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ORIGINAL ARTICLE

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 two (or possibly three) 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 Wägele et al. 2011; Pelletreau et al. 2011; Rumpho 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 (e.g. Mujer et al. 1996; Hanten and 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

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known to belong to the chloroplast long-term retention (LTR) forms, 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 lies on the known LTR forms, *Elysia chlorotica* (Mujer et al. 1996; Green et al. 2000; Rumpho et al. 2001) and *Elysia timida* (Marín and Ros 1992; Giménez-Casalduero and Muniain 2008; Giménez-Casalduero et al. 2011; Schmitt and Wägele 2011). Only recently *Plakobranchus ocellatus*, a widely distributed species in the Pacific and one of the most effective LTR taxa first investigated by Kawaguti and Yamasu (1965), has come into focus (Hirose 2005; Evertsen et al. 2007; Händeler et al. 2009; Wägele et al. 2011; 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, such as *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). This raises the question of which, if not all, chloroplasts 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

Fig. 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 1.5–2 cm

regimes to identify chloroplast source species sustained during the starvation period. *RbcL* (Curtis et al. 2006; Pierce et al. 2006; Maeda et al. 2010) and *tufA* (Händeler et al. 2009; 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. Five 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 12 h:12 h 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 pulse amplitude modulated (PAM) fluorometer (Walz, Effeltrich, Germany) after dark acclimatization (for details see Händeler et al. 2009). For this, two specimens were randomly selected from each treatment. After dark acclimatization, three separate yield values per animal were documented and the mean value for both specimens was 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 four in the control group, five in the high irradiance treatment (HT), five from the low irradiance treatment (LT) and three from darkness (DT). During the experiments, four 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) 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 36 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 0.5 µl of genomic DNA in a 20-µl final volume reaction supplied with 5.5 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. Amplification of *COI* was performed by denaturation for 15 min at 95 °C, 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. Amplification of *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 90 s).

For the barcoding of sequestered algal chloroplasts, the genes *rbcL* and *tufA* were analysed. PCR was performed using a touch-down protocol with ulvophycean-specific primers (see Table 2). These primers were tested a priori on a broad spectrum of various ulvophycean species with excellent results. 2.5 µl of genomic DNA was used as template in a 20-µl final volume reaction supplied with 3.5 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. PCR for 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,

Table 1 List of *Plakobranchus ocellatus* specimens used for phylogenetic analyses (columns for *CO1* and *16S*)

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
				JX272734	JX272775
				JX272735	
Ploc104 (C)	Philippines	JX272687	JX272650	–	–
Ploc105 (C)	Philippines	JX272688	JX272651	JX272736	JX272777
				JX272737	
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

Table 1 continued

Internal numbers (treatment)	Locality	GenBank accession numbers			
		Co1	*16S*	*rbcL*	*tufA*
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	–	–

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. Specimens designated with (pa) were not used in the experiments, but only for the phylogenetic analysis.

C control group; *HT*, *LT* and *DT* treatments in high light, low light and darkness, respectively. – indicates that genes were not analysed

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. For *tufA*, amplification was performed as follows: initial 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.

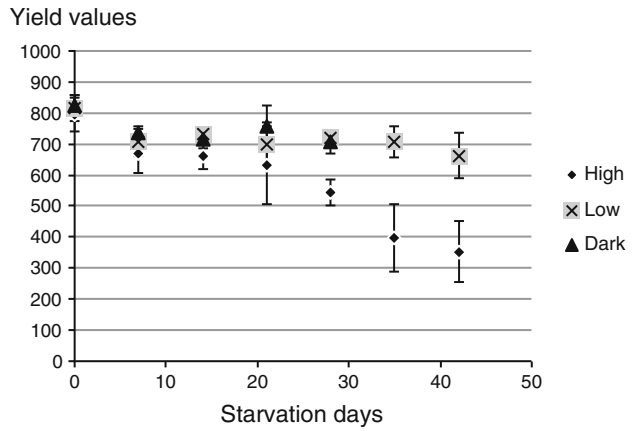

Fig. 2 Graphical depiction of the average weekly PAM-readings from two randomly picked individuals from each treatment group (see "Materials and methods"). Low intensity treatment group (low) received 40 µmol photons m^{-2} s^{-1}, high intensity group (high) received 200 µmol photons m^{-2} s^{-1}, 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

