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Functional kleptoplasty in a limapontioidean genus: phylogeny, food preferences and photosynthesis in *Costasiella*, with a 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 controversial. While some authors have suggested that it is a synapomorphy of the parapodia-bearing Plakobranchoidea, others have suggested an earlier origin at the base of the more inclusive clade Plakobranchacea. The latter is supported by the presence of kleptoplasts in *Costasiella ocellifera*, a ceras-bearing member of Limapontioidea, in which they remain functional for several weeks and fix CO_2. However, the phylogenetic relationships of *Costasiella*, especially with regard to the Plakobranchoidea, have not been satisfactorily demonstrated, and the photosynthetic ability and the importance of photosynthesis within the genus remain poorly studied. In this study we analyse the phylogenetic position, photosynthetic activity and importance of photosynthesis for survival during starvation of five *Costasiella* species, but focusing on *C. ocellifera*. We demonstrate that *Costasiella* is a basal member of the Limapontioidea, however a final conclusion on the origin of functional kleptoplasty within Sacoglossa is still not possible. Three *Costasiella* species maintain functional chloroplasts (of which *C. ocellifera* shows long-term retention, and both *C. kuroshimae* and *C.* sp. 1 short-term retention) and together form a monophyletic group, feeding mainly on *Avrainvillea*. The two nonphotosynthetic species, *C. nonatoi* and *C.* sp. 2, represent the sister clade and feed on algae other than *Avrainvillea*. Intriguingly, *C. ocellifera* survived under nonphotosynthetic conditions for a minimum of 38 d, demonstrating that photosynthates may not be essential in order to survive starvation. These findings support our previous suggestion that during starvation kleptoplasts primarily represent a sort of larder, whose function might benefit from ongoing photosynthesis.

INTRODUCTION

The Sacoglossa is a group of sea slugs represented by nearly 300 species. They comprise the shelled Oxynoacea and the non-shelled Plakobranchacea, the latter divided into 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 starvation periods by means of functional algal plastids sequestered from their food (Trench, Trench & Muscatine, 1972; Green *et al.*, 2000; Händeler *et al.*, 2009; Rumpho *et al.*, 2009). This sequestration of functional plastids, termed kleptoplasty (recently reviewed by 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 innovation that enhanced adaptive radiation within the Sacoglossa (Wägele, 2004). According to Händeler *et al.* (2009), this ability is a synapomorphy of the Plakobranchoidea and most likely involved delayed digestion of the sequestered plastids. In contrast, Maeda *et al.* (2010) assumed that functional kleptoplasty evolved at the base of the more inclusive clade Plakobranchacea and was lost multiple times within different genera. Two different retention levels of functional chloroplasts, introduced by Evertsen *et al.* (2007), can be distinguished based on maximum chlorophyll *a* fluorescence quantum yields (F_v/F_m) measured through a pulse amplitude modulated (PAM) fluorometer (see review by Maxwell & Johnson, 2000). Short-term retention (StR) is defined by PAM values higher than 0.5 for at least 1–2 d with decreasing values for up to 2 weeks, while long-term retention (LtR) is defined by PAM values higher than 0.4 for at least 20 d with retention of plastids for several weeks or months. The former is widespread among Plakobranchoidea and the latter is known in at least five different members of Plakobranchoidea (Händeler *et al.*, 2009). In contrast, almost all members of the Oxynoacea and Limpontioidea are considered nonretention (NR) species, in which PAM values are zero, with the exception of the limapontioidean *Costasiella ocellifera* (Simroth, 1895). Through CO_2 fixation experiments Clark *et al.* (1981) demonstrated in this species the incorporation of functional plastids and an ability to survive starvation for up to 65 d. Photosynthetic capability and efficiency during starvation was, however, not documented by PAM measurements. Moreover, for *C. kuroshimae* (Ichikawa, 1993), Händeler *et al.* (2009) reported PAM values similar to those of the plakobranchoidean *Thuridilla hopei* and suggested that functional kleptoplasty may be more widely distributed within the genus *Costasiella* than previously thought. According to the two most recent sacoglossan phylogenies, functional kleptoplasty evolved either once at the base of the Plakobranchoidea and once independently within *Costasiella* (Händeler *et al.*, 2009) or, alternatively, at the base of the Plakobranchacea, but with multiple losses of functionality within different genera (Maeda *et al.*, 2010). However, the position of *Costasiella* within the Limapontioidea (a paraphyletic group according to molecular analyses) and its relation to the Plakobranchoidea is not clearly resolved in either phylogeny.

