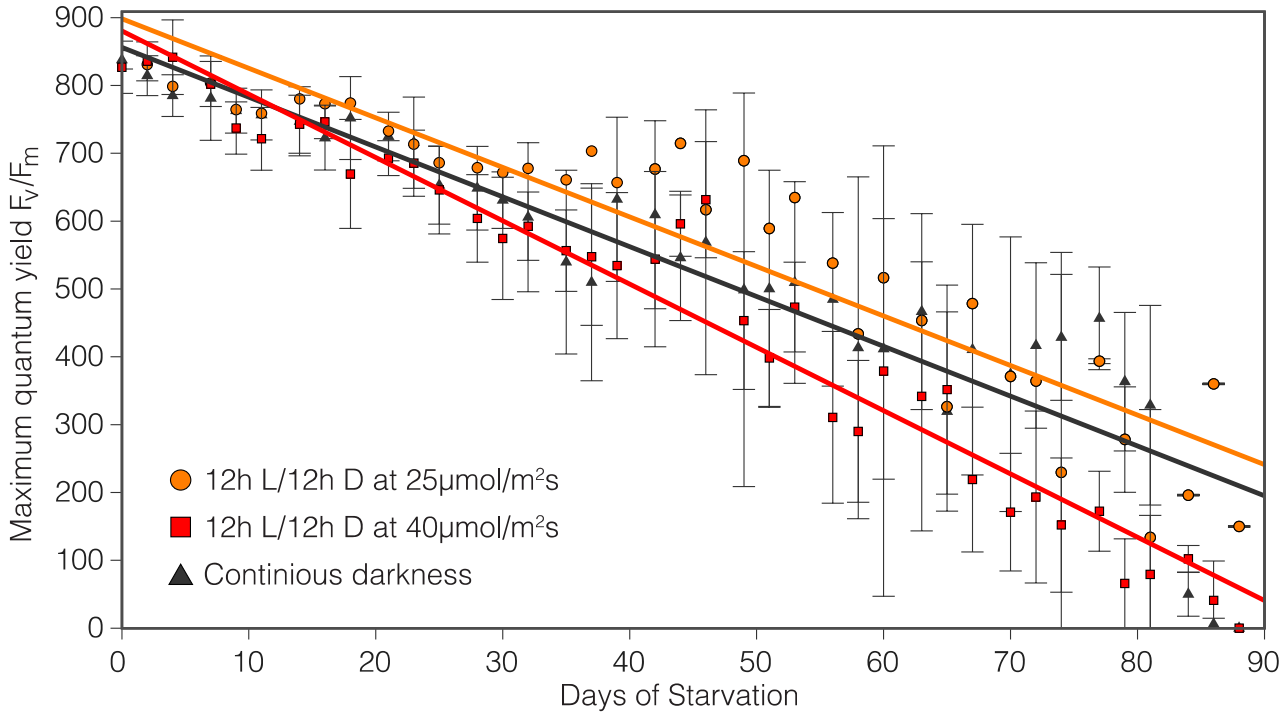

Figure 3 Pulse-Amplitude-Modulation (PAM) measurements of *Elysia timida*. The maximum quantum yields of slugs kept in the dark (black) was compared to slugs kept under low (orange) and high light (red) conditions. Those kept under high light show the strongest decrease over the three months measured, whereas the linear regression of those kept in the dark runs in parallel to that of those kept under low light conditions. Six specimens were used for each condition tested. The error bars present the standard deviation.

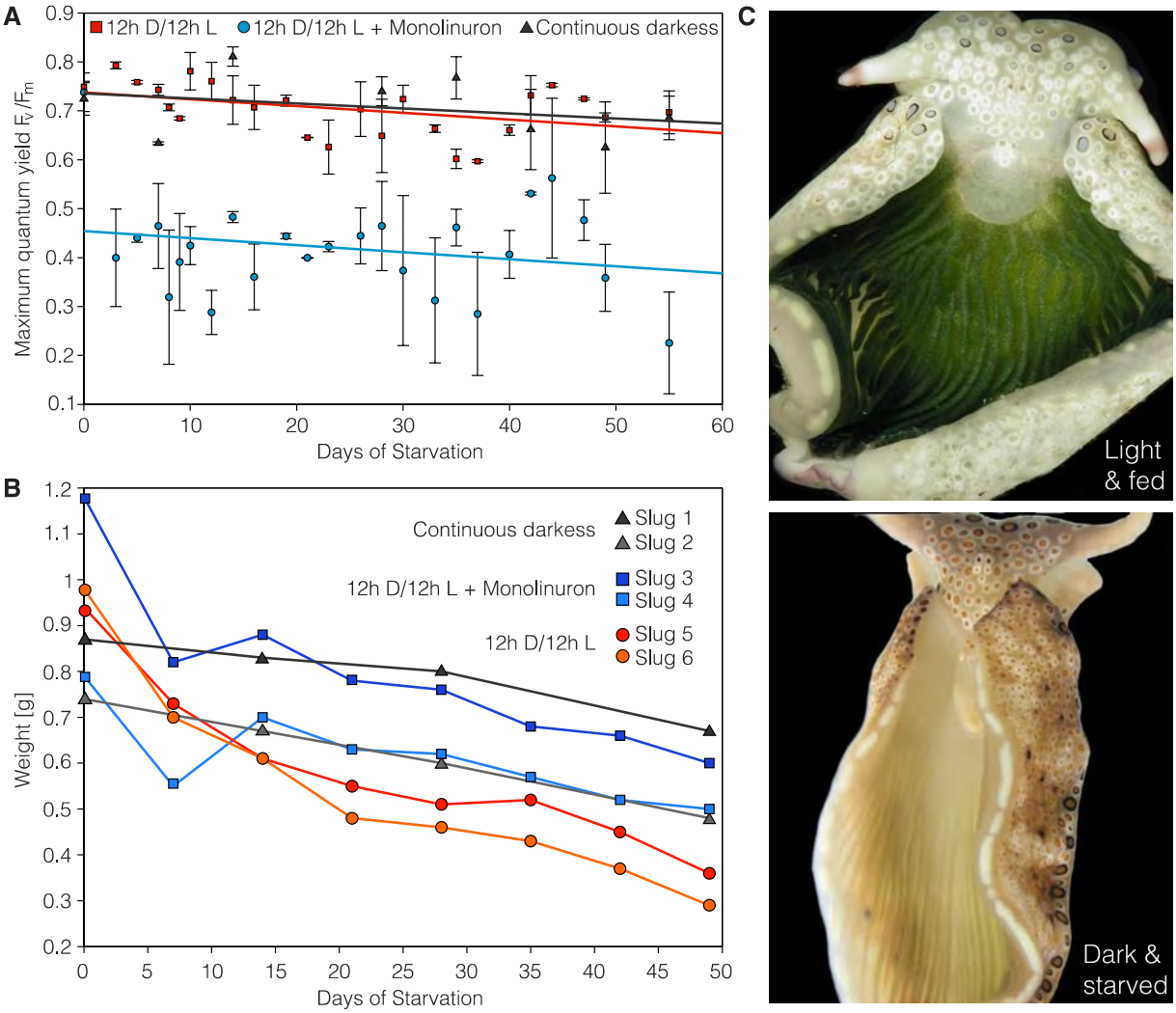

Figure 4 Influence of photosynthesis inhibition on *Plakobranchus ocellatus*. A) Pulse-Amplitude-Modulation (PAM) measurements of Monolinuron treated slugs in a 12h/12h day/night cycle (25µmol quanta $m^{-2}s^{-1}$; blue) in comparison to those kept in the dark (black) and under at a 12h/12h day/night cycle (25µmol quanta $m^{-2}s^{-1}$; red). Two specimens were used for each condition tested and the error bars present the standard deviation. B) Weight measurements of the *P. ocellatus* specimens shown in A. C) Exemplary images of *P. ocellatus* specimens. The top image shows a slug kept in the light and which was regularly fed, hence best representing natural conditions. The bottom image shows a slug after 55 days of starvation in the dark.

CHAPTER 6

Phylogenetic evidence that functional kleptoplasty evolved twice in Sacoglossa (Heterobranchia, Gastropoda)

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ABSTRACT

Sacoglossa is a rather small taxon of marine slugs with about 300 described species, yet it is quite fascinating for scientist for decades. This is mainly because of the ability of certain species to incorporate still photosynthetic active plastids of their algae prey, a phenomenon known as functional kleptoplasty. With the stolen plastids these slugs endure weeks (short-term-retention) or months (long-term-retention) of starvation, though contribution of the plastids to the survival and factors enhancing plastid longevity are unknown. Likewise contrasting hypotheses on evolution of functional kleptoplasty exist and the phylogenetic relationship of Sacoglossa taxa are still under debate. We analyzed the phylogenetic relationship of 105 sacoglossan species to address the question of the origin of functional kleptoplasty. Based on our phylogenetic analysis and the ancestral character state reconstruction we conclude that functional short-term-retention more likely arose twice. Previous suggestions that functional long-term-kleptoplasty is established with specific plastids are supported by our food analyses in *E. clarki* that finally harbors only plastids of the genus *Halimeda*, an unknown Rhipiliaceae and an unknown Pseudocodiaceae after a distinct starvation periods.