DNA purification, cloning and sequencing

PCR products were size-fractionated in a 1.5 % agarose gel for 90 min at 70 V. 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 the manufacturer's instructions. Isolated fragments were ligated into pGEM t-easy Vector (Promega) and cloned into competent *E. coli* XL1-blue cells (Stratagene). For each specimen, 12 clones were sequenced by Macrogen Inc, (Amsterdam, The Netherlands). 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

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)

	CO1								
Clade:	D	B	C	A	A	C	A	A	A
Specimens:	PlocLiz1	PlocLiz2	PlocGm1	PlocGm2	PlocGm3	PlocGm4	Ploc131 (Ph)	Ploc137 (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	–

Upper part designates *CO1* distances, lower part the concatenated dataset. Bold values are the highest distances found in the analyses

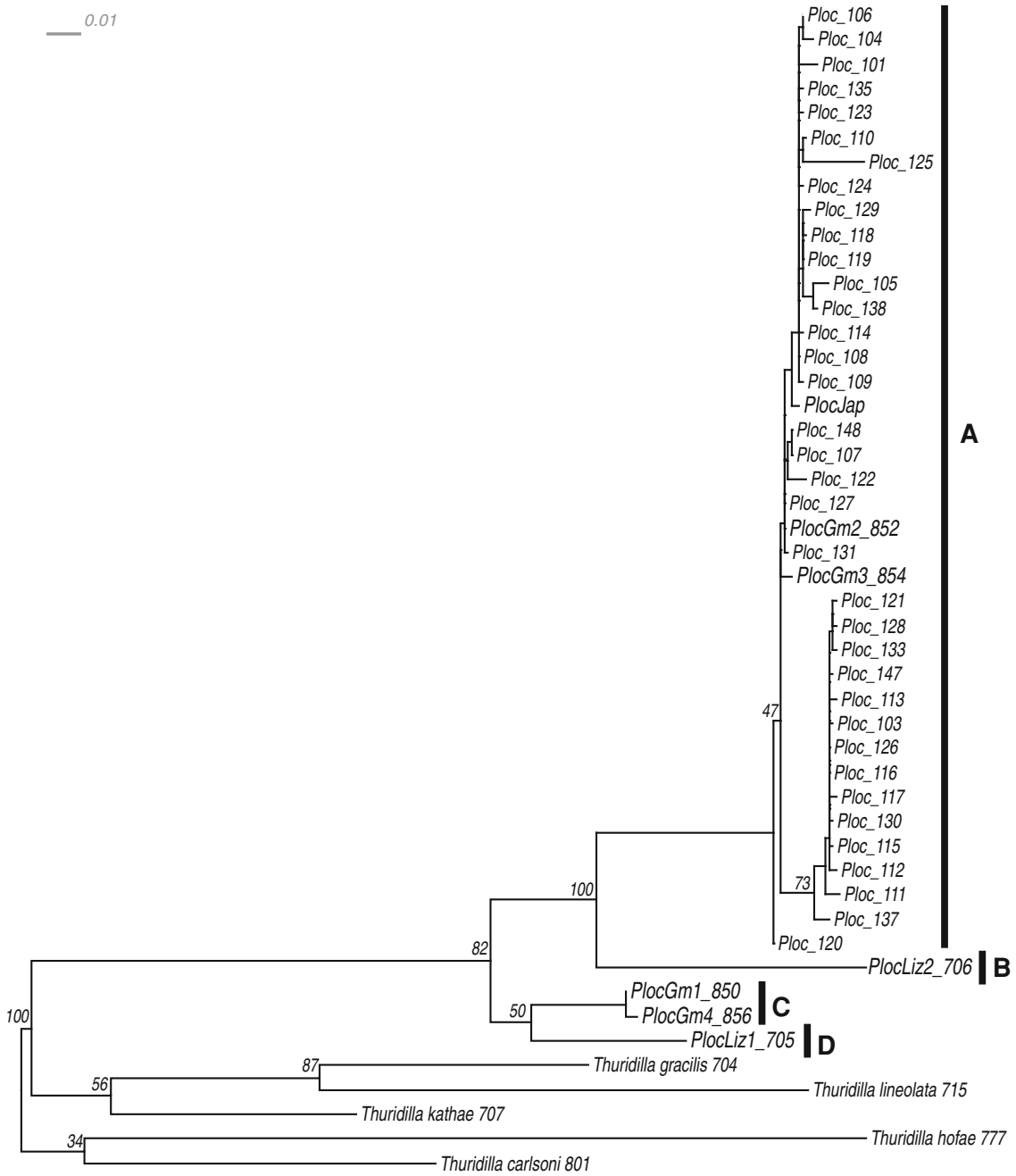

Fig. 3 Maximum Likelihood tree (GTR + I + G) of *CO1* and *16S* gene fragments (concatenated dataset) using haplotypes of *Plakobranchus ocellatus* found on the Philippine Islands (smaller letters), Guam (Gm), Japan (Jap) and Lizard Island (Liz). As outgroups several *Thuridilla* species were included

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 1,030 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 et al. 2008). Sequence divergence was determined by calculating p-distances using HyPhy (Kosakovsky Pond et al. 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

Table 4 Algal species obtained by *tufA* and *rbcL* analyses from Philippine specimens during light and starvation treatments

Light treatment	#	Days	Haplotypes per specimen		Algal species and number of clones per slug specimen	
			tufA	*rbcL*	*tufA* (no. of clones)	*rbcL* (no. of 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 sp 1 *(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 macroloba* (5)
					Halimeda borneensis (1)	*Halimeda* sp 2 (4)
						Udotea sp 1 (1)
DT	Ploc112	14	2	1	*Halimeda macroloba* (8)	*Halimeda macroloba* (10)
					Caulerpa fastigiata (1)	
	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)

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

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 668 bp for *tufA* (thus, we used shorter sequences than obtained for comparison with already available sequences) and 560 bp 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 to Guam cluster into one clade (Taxon A) with a sequence divergence between 0.2 and 1.5 % in the concatenated data set and 0.16–2.58 % 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 11–14 % and 14–17 %, 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 four 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 was much lower: 2 haplotypes could be assigned to 2 *Halimeda* species and 1 to *Caulerpa* (Tables 4, 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