Besides phylogenetic origin of kleptoplasty, the mechanism that allows plastid longevity is also unknown. Recent studies have rejected the possibility of lateral gene transfer (LGT), which for a long time appeared the most attractive explanation; transcriptomic analysis and sequencing of DNA from slug eggs 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 has likewise been 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.*, 2013; Christa *et al.*, 2014b). Regardless of the underlying mechanism, photosynthesis is thought to play a key role during starvation (Trench & Gooday, 1973; Trench, Boyle & Smith, 1973). Nevertheless, 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 required for the slugs to survive starvation periods. Recently, Klochkova *et al.* (2013) doubted that CO_2 fixation by kleptoplasts is necessary to survive starvation in the StR species *Elysia nigrocapitata*. Plastids may function as a sort of a larder and photosynthesis may play a secondary role by increasing this internal larder without an immediate transport of photosynthates into the cytosol (Christa *et al.*, 2014b). Photosynthates would only become available once the plastids either 'autonomously' degrade or if the slug actively digests them when required.

Here we examine 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 analysed their food sources and present results on their phylogenetic relationships within Limapontioidea. For *C. ocellifera* we analysed the importance of photosynthesis for survival during starvation. Our results support the hypothesis that functional kleptoplasty evolved more basally within Sacoglossa and we question the direct contribution of kleptoplasts to the survival of the slugs during periods of starvation.

MATERIAL AND METHODS

Species collection and starvation experiments

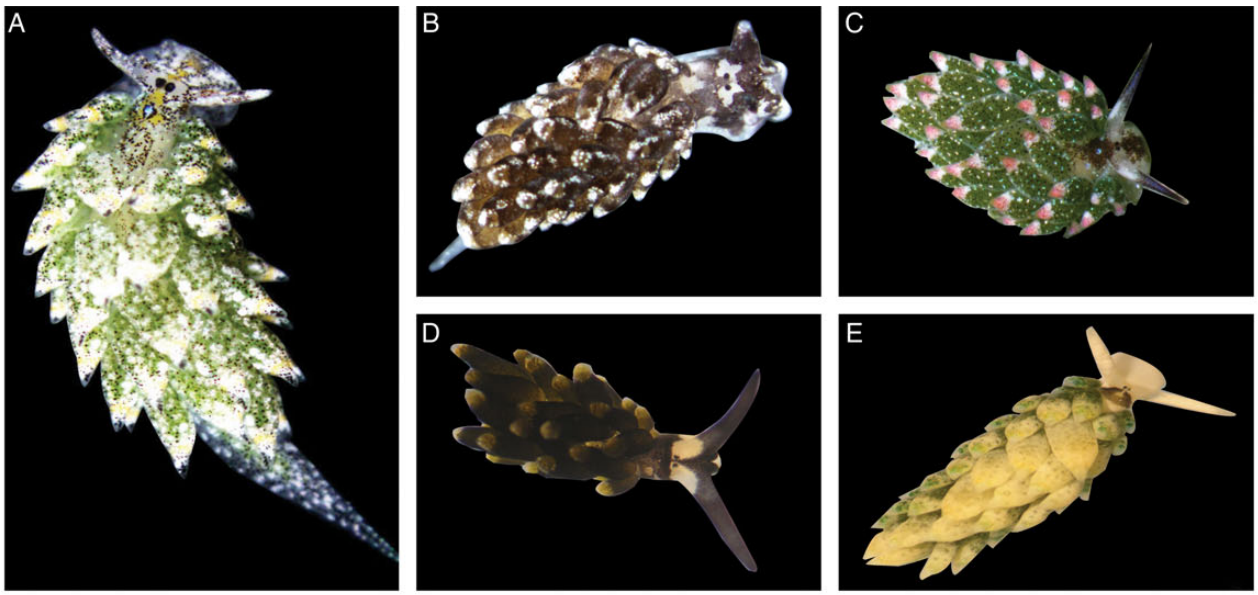
Specimens of *Costasiella* were collected at several places (Supplementary Material Table S1) by snorkeling. They were either fixed immediately in 70% ethanol for food barcoding, or examined alive at Guam or the Florida Keys (USA), or transferred alive to Bonn (Germany). At Guam and the Florida Keys, specimens were kept under a natural day-night rhythm with light intensities up to 600 μmol quanta/m^2 s in individual tanks at 24°C with natural sea water and the water changed every second day. In Bonn, all *Costasiella* specimens were starved in individual tanks with artificial seawater at 20–22°C, under different light conditions and experimental setups (see Table 1), with water changed every second day. We set up laboratory light conditions using a full-spectrum daylight lamp (Androv Medical, Model AND1206-CH). The photosynthesis-blocking agent Monolinuron (available at a concentration of 4,000 mg/l as Algol; JBL GmbH & Co KG, Neuhofen, Germany) was used at a final concentration of 2 μg/ml seawater. Monolinuron inhibits the plastoquinone (Q_A) binding site of the D1 protein, thus blocking the electron transport between Q_A and photosystem II (Arrhenius *et al.*, 2004). Its effectiveness is shown by a rapid increase in chlorophyll *a* fluorescence and a marked decrease in maximum quantum yield (if any is measurable).