INTRODUCTION

Symbiosis between heterotrophic and phototrophic organisms is widely distributed among metazoan such as porifera, cnidarians or mollusks (Johnson, 2010). A special form of symbiosis is found in a peculiar taxon of marine sea slugs: the Sacoglossa (Gastropoda, Heterobranchia). These slugs usually feed upon siphonous green algae by piercing the cell wall, sucking out the cell content and sequestering the plastids in cells of the digestive glands (Jensen, 1980; Händeler et al., 2009). The plastids of the algal food are then maintained by the slug in a symbiotic-like association, for which the term functional kleptoplasty was established (Waugh & Clark, 1986; Rumpho et al., 2006; Wägele & Martin, 2013). How the slugs benefit from the kleptoplasts, e.g., via transfer of photosynthates from plastids into the slugs (Trench & Gooday, 1973; Gallop, 1974) is still unknown. Despite results of former studies (Hinde & Smith, 1975; Trench, 1975; Giménez-Casalduero & Muniain, 2008) there is nowadays evidence that the slugs are not photoautotrophic (Christa et al., 2014).

The taxon Sacoglossa, with about 300 described species, comprises two major sister-clades: the shelled Oxynoacea with about 20% of all known species and the shell-less Plakobranchacea (Jensen, 1997; Kohnert et al., 2013). The ability to retain functional plastids varies among sacoglossan species and some taxa digest the plastids instead of maintaining them, like taxa of the Oxynoacea (Händeler et al., 2009). While functional kleptoplasty is only known from the genus *Costasiella* within the Limapontioidea, it is fairly common within the Plakobranchoidea (Clark et al., 1981; Händeler et al., 2009; Yamamoto et al., 2009; Johnson, 2010; Klochkova et al., 2010; Wägele & Martin, 2013).

Three categories of functional kleptoplasty are differentiated based on chlorophyll a fluorescence measurements (Maxwell et al., 2000) and the change (mostly the decrease) of the maximum quantum yield value (F_v/F_m) during starvation (Händeler et al., 2009): i) non-retention (NR), ii) short-term-retention (StR), iii) long-term-retention (LtR). We use this classification despite the fact that the limitation of the three states is to some degree difficult and that the photosynthetic capability of species can be influenced by various factors [see review of (Cruz et al., 2013)].

The phylogenetic relationships within Sacoglossa are still not satisfactorily resolved. Previous analyses used the shelled genus *Cylindrobulla* as outgroup (Jensen, 1980; 1996; Händeler & Wägele, 2007; Händeler et al., 2009), but recently it is assigned to the Oxynoacea based on molecular analyses (Jörger et al., 2010; Maeda et al., 2010; Neusser et

al., 2011). Sacoglossa however are now considered as a group within the Panpulmonata (Jörger et al., 2010; Neusser et al., 2011; Kocot et al., 2013). Relationships in Plakobranchacea (Limapontioidea and Plakobranchoidea), as well as in Limapontioidea could not be resolved so far (Händeler & Wägele, 2007; Händeler et al., 2009; Maeda et al., 2010), but this is in particularly important to reconstruct the evolution of functional kleptoplasty: Händeler et al. (2009) hypothesized its origin at the base of the Plakobranchoidea, but did not consider the genus *Costasiella* that exhibits functional kleptoplasty (Clark et al., 1981). Maeda et al. (2010), including *Costasiella* in their analysis, estimated the evolution of functional kleptoplasty at the base of the Plakobranchacea. Though, the relationship of Limapontioidea and Plakobranchoidea was not resolved in this analysis; thus they probably overestimated the evolution of functional kleptoplasty.

The mechanisms that enhance functional kleptoplasty are still unknown. Studies on LtR forms have convincingly shown that no algal genes were transferred to the slugs' genome that could have provided genetic information to support plastid longevity (Pelletreau et al., 2011; Wägele et al., 2011; Bhattacharya et al., 2013). Hence, mechanisms must have evolved to at least somehow control the digestion, since LtR forms do not digest the plastids in the same way as NR forms do (Evertsen & Johnsen, 2009; Händeler et al., 2009). The plastids on the other hand must also posses some features enabling survival outside the algal cell environment (Trench et al., 1973b; Gallop, 1974; Wright & Grant, 1978; Clark et al., 1990; Evertsen & Johnsen, 2009). Recently FtsH, a proteolytic enzyme important in the repair of the photosystem II, was discussed as such a factor. It is plastid encoded in at least two algal food sources of LtR forms (*Acetabularia* and *Vaucheria*), but not in another alga (*Bryopsis*) consumed by StR and NR forms (de Vries et al. 2014). Eventually, this can help to explain recent results on longevity of plastids in the polyphagous species *Plakobranchus ocellatus* van Hasselt, 1824, that only harbors plastids of *Halimeda* after a distinct starvation period (Christa et al., 2013). Thus, the correct identification of plastid origin in sacoglossan sea slugs with knowledge on their ability for short- and long-term-retention is therefore necessary to understand the role of FtsH in this enigmatic system. Especially polyphagous species, known to perform functional kleptoplasty, like *Elysia clarki* Pierce, Curtis, Massey, Bass, Karl & Finney, 2006 are of particular interest.

Here we present and include new results on the distribution of functional kleptoplasty within the Sacoglossa by measuring the chlorophyll a fluorescence. We

identified food sources of some unprocessed taxa by DNA-barcoding using *rbcL* and examined, if some plastids remain longer periods than others during starvation on *Elysia clarki*. We performed a comprehensive phylogenetic analysis of Sacoglossa to examine the relationship between Limapontioidea and Plakobranchoidea and executed an ancestral character state analysis (ACR) to investigate the most likely origin of functional kleptoplasty.

MATERIALS AND METHODS

Specimen collection and maintenance

Specimens for phylogenetic analysis and food barcoding were collected at different localities and immediately fixed in 96% EtOH and stored at -20°C (see Supplementary Data 1 and 2).

For analysis of functional kleptoplasty specimens were collected from February-May 2012 and May 2013 on the Florida Keys by snorkeling and examined either at Mote Marine Laboratory, Summerland Key or at Keys Marine Laboratory, Long Key (see Supplementary Data 3). *Alderia willowi willowi* Krug, Ellingson, Burton & Valdes, 2007 and *Alderia modesta* (Loven, 1844) were collected at San Francisco Bay and gently provided by Dr. Patrick Krug (California State University, Los Angeles, USA).

Depending on the size, animals were cultivated under natural light conditions in individual petri dishes or 300ml plastic container, with daily water change at 22-24 °C. Individuals of *Elysia clarki*, *Alderia* and *Alderia modesta* were transferred to Bonn, Germany, examined under laboratory light conditions with light intensity of 40µmol quanta $m^{-2}s^{-1}$ and cultivated in artificial seawater (Aqua Marin) at 22 °C. Light conditions were set-up by a full spectrum day light lamp (Androv Medical, Model AND1206-CH), and water changed on a daily base.

Phylogenetic analysis

Phylogenetic relationships of Sacoglossa were reconstructed using two partial nuclear marker (*H3* and *28S*) and two partial mitochondrial marker (*COI* and *16S*). Sequences were either generated *de novo* or retrieved from GenBank (Supplementary Data 1). For specimens that were processed *de novo* DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions and stored at -20 °C.

Sequences were generated with Sacoglossa specific primers (Händeler et al., 2009; Krug et al., 2013, Christa et al., submitted) under the following conditions: 1 µl of genomic DNA was used in a 20 µl final volume reaction supplied with 5 µl sterilized water, 2 µl Qiagen Q-Solution, 10 µl of double concentrated Qiagen Multiplex PCR Master Mix and 1 µl of 10 pmol/µl concentrated gene specific primer each. Amplification of partial *COI* was performed by denaturation for 15 min at 95 °C, followed by 25 standard cycles (94°C for 90 s, 48 °C for 90 s and 72 °C for 90 s) and a final extension at 72°C for 10 min. Amplification of partial *16S* was performed by denaturation for 15 min at 95 °C, followed by 9 touch-down cycles (94 °C for 90 s, 58 °C (-1 ° per cycle) for 90 s, 72 °C for 90 s) followed by 25 standard cycles (94 °C for 90 s, 49 °C for 90 s and 72 °C for 90s). Amplification of partial *H3* was performed by denaturation for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 90 s, 50 °C for 90 s and 72 °C for 90 s). Amplification of partial *28S* was performed by denaturation for 15 min at 95 °C, followed by 9 touch-down cycles (94 °C for 90 s, 65 °C (-1 °) for 90 s, 72 °C for 90 s) followed by 25 standard cycles (94 °C for 90 s, 56 °C for 90 s and 72 °C for 90 s).