Table 5 Chloroplast haplotypes identified by DNA-barcoding in various specimens

Specimen's origin	*Halimeda* spp.		*Caulerpa* spp.		*Udotea* spp.		*Acetabularia acetabulum*		Unidentified algae (*Pseudochlorodesmis*, *Pseudocodium*)	
	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

DNA-barcoding 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

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 detected only 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 four 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; Rudman 1998; Hirose 2005; Evertsen et al. 2007; Händeler et al. 2009; Maeda et al. 2010; Trowbridge et al. 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 synonymization 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 and 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. Wägele et al. 2010; Huelsken et al. 2011; Weigand 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. (2010) 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, personal communication). 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 and Martin 2013, in press), no study to date has investigated which algal chloroplast type contributes to long-term photosynthesis under starvation conditions or 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 2 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 et al. 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 1,000 μmol m^{-2} s^{-1} during midday in the few centimetre depths where specimens were collected on Lizard Island. Usually the animals burrow in the sand to avoid high irradiances (personal 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 no reports are known to us. 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 (28 μmol m^{-2} s^{-1}) in their so-called high light treatments all differed considerably from our settings. Our LT with 40 μmol m^{-2} s^{-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, four 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 four different chloroplast types. Also, in animals kept in darkness and exhibiting still two 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 and 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 et al. (1996) and Haugen et al. (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 et al. (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 (Famá et al. 2002; O'Kelly et al. 2004; Verbruggen et al. 2009; Händeler et al. 2010; Saunders and 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 analyse more than one gene (hopefully finding one that is also 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 LTR of kleptoplasts in these enigmatic sacoglossan slugs.

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