PAM measurements

The presence of functional photosynthesis was measured for all *Costasiella* species by analysing the maximum quantum yield (F_v/F_m) values with a Diving-PAM (Walz, Germany) (Table 1 and Supplementary Material Table S3). Regular PAM measurements of all individuals were taken, first keeping the slugs in darkness for 15 min prior to each measurement (individual measurements available upon request). After background calibration (ground fluorescence set to zero), the optic fibre was placed 3–5 mm above the slug to obtain F_0 around 200–500 (measured with far-red light of 0.15 μmol photons/m^2 s emitted by a red LED at 0.6 kHz). The maximum chlorophyll *a* fluorescence (F_v/F_m) was subsequently measured with white light emitted by a halogen lamp at an irradiance of 10,000 μmol photons/m^2 s for 0.8 s at 20 kHz. For *C. ocellifera* and *C. nonatoi* we set up additional starvation conditions (Table 1) to determine the importance of photosynthesis during starvation. Experiments were stopped at day 38 (see Results).

Identification of food sources by barcoding

In recent works the molecular identification of food sources using the *rbcL* gene has proved to be a rapid and precise tool (Curtis, Massey & Pierce, 2006; Händeler *et al.*, 2010; Maeda



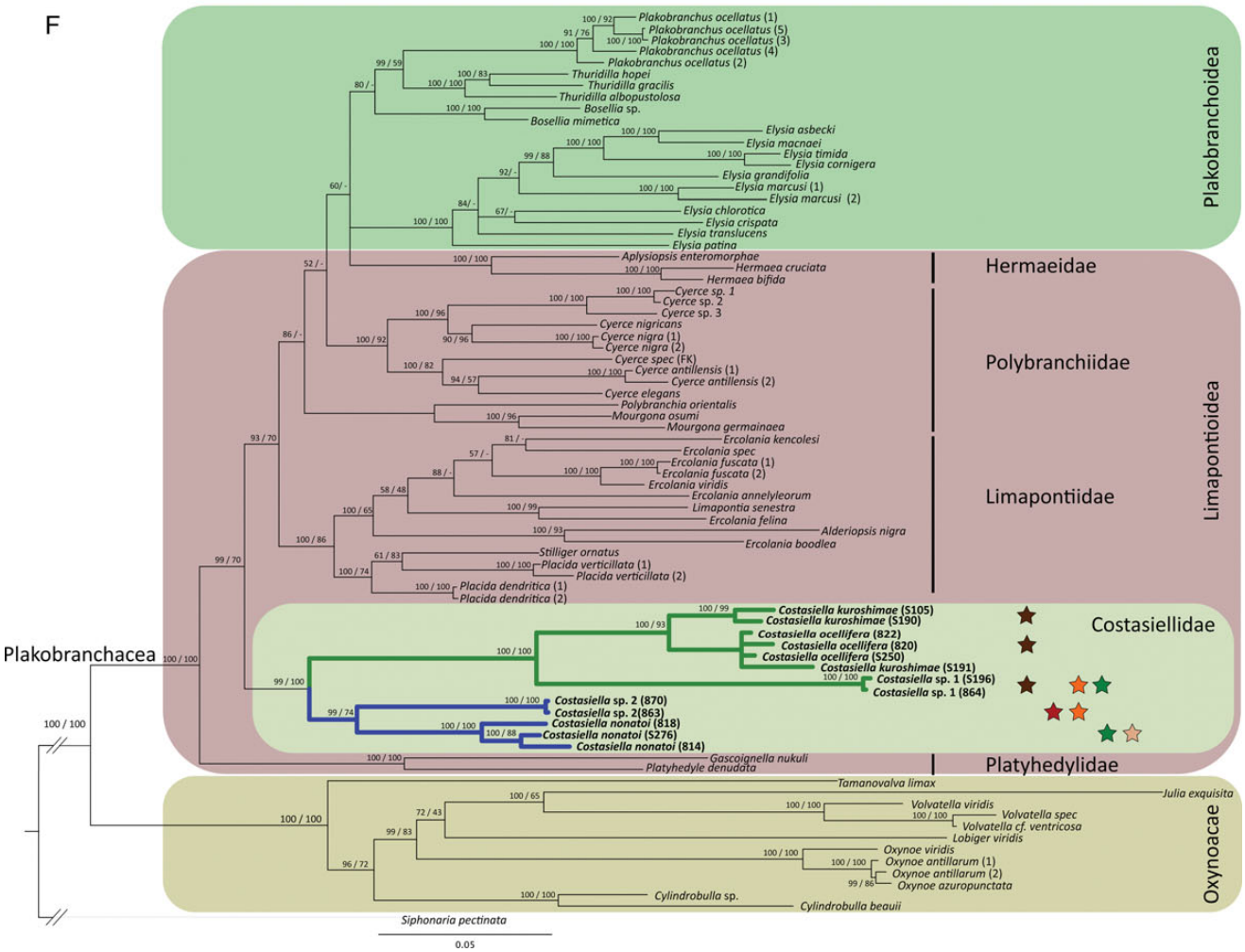