Each gene was aligned separately using MAFFT (Katoh et al., 2002) (lengths of single gene alignments were 269bp for H3, 1351bp for *28S*, 657bp for *COI* and 537bp for *16S,* respectively), subsequently concatenated using Fasconcat (Kück & Meusemann, 2010) and manually improved when necessary (total alignment length 2480bp). Based on previous analysis (Händeler et al., 2009) only the 1st and 2nd position of *COI* was used for phylogenetic reconstruction in the concatenated alignment. Bayesian analysis was performed with MrBayes [v. 3.2, (Ronquist et al., 2012)] using the CIPRES Science Gateway server (http://www.phylo.org/portal2/login) (v. 10.7.5) with the GTR model and four random starting trees. For each tree three heated and one cold Markov chain were used for 5.000.000 generations with sampling each 1000th generation. Runs stopped after 1.687.000 generations (average standard deviation of split frequencies was lower than 0.005 and log likelihood values of the cold chain did not further increase). The first 1000 trees of all runs were discarded as "burn-in" and a majority rule consensus tree, of the remaining 2748 trees (687of each run), calculated. Posterior Probabilities (PP) were calculated to determine nodal support of the 50% majority rule consensus tree.

Reconstruction of ancestral state characters

To estimate the origin of functional kleptoplasty an ancestral state reconstruction (ASR) was performed. Maximum likelihood and maximum parsimony ASR were carried out using Mesquite [v. 2.75,(Maddison & Maddison, 2001)]. Analyses were performed over the 50% majority rule consensus tree generated by the Bayesian analysis. The Markov k-state 1 (Mk1) model, with equal probability for any particular character change, was used as model of evolution for both analyses. We coded information on specimens known for their retention form (see Christa et al. submitted) as having one of the three characters: NR (0), StR (1) and LtR (2). When no information was available specimens were coded as missing data. To address the question whether *Halimeda* or *Caulerpa* is the most likely ancestral food source of Sacoglossa we performed an ASR by coding information on specimens known for their food sources as follows: *Halimeda* (0), *Caulerpa* (1). When no information was available or *Halimeda* or *Caulerpa* is not the food item the respective species was coded as missing data.

PAM-measurements

Maximum quantum yield (Fv/Fm) values were measured for all examined species during starvation with a Diving-PAM (Walz, Germany) with set-ups as described elsewhere (Supplementary Data 2; Christa et al., submitted). One measurement of every individual was taken on a regular base. Specimens starved in the light were dark acclimated for 15 min prior to measurements.

Food analyses

For barcoding of sacoglossan food sources, several non-starved slug specimens were directly fixed in 96% EtOH after collection. For *E. clarki* a starvation experiment under two different light conditions was set up: four individuals were starved in the light at 40µmol quanta/m^2s^1 and four individuals starved in complete darkness. After seven, 28, 35 and 49 days one individual per starvation experiment was fixed in 96% EtOH and used for food analysis. *rbcL* was used as Barcoding marker with Ulvophyceaen specific primer [forward primer rbcLF: 5' AAA GCN GGK GTW AAA GAY TA 3' and reverse primer rbcLR: 5'-CCA WCG CAT ARA NGG TTG HGA-3'; (Pierce et al., 2006)]. 1 µl of genomic DNA was used as

template in a 10 µl final volume reaction supplied with 1 µl sterilized water, 1 µl Qiagen Q-Solution, 5 µl of double concentrated Qiagen Multiplex PCR Master Mix and 1 µl of 10 pmol/µl concentrated primer each. Amplification of *rbcL* was performed by an initial denaturation for 15 min at 95 °C, followed by 9 touch-down cycles at 94 °C for 45 s, 60 °C (-1 °C per cycle) for 45 s, 72 °C for 90 s, followed by 25 standard cycles (94 °C for 45 s, 51 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. Afterwards PCR products were size-fractionated in a 1.5 % agarose gel for 90 min at 70 V and bands extracted from the gel and purified using Machery-Nagel Nucleo Spin Extract II kit. Isolated fragments were ligated into pGEM t-easy Vector (Promega) and cloned into competent *E. coli* XL1-blue cells (Stratagene). Of each individual 12 clones were amplified in a 20 µl final volume reaction supplied with 14 µl sterilized water, 5 µl of double concentrated Larova PCR Master Mix (Berlin, Germany) and 1 µl of 10 pmol/µl concentrated primer each (Forward primer T7Promoter 5'TAA TAC GAC TCA CTA TAG GG 3' and reverse primer SP6Promoter 5' ATT TAG GTG ACA CTA TAG 3'). Amplification was performed by an initial denaturation for 15 min at 95 °C, followed by 25 standard cycles (94 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. Amplification products were purified using Machery-Nagel Nucleo Spin Extract II kit and all samples were sequenced by Macrogen Inc, (Amsterdam, The Netherlands). All obtained sequences were verified by BLAST search using the NCBI homepage. Subsequent, consensus sequences for one individual were generated when sequence divergence of chloroplast genes was lower than 1 %. For identifying origin of plastid sequences obtained of the slugs we used a hybrid of similarity and tree-based DNA-barcoding method (Little, 2011; Tanabe & Toju, 2013). Sequence identity was verified first by BLAST search using the NCBI homepage and assigned to an algae species if sequence similarity was 99-100%. When sequence similarity was lower than 99% or when multiple hits with 99-100% were obtained sequences were assigned to higher algal taxa according to a tree-based DNA-barcoding approach. For this purpose we combined all sequences created by ourselves with a set of corresponding algal sequences (Alignment available upon request, 561bp in length). A maximum likelihood tree was generated using RaxML server with substitution model GTR + G + I (http://embnet.vital-it.ch/raxml-bb/; Stamatakis et al., 2006) to finally assign obtained plastid sequences to certain algal taxa. Sequence assingment to the according higher taxon based on forming monophyletic groups with distinct algal species. When no monophyletic

grouping was present sequences were defined as “Ulvophyceae sp.” (Supplementary Figure 1-3).

RESULTS

PAM-measurements and survival of starvation periods

We measured the chlorophyll a fluorescence of 16 sacoglossan species and noted the maximum starvation period that individuals of each species were capable.

Oxynoe antillarum Mörch, 1863 (n=13) survived a maximum starvation period of 14 days, though F_v/F_m values indicate no capability of functional kleptoplasty at all (Fig. 1 A). *Cyerce antillensis* Engel, 1927 (n=5) and *C*. sp. 4 (n=6) showed only minor chlorophyll a fluorescence (if any) and survived a maximum of four and five days of starvation, respectively (Fig. 1B). No initial fluorescence was measured for *Alderia modesta* (n=4), *A. willowi* (n=4), *Mourgona germaineae* Marcu & Marcus, 1970 (n=4), *Ercolania* sp. 1 (n=2) and *Ercolania fuscata* (Gould, 1870) (n=2) (we did not record maximum survival rates of these five species). Overall eight plakobranchoid species were investigated: F_v/F_m values of *Bosellia marcusi* (n=3), *Elysia tuca* Marcus & Marcus, 1967 (n=22) and *Elysia zuleicae* Ortea & Espinosa 2002 (n=7) were always lower than 0.4 during starvation, while the species are able to survive nine days, or in the case of *E*. *tuca*, even 20 days of starvation (Fig. 1 F). According to the classification introduced by Händeler et al (2009) we assign these species as NR forms.

Elysia cornigera Nuttall, 1989 (n=4), *E*. *patina* Marcus, 1980 (n=4), *E. papillosa* Verill, 1901 (n=4) and *E*. *pratensis* Ortea & Espinosa, 1996 (n=5) are classified as StR forms because the F_v/F_m values are above 0.4 for at least one day of starvation. However, starvation periods differ between these species (12, 8, 22 and 8 days, respectively) (Fig. 1 C, E, F). In our analysis *Elysia clarki* (n=6) suvived for at least 49 days while F_v/F_m values were always above 0.5 (Fig. 1 E). We include here a non-sacoglossan species, *Phyllaplysia* sp. (Anaspidea, Heterobranchia), for comparison that feeds on epiphytic algae on sea grasses but does not incorporate plastids. *Phyllaplysia* sp. (n=3) is able to starve up to 10 days with F_v/F_m values between 0.1 – 0.2 (Fig. 1 A).

Food analysis

We analyzed the food source of overall nine species of the Florida Keys, out of which eight species were not processed to date (Tab. 1).

For both individuals of *Oxynoe antillarum* we exclusively found plastids of *Halimeda* (*Halimeda incrassata* and *H*. sp. 1). All individuals of the limapantiodean *Cyerce antillensis* fed on *Halimeda* sp. 1, but differences in additive food sources depending on collection locality were found: the individual collected at Sunset Drive also fed upon *Udotea* sp. 1 and on *Udotea* sp. 2, whereas the individual collected at Geiger Beach fed additionally upon *Halimeda* sp. 2, Rhipiliaceae sp. 2, *Avrainvillea* sp., *Bryopsis hypnoides*, *B*. sp. 1 and *Pseudochlorodesmis* sp. 2. The individual we collected at Henry Street additionally sequestered plastids of *Udotea* sp. 2, while in the individual collected at Niles Road only plastids of *Halimeda* sp. 1 were detected. *Cyerce* sp. 4, which was only found on a single locality, ingested almost exclusively *Halimeda* sp. 1, besides single findings of *Polyphysa* sp. (S251) and *Udotea* sp. (S252, see Tab. 1). *Elysia papillosa* only consumed *Halimeda* spp (*H*. *incrassata*, *H*. *monile*, *H*. *simulans* and *H*. sp. 2), while *Elysia patina's* identified food source is *Udotea* sp. 1 and Udoteaceae sp. 1. *Elysia subornata* incorporated plastids of *Rhipocephalus phoenix*, whereas *Elysia* cf. *zuleicae* those of *Halimeda* sp. 1 and *H*. sp. 3, *Pseudochlorodesmis* sp. 1, *Avrainvillea* sp. and *Rhipocephalus phoenix*. *Elysia zuleicae* on the other hand sequestered *Halimeda incrassata*, *Bryopsis hypnoides*, *Udotea flabellum*, Pseudocodiaceae sp. and *Polyphysa parvula.* Specimens of *Elysia clarki* fed upon different food sources depending on collection site, similar as observed in *Cyerce antillensis*. The individual from Niles Road only fed upon *Halimeda incrassata* and Pseudocodiaceae sp., while the specimens from Mote Lab fed on *Halimeda incrassata*, *H. sp. 2*, *Bryopsis* spp, Rhipiliaceae sp. 1, Pseudocodiaceae sp. and one unidentified ulvophyceaen species (Ulvophyceae sp. 1). The specimen from Geiger Beach only consumed *Halimeda incrassata* and *Acetabularia crenulata*, however the specimen from Henry Street sequestered *Halimeda monile*, *H*. *incrassata*, Pseudocodiaceae sp. and Rhipiliaceae sp. 1; those from Sunset Drive *Halimeda monile*, Pseudocodiaceae sp. and Rhipiliaceae sp. 1.

Food analysis in Elysia clarki during starvation

Plastids in *Elysia clarki* individuals kept in the dark exhibit always higher F_v/F_m values during the 49 days of starvation than in the light, though in both cases values are generally higher than 0.4 (Fig. 2, Supplementary Data). *Halimeda incrassata*, *Rhipiliaceae* sp. and Pseudocodiaceae sp. are still incorporated after a starvation period of 49 days,

independent on light regime (Tab. 2). *Acicularia schenckii* and *Penicillus* sp. sequences were only retrieved in one animal after seven days of starvation (at 40µmol quanta $m^{-2}s^{-1}$, Tab. 3). *Bryopsis,* however, was not found in any of the starved specimens. Between individuals of both light regimes and various starvation periods, only minor differences are found in food composition (Tab. 2, Supplementary Data).

Phylogeny

The dataset presented here comprised 146 specimens of 105 species, with six species belonging to the outgroup. The monophyly of the Sacoglossa is well supported (PP 100, Fig. 3). Within Sacoglossa the Oxynoacea are monophyletic, as well as all families herein. *Cylindrobulla* represents the first offshoot and is sistertaxon to the remaining oxynoacean members (Fig. 3).

The Limapontioidea and the Plakobranchoidea, in the sense of Bouchet & Rocroi (2005) appear paraphyletic, in the latter because of the basal position of the Platyhedylidae (Fig. 3). The Costasiellidea form the most basal taxon of the Limapontiodea with high nodal support (PP >99). The monophyletic Limapontiidae is sister taxon to the paraphyletic Polybranchiidae (PP > 99, Fig. 3). Yet, within Limapontiidae the genus *Placida* is paraphyletic, including *Ercolania boodleae* (Baba, 1938) and *Stilliger ornatus* Ehrenberg, 1831 and is positioned as sister taxon to the remaining Limapontiidae (PP > 99, Fig. 3). *Alderiopsis nigra boodleae* (Baba, 1937), *Limapontia senestra* (de Qautrefages, 1844) and *Ercolania felina* (Hutton, 1882) are set as first offshoot within Limapontiidea (PP 86) and the genus *Alderia* forms the sister taxon of *Ercolania* with high support (PP 100, Fig. 3). *Ercolania* is found to be polyphyletic since *E. felina* and *E. boodleae* are not grouped within. The Polybranchiidae are paraphyletic, with monphyletic *Cyerce* branching off first (Fig. 3). Hermaeidae forms sister group to the monophyletic Polybranchiidae (with *Cyerce* excluded). Within the latter, relationship of *Caliphylla mediterranea* (da Costa, 1867), *Mourgona* and *Polybranchia* is well supported. The clade Hermaeidae and reduced Polybranchiidae is sister taxon to the Plakobranchoidea with high nodal support (PP > 95, Fig. 3). Within Plakobranchoidea, two major clades are supported with high PP values (both with 93). The first comprises *Bosellia* as sister taxon to monophyletic *Thuridilla* and *Plakobranchus* (PP 93, Fig. 3). This clade is with high nodal support the sister taxon to *Elysia* (PP 100, Fig. 3).

Evolution of functional kleptoplasty

Likelihood and Parsimony ancestral state reconstruction indicate that at the base of the sacoglossan lineage, no functional kleptoplasty existed (Fig 3). Thus, plastids engulfed during feeding are digested and not maintained in the digestive system. Then, short-term kleptoplasty originated twice, once at the base of the Costasiellidae and once at the base of Plakobranchoidea (Fig. 3). Long-term-retention arose five times independently, in *Costasiella ocellifera* (Simroth, 1895), *Plakobranchus ocellatus*, *Elysia chlorotica* Gould, 1870, *E. crispata* and *E. timida* (Risso, 1818), respectively (Fig. 3). Although *E. chlorotica* and *E. crispata* are closely related, the ancestral character state reconstruction support short-term-retention as ancestral for this group (Supplementary Data).

Our ARS analysis of the consumed food algae (*Caulerpa* versus *Halimeda*) gives evidence that *Halimeda* was the original food of the ancestors of the Sacoglossa (proportional likelihood 0.965, parsimony state 0).

DISCUSSION

Food sources of Sacoglossa

Due to new hypotheses on long-term survival of plastids outside their natural environment, de Vries et al. (2014) most recently stressed the importance of rapid and precise identification of plastid sources within Sacoglossa. Clearly, the food source is not exclusvively sufficient to establish functional kleptoplasty, but without adequate physiological pre-requisitions of target plastids this relationship may not have been established in the first place. Especially the results on starving experiments with polyphagous species such as *Plakobranchus ocellatus* (Christa et al., 2013) and *Elysia clarki* (this study) indicate that sequestered plastids of different algae are not maintained equally over time. In *E. clarki*, plastids of *Bryopsis* are obviously not maintained for more than seven days. However, *Elysia ornata* (Swainson, 1840) also feeds upon *Bryopsis* and starves up to 13 days but looses photosynthetic activity within six days [see Supplementary Data of (Händeler et al., 2009)]. It is therefore necessary to analyze how fast plastids of *Bryopsis* degrade and how much they support the slug's survival. Their faster degradation may be based on the lack of a plastid encoded repair mechanism of PSII (de Vries et al. 2014), while this is still present in *Vaucheria litorea* and *Acetabularia acetabulum*, the food sources of two LtR forms (*Elysia chlorotica* and *E. timida,* respectively). The slugs, however,

do not need to actively discriminate between plastid valuably, they would most probably digest degrading plastids by autophagy, as in the case of mitochondria (Kim, Rodriguez-Enriquez & Lemasters, 2007; Kiššová et al., 2007).

Besides targeting plastids that are essential for establishing functional kleptoplasty, food analyses in Sacoglossa are an important task in understanding ecological associations and the reconstruction of the ancestral food source to examine host shift processes. Our results indicate that, depending on collection locality, food sources may differ to some degree and can also differ between specimens of the same collection locality, as was previously also reported for *Plakobranchus ocellatus* (Maeda et al., 2012). This can be based on seasonality of algae or differences in the abundance of certain algae (Marìn & Ros, 1993). The latter hypothesis is more supported by the results of Gallop (1980) who showed that the slugs most likely are not able to actively find their algal prey by chemotaxis but by a "try-and-error" system (Gallop, Bartrop & Smith, 1980).

Our reconstruction of the ancestral food source supports assumption of Jensen (1996) that *Halimeda* is the ancestral food source, yet a final conclusion is not possible because of too less data on food sources. Furthermore, differences in food source identification between feeding experiments or observation and DNA-barcoding occur. This can be clearly seen in the case of *Oxynoe*: results obtained by barcoding revealed *Halimeda* as food source, however they were exclusively collected from *Caulerpa* and said only to feed on *Caulerpa* (Jensen, 1996; Händeler & Wägele, 2007). These findings implement that Oxynoacea may also feed upon *Halimeda* not covered so far and that the algae upon they were collected does not necessarily have to be their food algae. Even if the slugs feed upon these algae under laboratory conditions, in natural environment they may not. For example, de Vries et al. (2013) fed juvenile *Elysia cornigera* with *Acetabularia acetabulum* Silva, 1952 in the laboratory and they developed just fine, although this food source does not occur in the habitat of *E. cornigera*. Not knowing environmental conditions may hence lead to misidentification of results during feeding experiments in the laboratory.