Figure 1. Species of *Costasiella* investigated. **A.** *C. ocellifera* (Florida Keys). **B.** *C. nonatoi* (Florida Keys). **C.** *C. kuroshimae* (Guam). **D.** *C.* sp. 2 (Guam). **E.** *C.* sp. 1 (Guam). **F.** Phylogenetic relationship of *Costasiella* based on partial sequences of 16S, 1st and 2nd positions of COI, H3 and 28S. Shown is a 50% majority-rule tree based on a Bayesian analysis. Numbers at nodes represent posterior probabilities (Bayesian analysis) and bootstrap values (maximum-likelihood 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*. Blue clade shows *Costasiella* with no functional retention of kleptoplasts, green clade indicates species with functional retention.

Table 1. Setups of starvation experiments using *Costasiella ocellifera* and *C. nonatoi*.

Species	Light condition	Light intensity	Condition	*N*	d	Mean ± SD, starting F_v/F_m	Mean ± SD, final F_v/F_m
C. ocellifera	12 h/12 h (day/night)	25 μmol photons/m^2 s	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
C. nonatoi	12 h/12 h (day/night)	25 μmol photons/m^2 s	Control	3	28	–	–
			Monolinuron	3		–	–
	Complete darkness		Control	3		–	–

Abbreviations: *N*, number of individuals; d, days of starvation; SD, standard deviation.

et al., 2012; Christa *et al.*, 2013). We applied barcoding to 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.