Functional kleptoplasty in Sacoglossa

Including the results presented in this study 77 sacoglossan species are investigated with regard to their photosynthetic capability by means of PAM-measurements to data (Supplementary Data 4). Considering *Elysia clarki* and *E. crispata* as two distinct species (Pierce et al., 2006), six LtR species are now known to science. These additionally comprise the plakobranchoid species *Elysia timida*, *Elysia chlorotica* and *Plakobranchus ocellatus* and

the limapontioid species *Costasiella ocellifera* (Händeler et al., 2009; Rumpho et al., 2011). Short-term-retention is now proven for 34 species of the Plakobranchoidea and two species of *Costasiella,* but is not from taxa of the Oxynoacea. Only about 25% of known Sacoglossa species are investigated by means of PAM-measurements. Especially within the Limapontioidea many taxa are unfortunately not covered, although former investigations indicate a possible kleptoplasty. E.g., *Hermaea* is considered to be potentially photosynthetic based on CO_2 fixation experiments, although evidences are not conclusive (Taylor, 1971b; Kremer & Schmitz, 1976). Higher F_v/F_m values in the Japanese oxynoacean *Julia zebra* Kawaguchi, 1981, measured by Yamamoto et al. (2009) certainly need to be repeated, since no further information on the time frame and conditions of the slugs after collection were given. To our knowledge, the Oxynoacea are not able to incorporate functional chloroplasts. Their digestive gland surrounds the stomach as a compact mass without branching and does not show any adaptation for functional plastid retention. Even the surrounding shell would reduce efficiency of plastids. Therefore, it is more likely that the values obtained from *J. zebra* do not reflect functional kleptoplasty but rather display the functionality of plastids within the stomach shortly after feeding. For these reasons, *J. zebra* is handled as NR form. Considering especially these conflicting data, the identification of functional kleptoplasty within Sacoglossa is still an ongoing task, but some basic requirements should be considered or mentioned, including the time in between animals were collected and the measurements were taken.

Evolution of functional kleptoplasty within Sacoglossa

Händeler et al. (2009) estimated the evolution of functional kleptoplasty at the base of the Plakobranchoidea, although *Costasiella* was not included as retention-form. On the other hand, *Costasiella* was included as retention-form in the study by Maeda et al. (2910) that estimated the evolution of kleptoplasty in the stemline of Plakobranchacea. However, in both studies the taxa *Platyhedyle denudata* Salvini-Plawen, 1973 and *Gascoignella nukuli* Swennen, 2001 were not included. According to our analysis, which based on a broader taxon sampling and included the two latter species, functional short-term kleptoplasty probably originated two times (once in *Costasiella* and once in the Plakobranchoidea), whereas LtR forms evolved five times independently. The results underline the importance in identifying functional kleptoplasty in Sacoglossa in order to reveal a robust hypothesis of the evolution of functional kleptoplasty. Therefore, our conclusions may eventually be superseded when more information on hardly investigated taxa becomes available.

Irrespective of this general uncertainty when working on historical aspects, our results broaden our knowledge with regard to importance of plastid origin, evolutionary food traits within Sacoglossa and functional kleptoplasty. These results will help to specify further experiments and investigations to finally explain evolution of longevity of chloroplasts and their relevance in survival of the slugs.

AUTHORS CONTRIBUTION

GC, KH and HW planned the experiments and collected the material. GC, HW, DK, JF, MV, PK performed the experiments and analyzed the molecular data. GC, KH, DK, JF, MV, PK and HW analyzed the data and wrote the paper.

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TABLES

Table 1 Food analysis in freshly collected Sacoglossa. Food sources are identified with the DNA-barcoding marker *rbcL*. Dario Karmeinsky processed specimens in grey boxes within his Bachelor thesis.

Species	Collection place	Specimen	Identified food	Accession Nr.
Oxynoe antillarum	Sunset Drive, Summerland Key	S271	*Halimeda* sp. 1	
		S272	*Halimeda incrassata*	
			Halimeda sp. 1	
Cyerce antillensis	Sunset Drive, Summerland Key	S216	*Halimeda* sp. 1	
			Udotea sp. 1	
			Udotea sp. 2	
	Geiger Beach, Boca Chica Key	S217	*Halimeda* sp. 1	
			Halimeda sp. 2	
			Rhipiliaceae sp. 2	
			Avrainvillea sp.	
			Bryopsis hypnoides	
			Bryopsis sp. 1	
			Pseudochlorodesmis sp. 2	
	Henry Street, Summerland Key	S218	*Halimeda* sp. 1	
			Udotea sp. 2	
	Niles Road, Summerland Key	S219	*Halimeda* sp. 1	
Cyerce sp. 4	Horseshoe Pit, Spanish Harbour Key	S214	*Halimeda* sp. 1	
		S215	*Halimeda* sp. 1	
		S251	*Halimeda* sp. 1	
			Polyphysa sp.	
		S252	*Halimeda* sp. 1	
			Udotea sp. 2	
Elysia clarki	Niles Road, Summerland Key	N1	*Halimeda incrassata*	
			Pseudocodiaceae sp.	
	Mote Laboratory, Summerland Key	48	*Halimeda incrassata*	
			Halimeda sp. 2	
			Rhipiliaceae sp. 1	
			Bryopsis sp. 1	
			Bryopsis sp. 2	
			Pseudocodiaceae sp.	
			Ulvophyceae sp. 1	

		71	Rhipiliaceae sp. 1
			Bryopsis sp. 3
			Ulvophyceae sp. 1
			Pseudocodiaceae sp.
	Geiger Beach, Boca Chica Key	49	*Halimeda incrassata*
			Acetabularia crenulata
	Henry Street, Summerland Key	50	*Halimeda monile*
			Halimeda incrassata
			Pseudocodiaceae sp.
			Rhipiliaceae sp. 1
	Sunset Drive, Summerland Key	72	*Halimeda monile*
			Pseudocodiaceae sp.
		73	Pseudocodiaceae sp.
			Rhipiliaceae sp. 1
Elysia papillosa	Sunset Drive, Summerland Key	S223	*Halimeda incrassata*
			Halimeda monile
		S224	*Halimeda incrassata*
			Halimeda sp. 2
		S257	*Halimeda monile*
			Halimeda simulans
			Halimeda sp. 2
Elysia patina	Sunset Drive, Summerland Key	S258	*Udotea* sp. 1
			Udoteaceae sp. 1
		S259	*Udotea* sp. 1
		S260	*Udotea* sp. 1
Elysia subornata	Henry Street, Summerland Key	S263	*Rhipocephalus phoenix*
Elysia cf. *zuleicae*	Sunset Drive, Summerland Key	S220	*Halimeda* sp. 3
			Pseudochlorodesmis sp. 1
		S222	*Halimeda* sp. 1
			Avrainvillea sp.
			Rhipocephalus phoenix
Elysia zuleicae	Geiger Beach, Boca Chica Key	S269	*Halimeda incrassata*
			Bryopsis hypnoides
		S270	*Halimeda incrassata*
			Udotea flabellum
			Pseudocodiaceae sp.
			Polyphysa parvula

Table 2 Food analysis of *E. clarki* during starvation. All specimens were collected at Mote Lab and starved for overall 49 days und either light (40µmol quanta/m^2s^1) or complete darkness. Food analyses were carried out by DNA-barcoding after four different starvation periods, in each case on a single individual. *Halimeda incrassata* (highlighted in bold) was the only food source found in every sample.

Starvation condition	Starvation Period [days]	Specimens Nr.	Identified food by Barcoding	Accession Nr.
non-starved	-		***Halimeda incrassata***	
			Halimeda monile	
			Halimeda sp. 2	
			Rhipiliaceae sp. 1	
			Bryopsis spp	
			Ulvophyceae sp. 1	
			Pseudocodiaceae sp.	
			Acetabularia crenulata	
40µmol quanta/m^2s^1	7	B6	***Halimeda incrassata***	
			Acicularia schenckii	
			Penicillus sp.	
	28	B3	***Halimeda incrassata***	
			Halimeda sp. 2	
			Rhipiliaceae sp. 1	
	35	B4	***Halimeda incrassata***	
			Halimeda sp. 2	
			Rhipiliaceae sp. 1	
			Pseudocodiaceae sp.	
	49	B5	***Halimeda incrassata***	
			Halimeda sp. 1	
			Rhipiliaceae sp. 1	
			Pseudocodiaceae sp.	
darkness	7	B10	***Halimeda incrassata***	
			Ulvophyceae sp. 1	
	28	B12	***Halimeda incrassata***	
			Rhipiliaceae sp. 1	
	35	B9	***Halimeda incrassata***	
			Halimeda sp. 2	

		Rhipiliaceae sp. 1
		Pseudocodiaceae sp.
49	B15	***Halimeda incrassata***
		Rhipiliaceae sp. 1
		Pseudocodiaceae sp.