For barcoding of food sources of *C. nonatoi* and *C. ocellifera* we used the nonstarved animals that had been fixed in 70% ethanol after collection. We amplified the plastid gene *rbcL* with a pair of ulvophyceaen-specific primers (forward rbcLF: 5′-AAA GCN GGK GTW AAA GAY TA-3′ and reverse rbcLR: 5′-CCAW CGCATARANGGTTGHGA-3′; Pierce *et al.*, 2006). The gene *tufA*, as suggested by Christa *et al.* (2013), was not investigated, because no ambiguous food sources were found after analysis of *rbcL* sequences. One microlitre of genomic DNA isolate was used as template in a 10 μl final volume reaction composed of 1 μl sterilized water, 1 μl Qiagen Q-solution, 5 μl double concentrated Qiagen Multiplex PCR Master Mix and 1 μl each primer at a concentration of 10 pmol/μl. 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 previously determined gene-fragment length (560 for *rbcL*) using a 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 composed of 14 μl sterilized water, 5 μl double concentrated Larova PCR Master Mix (Berlin, Germany) and 1 μl each primer at 10 pmol/μl (forward T7Promoter 5′-TAA TAC GAC TCA CTA TAG GG-3′ and reverse 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). Sequence identity was verified by BLAST search using the NCBI homepage. Consensus sequences were generated when sequence divergence of chloroplast genes was <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 39 *rbcL* sequences (561 bp in length). A maximum-likelihood tree was generated using raxMLHPC v. 7.2.8 (Stamatakis, 2006) with substitution model GTR + G + I to identify plastid origin.

Feeding experiments

In the field, specimens of *C. ocellifera* are often not associated with their host alga *Avrainvillea*. Through feeding experiments using various algae found in the surrounding environment, we aimed to establish whether other food sources can also be consumed and potentially used by *C. ocellifera* for functional kleptoplasty. Specimens of *C. ocellifera* were starved under a natural day/night cycle with light intensities up to 600 μmol quanta/m^2 s at the Florida Keys in individual tanks with water changed ever second day. F_v/F_m values were measured daily and, after a decrease to zero, specimens were fed for 7 d with *Avrainvillea* ($n = 6$), *Udotea* ($n = 5$), *Penicillus* ($n = 3$) or *Pseudochlorodesmis* ($n = 5$), respectively. Change of F_v/F_m values was recorded by daily measurements (individual measurements available upon request). Out of each setup one specimen with the highest F_v/F_m value was used for barcoding (see below) to verify whether the provided food had been ingested or not.

Phylogenetic relationships

To investigate the phylogenetic relationships of *Costasiella* we analysed partial sequences of 16S, COI, H3 and 28S genes of 79 taxa of Sacoglossa, representing the major groups. 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 composed of 5 μl sterilized water, 2 μl Qiagen Q-Solution, 10 μl double concentrated Qiagen Multiplex PCR Master Mix and 1 μl of each primer at a concentration of 10 pmol/μl using sacoglossan-specific primer pairs for 16S, COI, H3 and 28S (Vonnemann *et al.*, 2005; Bass, 2006; Supplementary Material Table S2). 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 90 s). 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).

Newly generated sequences were supplemented by others downloaded from GenBank (Supplementary Material Table S1). *Siphonaria pectinata* was used as outgroup based on results of Jörger *et al.* (2010) and Neusser *et al.* (2011). Sequences of each gene were aligned individually using Mafft (Katoh *et al.*, 2002) and subsequently concatenated (alignment available upon request). For COI only first and second positions were used, following 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) with substitution model GTR + G + I and 1,000 replicates for bootstrapping analysis. Bayesian analysis were performed using MrBayes v. 3.2 (Ronquist *et al*., 2012) 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 at each 1,000th generation. After 3,148,000 generations the run was stopped, because average standard deviation of split frequencies was <0.005 and log-likelihood values of the cold chain did not increase further. The first 1,000 trees of both runs were discarded as 'burn-in' and a majority-rule consensus tree of the remaining 4,296 trees (2,148 from both runs) was calculated. Posterior probabilities were calculated to determine nodal support.

RESULTS

PAM measurements

The photosynthetic performance of the five *Costasiella* species (Fig. 1A–E) in a natural day/night cycle during starvation, measured by *in situ* PAM measurements, is shown in Figure 2. *Costasiella ocellifera* survived 52 d, the longest starvation period of all *Costasiella* species investigated so far (Supplementary Material Table S3) with maximum quantum yield (F_v/F_m) values exceeding 0.5 for 21 d and 0.2 for the following 28 d before declining to zero. Maximum quantum yield values in *C.* sp. 1 were higher than 0.4 during the 8 d of the starvation experiment (Fig. 2). In contrast, *C. kuroshimae* had lower maximum quantum yields, not exceeding 0.4 over the starvation period of 10 d (Fig. 2). 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 (Supplementary Material Table S3).

Starvation of C. ocellifera *during blocked photosynthesis*

Starvation experiments with *C. ocellifera* were carried out for a maximum of 38 d (Table 1), representing 76% of the maximum starvation time observed during our preceding experiments with *C. ocellifera*. Four animals were kept under a 12 h/12 h light/dark cycle under low light of 25 μmol m^{-2} s (LL) in Bonn and eight animals were kept in complete darkness in the Florida Keys. F_v/F_m values in both setups were nearly identical and continuously higher than those measured under natural conditions (Fig. 3A). In darkness and LL treatments F_v/F_m never declined <0.4, whereas in natural conditions after day 33 values were always lower than 0.4 (Fig. 3A). F_v/F_m values dropped immediately to <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 under LL plus Monolinuron treatments (with a similar slope to the animals starved under LL alone), which we did not observe in the animals kept in darkness with Monolinuron (Fig. 3B).

Nearly all investigated specimens survived for the full 38 d regardless of experimental setup. The death of seven individuals (two in the control group, two with Monolinuron, one in darkness, two in darkness with Monolinuron) is most likely attributable to individual fitness, rather than experimental conditions. To test if there is a general ability of *Costasiella* to survive starvation, we conducted starvation experiments under LL, LL plus Monolinuron and in complete darkness for the NR form *C. nonatoi*, a sympatric congener of *C. ocellifera* (Supplementary Material Table S3). Since *C. nonatoi* did not exhibit any fluorescence in the previous experiments (see above), PAM measurements were not performed. Six of the 9 specimens used in the experiments survived 20 d without performing any photosynthesis at all, with a maximum starvation period of 28 d. Three specimens left the petri dishes and died (Supplementary Material Table S3).

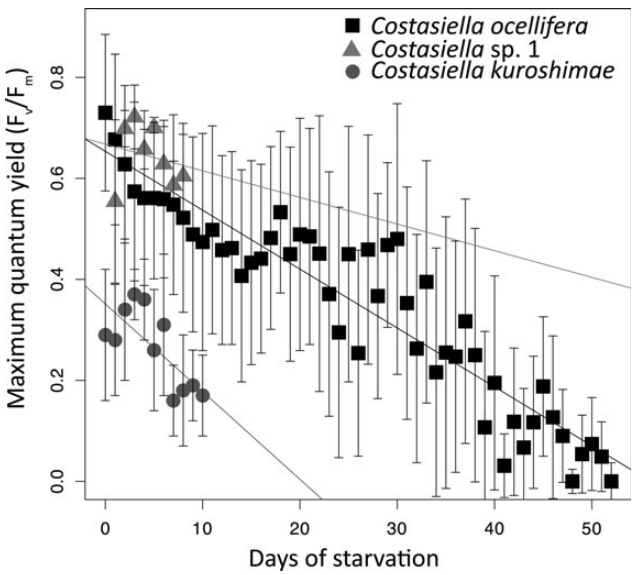


Figure 2. PAM measurements of *Costasiella* species. Maximum quantum yield values (F_v/F_m) of *C. ocellifera* (squares; $n = 31$), *C.* sp. 1 (triangles; $n = 2$) and *C. kuroshimae* (circles; $n = 6$) during starvation periods under natural light conditions. Shown are mean and standard errors of measured specimens.

Identification of food by barcoding

This technique revealed more information on food sources than feeding experiments conducted previously (Christa *et al*., 2013). According to our results, the species that retained plastids (StR and LtR) fed on at least one species of *Avrainvillea*, with *C. ocellifera* exclusively feeding on *A. mazei*, *C.* sp. 1 and *C. kuroshimae* on an unidentified species of *Avrainvillea*, and *C.* sp. 1 additionally on an unidentified *Rhipilia* and a *Pseudochlorodesmis* species (Supplementary Material Fig. S1). *Avrainvillea* was absent from the diet in NR species; *C. nonatoi* fed upon unknown taxa related to *Bryopsis* and a *Pseudochlorodesmis* species, *C.* sp. 2 on *Tydemania expeditionis* and the same unknown *Rhipilia* species as *C.* sp. 1 (Supplementary Material Fig. S1).