FIGURES

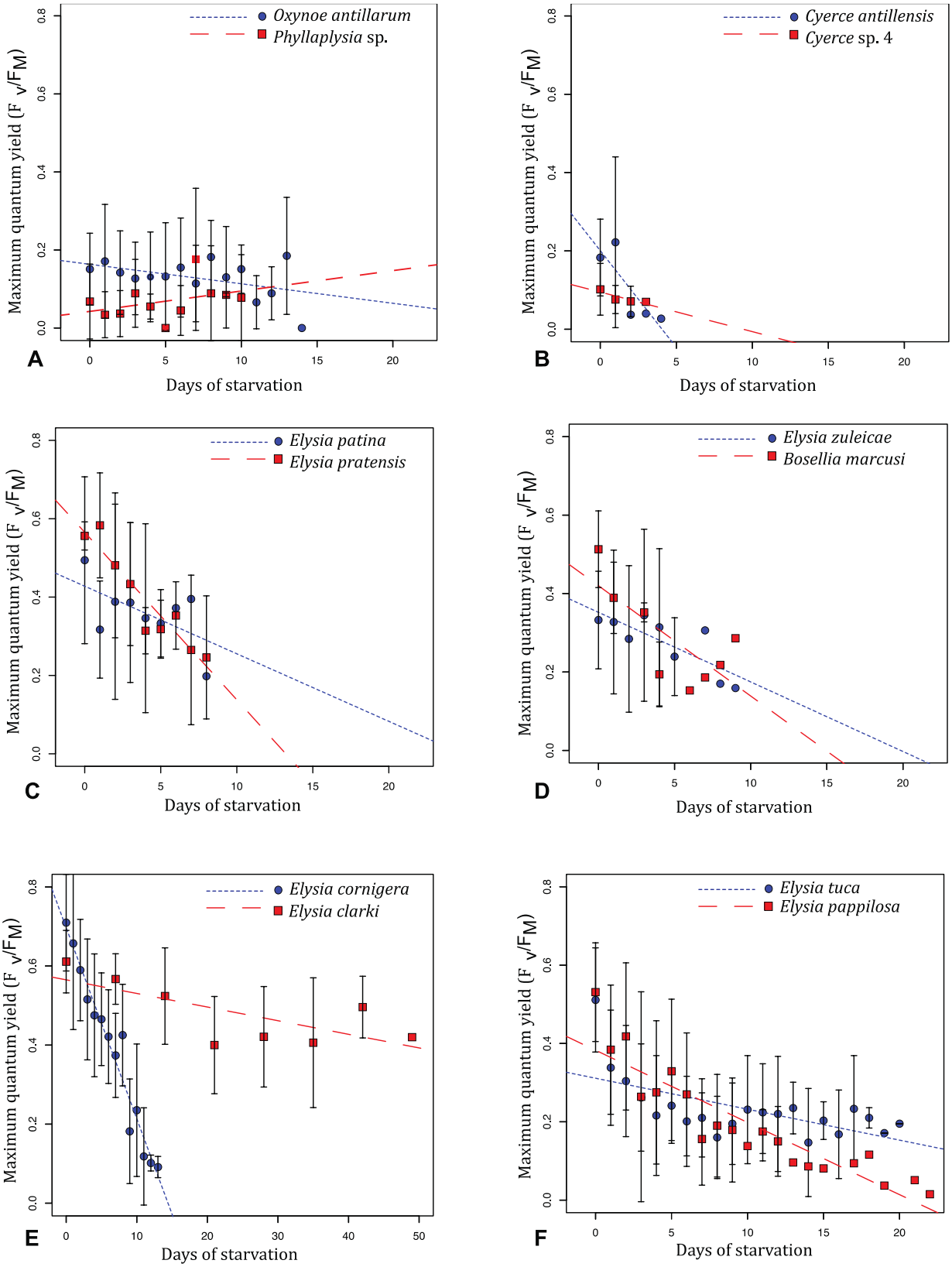
Oxynoe antillarum
Phyllaplysia sp.
Maximum quantum yield (F v/FM)
Days of starvation
A
Cyerce antillensis
Cyerce sp. 4
B
Elysia patina
Elysia pratensis
C
Elysia zuleicae
Bosellia marcusi
D
Elysia cornigera
Elysia clarki
E
Elysia tuca
Elysia pappilosa
F

Figure 1 Chlorophyll a fluorescence measurements of several sacoglossan species. The anaspidean *Phyllaplysia* sp. was measured in order to compare accuracy of F_v/F_m measurements for Sacoglossa with a slug that feeds on algae but digests at once. Error bars indicate standard deviation between different species for each measuring point.

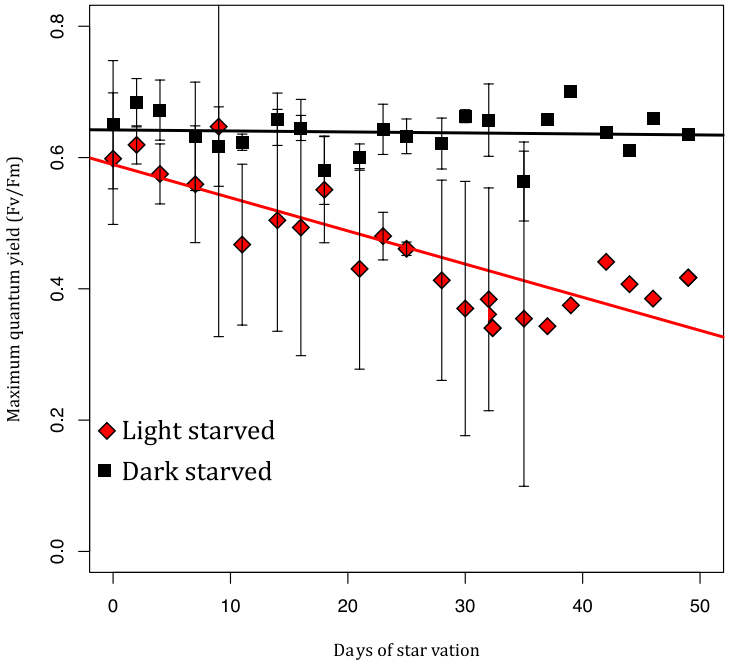

Figure 2 Maximum quantum yield measurements (F_v/F_m) of *Elysia clarki*. Specimens of *E. clarki* were either starved under 40µmol quanta/m^2s^1 (diamonds) or in complete darkness (rectangles) over a period of 49 days. Error bars indicate standard deviation between different species for each measuring point. Because specimens were fixed at certain intervals, finally only one individual was left at day 49.