Feeding experiments

Of the algae offered, only consumption of *Avrainvillea mazei* resulted in a constant increase of F_v/F_m without any specimens of *C. ocellifera* dying (Fig. 4, Supplementary Material Table S4). Provision of *Udotea* sp. resulted in a brief increase of F_v/F_m, but three out of the five investigated animals did not survive the 7-d duration of the experiment. Neither *Pseudochlorodesmis* sp., nor *Penicillus dumestosus* resulted in an increase in F_v/F_m (Fig. 4) and some animals died during the feeding period (Fig. 4, Supplementary Material Table S4). Barcoding revealed that *Udotea*, *Penicillus* and *Pseudochlorodesmis* plastids were not incorporated by *C. ocellifera* during these feeding experiments (Supplementary Material Table S4). Single sequences of *Bryopsis* (specimen CoocPe1) were identified after starvation and subsequent feeding experiments, although this alga was not provided as a food source (Supplementary Material Table S4).

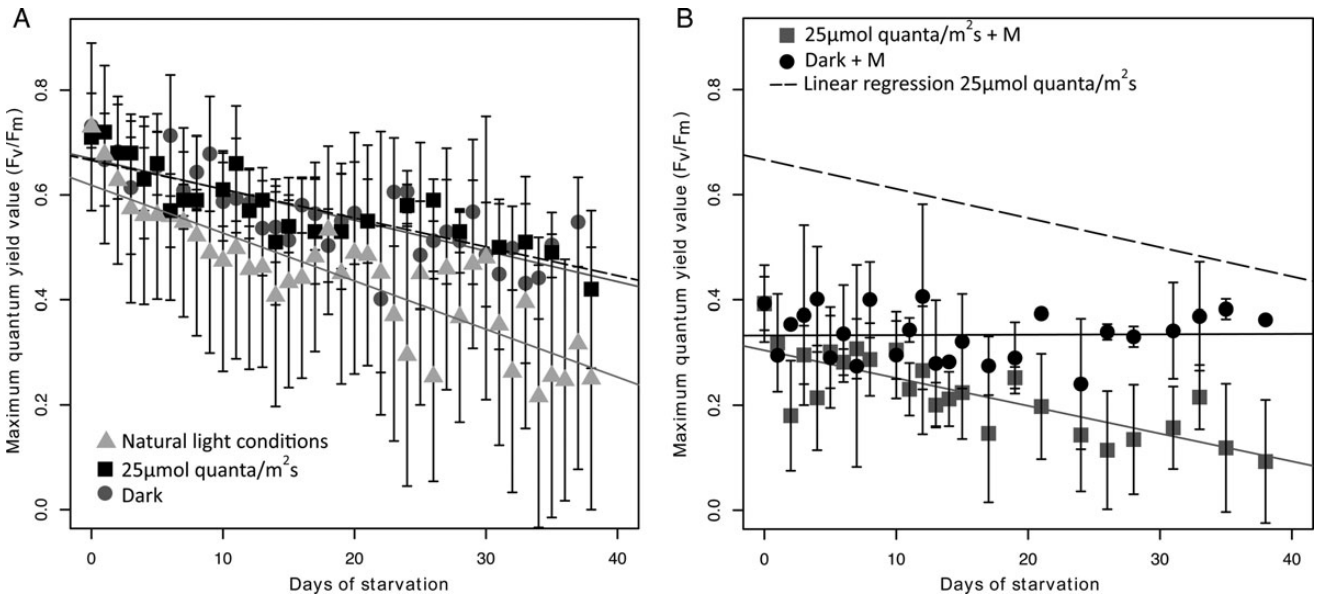


Figure 3. PAM measurements of *Costasiella ocellifera*. Maximum quantum yield values (F_v/F_m) during starvation for 38 d under different light conditions. **A.** Various light conditions: natural light (triangles; $n = 31$); low light (LL) at 25 µmol quanta/m^2 s (squares; $n = 4$); complete darkness (circles; $n = 8$). Lines indicate linear regression. **B.** Specimens treated with photosynthesis blocker Monolinuron under various light conditions: LL at 25 µmol quanta/m^2 s + Monolinuron (squares; $n = 6$); complete darkness + Monolinuron (circles; $n = 4$). Shown are mean and standard deviation of measured specimens. Linear regression for LL at 25 µmol quanta/m^2 s (dashed line) is shown for comparison.