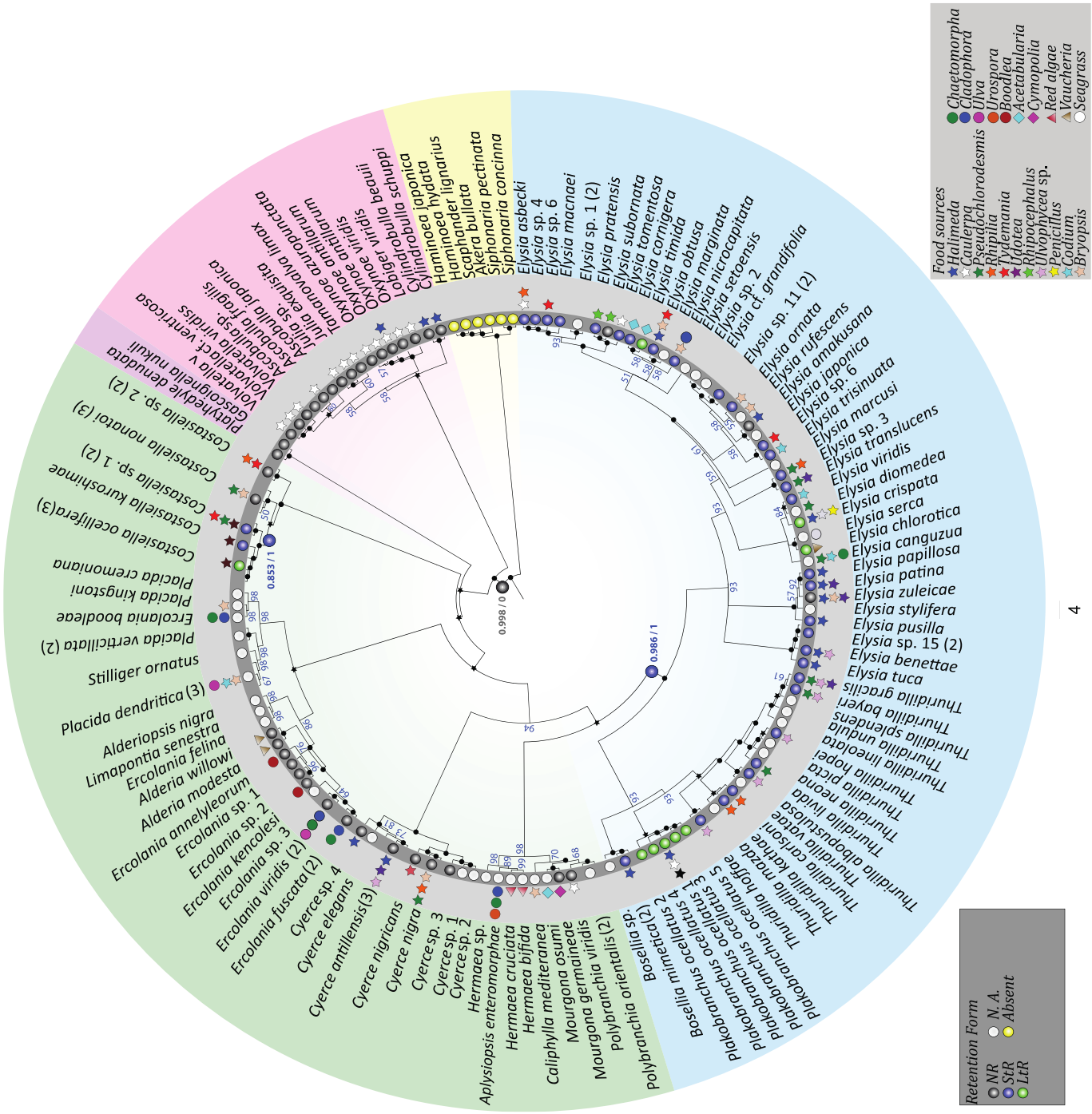

Figure 3 Phylogeny of the Sacoglossa based on Bayesian analysis. Shown is a 50% majority rule consensus tree. Numbers at nodes indicate Posterior Probability (PP), black circles indicate PP = 100, black asterisk indicate PP = 95-99. The inner dark grey circle borders functional-retention information of taxa, the outer food sources (displayed only three major food sources per species). The scale bar displays substitutions per site. Yellow highlighted is the "outgroup", pink the Oxynoacea, purple the Platyhedylidae, green the "Limapontioidea", and blue the Plakobranchoidea with Platyhedylidae as sistergroup. Numbers in brackets behind species names indicate number of investigated individuals for this species. Grey circle in the tree

displays the evolution of non-functional retention at the base of the Sacoglossa obtained by Ancestral Character State reconstruction (ASR), blue circles displays the evolution of short-term-retention. Numbers at circles display the proportional likelihood values of the likelihood reconstruction and the values of the parsimony reconstruction (0 = non-retention, 1 = short-term-retention) for ancestral state reconstruction (ASR). Additional information on food sources and retention form was taken out of literature (see Christa et al. submitted) or generated in this study.

SUPPLEMENTARY DATA

For the Supplementary Data please see the attached CD. There is a folder named "Chapter 6 - Phylogenetic evidence that functional kleptoplasty evolved twice in Sacoglossa" containing all additional information.

Supplementary Data 1 – List of species used for phylogenetic analysis
Supplementary Data 2 – PAM-measurements of *Elysia clarki* during starvation in light and in dark condtions
Supplementary Data 3 – Food sources and PAM-measurements of sacoglossan sea slugs
Supplementary Figure 1 – Food sources of sacoglossan slugs identified by DNA-barcoding in this study
Supplementary Figure 2 – Food sources of non-starved *Elysia clarki* by DNA-barcoding in this study
Supplementary Figure 3 – Food sources of starved *Elysia clarki* by DNA-barcoding in this study

CHAPTER 7

General Discussion

The subject of this thesis was to investigate the evolution of chloroplasts sequestration in Sacoglossa. Surprisingly, some results presented here build a contrast to former studies. A major assumption in the past was the photoautotrophy of the kleptoplastic slugs, but with the results of this sudy it became obvious that they use the plastids rather as food depot than as solar panel. Aspectes, like plastid physiology, carbon fixation of plastids in starving slugs and how the slugs benefit from the plastids need to be re-investigated. These topics were not investigated in the past 10-20 years but seem likely to hold the key to establish functional kleptoplasty. Furthermore, the work presented here elucidates the importance to include a broad spectrum of species to reveal important questions, like the evolution of functional kleptoplast, thus underlying the importance of biodiversity research in this field.

Functional chloroplasts among Sacoglossa

The survey of plastid retention within Sacoglossa is nowadays mainly based on PAM-measurements. This non-invasive technique allows to analyze the chlorophyll a fluorescence in individuals during starvation periods (Wägele & Johnson, 2001; Evertsen et al., 2007; Giménez-Casalduero & Muniain, 2008; Evertsen & Johnsen, 2009; Händeler et al., 2009; Jesus, Ventura & Calado, 2010; Serôdio et al., 2010; Wägele et al., 2010; Middlebrooks, Pierce & Bell, 2011; Pelletreau et al., 2011; Wägele et al., 2011; Bhattacharya et al., 2013; Ventura, Calado & Jesus, 2013; Wägele & Martin, 2013; Christa et al., 2014). The study of functional kleptoplasty within Sacoglossa is particularly important to reveal its evolution in the lineage of the Sacoglossa and the photosynthetic quality of plastids outside the algal cell. Like every technique, PAM-measurements also posses some drawbacks [see review of (Cruz et al., 2013)], but individuals can be used for further analyses, e.g. food analyses during starvation, phylogenetic studies, change of photosynthesis after re-feeding with different food sources, etc. (Christa et al., 2013).

Along with the results of this thesis altogether 77 sacoglossan species were investigated with regard to functional kleptoplasty by means of PAM measurements to date. No taxa of the Oxynoacea posses any chlorophyll fluorescence at all and except the genus *Costasiella* no Limapontiodea taxa exhibit functional kleptoplasty either. On the other hand it is fairly common in the Plakobranchoidea and only a handful of species are not able to incorporate functional plastids, like *Elysia serca*, *Elysia zuleicae*, *Elysia subornata* and *Bosellia marcusi*.
So far six long-term-retention forms are identified (*Costasiella ocellifera*, *Elysia clarki*, *E. crispata*, *E. chlorotica*, *E. timida* and *Plakobranchus ocellatus*), but *Elysia viridis* could be another long-term retention form as well. Händeler et al. (2009) investigated specimens collected from the Mediterranean and considered *E. viridis* as short-term-retention form. Other studies, however, analyzed specimens of *E. viridis* from the Northern Atlantic and demonstrated that it is a long-term-retention form (Trench, 1969; Hinde & Smith, 1972; Evertsen & Johnsen, 2009). Differences in the photosynthetic retention may be based for example on differences in temperature, light intensity and food source between both habitats (Waugh & Clark, 1986; Marin & Ros, 1992; Maeda et al., 2012). The latter is supported by results, that individuals of *Elysia viridis* from the Mediterranean feed upon *Bryopsis* (Händeler et al. 2010), whereas specimens of *E. viridis* from the Atlantic feed upon *Codium* (Trench, 1969; Hinde & Smith, 1972; Evertsen & Johnsen, 2009). It is likely that the capability of functional kleptoplasty is based on physiological specialites of certain plastids, thus plastids degrade faster when these adaptations are missing. Interstingly, *Bryopsis* was shown not to posses FtsH in its plastid genome, which is assumed as such a speciality to contribute to plastid longevity (de Vries et al., 2014), however there is no information on its presence in *Codium*. Hence, only evidences exist that the differences between these two *Elysia viridis* populations may be nutrition based and it is mandatory to investigate if FtsH is plastid encoded in *Codium*. Furthermore, it cannot be ruled out that *Elysia viridis* is a species complex, like shown for *Plakobranchus ocellatus* (Christa et al., 2013; Krug et al., 2013). Unfortunately no molecular data is available for the Atlantic *Elysia viridis* so that a genetic comparison was not possible to date. As a consequence, researchers may not have recognized that they were studying different species.
Overall only about 25% of Sacoglossa species are examined by using a PAM with regard to their ability to retain plastids. As shown in the phylogenetic analysis (Chapter 5), including *Costasiella* as retention form changed the hypothesis of the evolution of functional

kleptoplasty considerably: while it was either calculated at the base of the Plakobranchoidea or at the base of the Plakobranchacea (Händeler et al. 2010; Maeda et al. 2010), the hypothesis of two independent origins is now more supported. This underlines the importance of including as many species as possible to get a more detailed knowledge about the distribution of functional kleptoplasty and its evolution in Sacoglossa.

The classification of functional kleptoplasty

To examine the functional retention of plastids in Sacoglossa, various methods were applied such as CO_2 fixation experiments, O_2 evolution determination or PAM-measurements (Evertsen et al., 2007; Händeler et al., 2009; Rumpho et al., 2011; Cruz et al., 2013). Usually, the division of Händeler et al. (2009) in three states based on PAM measurements is used to classify the retention ability of Sacoglossa, although other systems exist (Clark, Jensen & Stirts, 1990; Evertsen et al., 2007).

Comparing results between the different measuring techniques, a discrepancy between CO_2 fixation experiments and measuring of functional kleptoplasty by chlorophyll a fluorescence in Sacoglossa becomes obvious. While PAM-values often decline linear and more slowly over a distinct period, the CO_2 fixation rate decreases almost exponentially (Trench, 1969; Greene, 1970; Hinde & Smith, 1972; Clark et al., 1981): for *C. ocellifera* CO_2 fixation ceased after about 10 days (Clark et al., 1981), while the PAM-measurements (Chapter 3) still showed yield values of around 0.5. Similar results were obtained for *Plakobranchus ocellatus* and *Elysia timida* with a considerable decline of CO_2 fixation within 27 and 5 days, respectively (Greene, 1970; Marìn & Ros, 1989), while maximum quantum yield values stayed high for over several months (Giménez-Casalduero & Muniain, 2008; Yamamoto et al., 2012; Christa et al., 2013; 2014). Thus, photosynthesis in sacoglossan slugs measured by PAM with yield values <0.5 may be regarded carefully as functional kleptoplasty. It is conceivable that there is no photosynthetic CO_2 fixation although chlorophyll a fluorescence is measured. It is therefore feasible that the ability of functional kleptoplasty in many of the StR forms (especially within *Thuridilla*) and the duration of photosynthetic active plastids in LtR forms based on PAM measurements is overestimated and needs to be re-investigated by combining several approaches.

Importance of photosynthesis in the survival of starvation periods

Experiments of Trench & Gooday (1973) showed that photosynthates are recorded outside the chloroplast within slug tissue and a "factor" in *Elysia timida* shall stimulate the release of photosynthates (Gallop, 1974). However, a differentiation if photosynthates are released actively (Trench & Gooday, 1973) by making the plastid envelope more permeable (Gallop, 1974) or by degrading and/or digested plastids is not possible. Although we do not know how the plastids actually provide the new host with energy, the slugs are commonly termed as "leaves that crawl" or "solar powered" and the plastids "…enabling their animal host to survive photo-autotrophically." (Trench, 1975; Rumpho, Summer & Manhart, 2000; Pelletreau et al., 2011). Evidences that photosynthesis is essential for surviving starvation periods come from starvation experiments under different light conditions (light/dark) as well as the observation that specimen in dark conditions died earlier and lost weight faster than those in light (Hinde & Smith, 1972; 1975; Giménez-Casalduero & Muniain, 2008; Yamamoto et al., 2012). In other experiments no difference in weight lost and survival rate between photosynthetic and non-photosynthetic starvation periods was found (Klochkova et al., 2010; Christa et al., 2014). Furthermore in every starvation experiment conducted so far, independent of starvation condition, the slugs shrink and are not able to maintain their biomass (Hinde & Smith, 1972; Giménez-Casalduero & Muniain, 2008; Evertsen & Johnsen, 2009; Pelletreau et al., 2011; Rumpho et al., 2011; Klochkova et al., 2012; Cruz et al., 2013; Christa et al., 2014). Especially CO_2 fixation experiments clearly showed that in some of the long-term-retention forms the phototrophic CO_2 fixation declines rapidly and considerably within the first days of starvation (e.g. by about 87% in seven days*C*. ocellifera or about 55% in five days in *E*. *timida*), but the slugs were still able to survive extended starvation periods (Trench, 1969; Greene, 1970; Hinde & Smith, 1972; Marìn & Ros, 1989). Low CO_2 fixation values were often interpreted as still phototrophic CO_2 fixation, however similar values of dark starved animals, or non-retention forms interpreted differentially (Trench, 1969; Hinde & Smith, 1972; Marìn & Ros, 1989; Clark et al., 1990).

It is still unknown in which way the slugs benefit from the kleptoplasts or if the slugs only use the sequestered plastids as food storage (Christa et al., 2014). As long as the plastids continue to photosynthesize and synthesizing proteins important for PSII repair, for example, they may stay healthy and proceed synthesizing lipids and amino acid, for example, thus being more valuable for the slugs (Trench & Gooday, 1973; Pierce, Biron & Rumpho, 1996).

Outlook

The study on "solar-powered" (Wägele et al., 2010) consist of conflicting data and especially the survey of the phenomenon of functional kleptoplasty requires expertise of different research fields, such as animal physiology, plant physiology, photobiology, genome biology, evolution and biodiversity research. A combination of these fields by using the latest techniques and standardized experimental set-ups is needed to reveal the mechanisms enabling and enhancing functional kleptoplasty.

The phylogenetic reconstruction is now based on a four-gene analysis instead of three as used in former studies [(Händeler et al., 2009; Jörger et al., 2010; Händeler, 2011; Neusser et al., 2011; Maeda et al., 2012), Chapter 2 and 5]. It inclduded about one third of known species and provides a more profound insight in the relationship of sacoglossan families. However, in recent years the application of transcriptomic data improved the reliability of molecular based phylogenetic reconstructions considerably. Especially highly derived taxa, like the Platyhedylidae or the paraphyletic "Limapontioidea", may result in wrong affiliations in phylogenetic reconstruction when using single genes. To understand the evolution of functional kleptoplasty within Sacoglossa, a phylogeny based on transcriptomic data is notably important.

Likewise important to understand the evolution of functional kleptoplasty is the investigation of as many species as possible with regard to their retention ability. In particular, non-plakobranchoid species have to be more intensively investigated in future to reveal possible retention-forms other than Plakobranchoidea, as shown for *Costasiella*. Still, PAM-measurements are the fastest and the least harmful way to investigate Sacoglossa, though its application alone may not reveal the "real nature" of photosynthetic capability of the investigate species. Additively, CO_2 fixation should be applied.

The identification of food sources sets the basic knowledge to search for genomic and physiological properties of plastids that enhance functional kleptoplasty, especially in long-term-retention forms. Yet, plastids that are not incorporated by LtR forms should also be addressed in order to exclude false interpretation of revealed data.

On the other hand the slugs need to be more intensively investigated with regard to genomic adaptions that enhance the sequestration of plastids and the subsequent maintaining in the slug's body without digestion. Therefore comparative transcriptomic analyses of sister taxa species that feed on the same algal source seem to be the most promising way.

The influence of photosynthetic active plastids for the development of sacoglossan sea slugs is hardly studied. Recently, Pelletreau et al. (2012) showed that in early stages of the development in *Elysia chlorotica* plastids are completely digested and the juveniles develop abnormal when starved early during the development. However, it is not known if the slugs just need to feed or if they need to feed on photosynthetic active plastids (because they need the photosynthesis) for a normal development. Studies under blocked photosynthesis are thus coactive needed to reveal the importance of photosynthetic active plastids for the development.

Still, the survey of functional klepotoplasty in Sacolgossa, its physiological basics and its extent to which it occurs remains fascinating (also to non-scientific readers) and the enthusiasm that comes along with the study of Sacoglossa will increase even more.

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Scientific talks and poster presentations at national and international conferences

09.2010	Poster presentation at the „3rd International Workshop on Opisthobranchs“, Vigo, Spain
02.2011	Oral presentation at the 12th annual meeting of the “Gesellschaft für Biologische Systematik (GfBS)”, Berlin, Germany
10.2012	Poster presentation at “15th international Colloquium on Endocytobiology and Symbiosis”, München, Germany
02.2013	Oral presentation at the 14th annual meeting of the “Gesellschaft für Biologische Systematik (GfBS)”, Wien, Austria
07.2013	Oral presentation at the “World Congress of Malacology”, Ponta Delgada, Portugal

Grants and honors

02.2011	Travel grant of the “Gesellschaft für Biologische Systematik (GfBS)”
02.2013	Travel grant of the “Gesellschaft für Biologische Systematik (GfBS)” and of the “Alexander Koenig Gesellschaft”
07.2013	Travel grant of the “Unitas Malacologia”
07.2013	Student award for the “most interesting and informative talk” at the “World Congress of Malacology”, Ponta Delgada, Portugal assigned by “The Malacological Society of London”

Graduate training

10.2013	“Bioinformatics for Beginners”, students course to learn basics of Perl programming at “Zoologisches Forschungsmuseum Alexander Koenig”, Bonn

Research trips

02.2012 – 05.2012	Research stay at “Mote Marine Laboratory”, Florida Keys, USA
02.2013	Research stay at “Keys Marine Laboratory”, Florida Keys, USA

Publications

Christa, G., Wescott, L., Schäberle, T.F., König, G.M. & Wägele, H. (2013). What remains after 2 months of starvation? Analysis of sequestered algae in a photosynthetic slug, *Plakobranchus ocellatus* (Sacoglossa, Opisthobranchia), by barcoding. *Planta* **237**, 559–572.

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