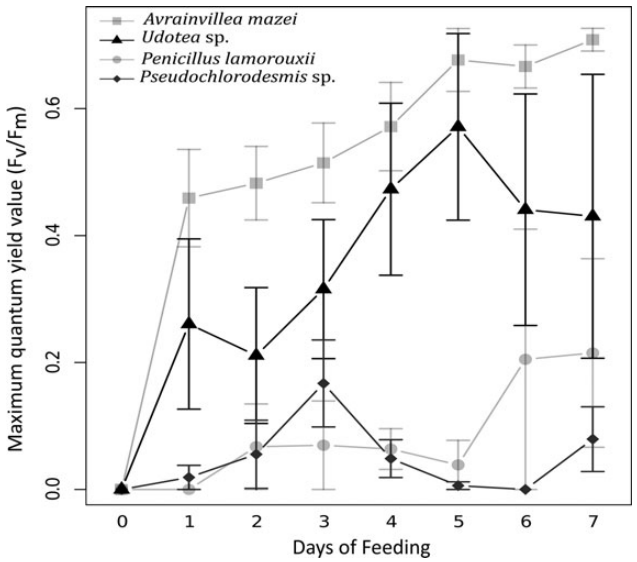


Figure 4. Feeding experiment with *Costasiella ocellifera*. Maximum quantum yield values (F_v/F_m) during 7 d of feeding with different food sources under a natural day-night rhythm with light intensities up to 600 µmol quanta m^{-2} s^{-1}. Food sources were provided after specimens were starved until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

Phylogenetic relationship

In our analysis the monophyletic genus *Costasiella* is a relatively basal group within a paraphyletic Limapontioidea, and NR species of *Costasiella* and those with functional retention appear as sister clades (Fig. 1F). Platyhedylidae is the sister taxon of the Limapontioidea and the Plakobranchoidea, thus placed at the base of the Plakobranchacea (Fig. 1F). Some limapontioidean genera and families are well supported: the Limapontiidae form a monophyletic group, as do the Hermaeidae. *Cyerce* is the monophyletic sister taxon of *Mourgona* and *Polybranchia*, resulting in a paraphyletic *Polybranchiidae* (Fig. 1F).

DISCUSSION

Functional kleptoplasty and phylogenetic relationships

Our PAM measurements confirm the earlier hypothesis that functional kleptoplasty is not limited exclusively to Plakobranchoidea (Clark *et al.*, 1981; Händeler *et al.*, 2009), but is also found in the limapontioidean genus *Costasiella*. *Costasiella ocellifera* incorporates functional plastids that remain functional for several weeks, similar to LtR species of the Plakobranchoidea [e.g. *Plakobranchus ocellatus*, *Elysia chlorotica*, *E. crispata*, *E. clarki* and *E. timida* (Händeler *et al.*, 2009; Middlebrooks *et al.*, 2011)]. Our results demonstrate that two other *Costasiella* species, *C. kuroshimae* and *C.* sp. 1, are StR forms, similar to *Thuridilla hopei* or *E. nigrocapitata*, respectively (Händeler *et al.*, 2009; Klochkova *et al.*, 2010). The remaining two investigated *Costasiella* species, *C. nonatoi* and *C.* sp. 2, appear to be NR species, digesting the plastids rapidly upon incorporation.

Findings on functional kleptoplasty based on carbon fixation rates in other limapontioidean genera, e.g. *Mourgona* (Evertsen & Johnson, 2009), have until now not been confirmed through *in situ* PAM measurements. The latter method, although commonly used for studying photosynthetic ability of Sacoglossa (reviewed by Cruz *et al.*, 2013), has only been applied to only about 40 sacoglossan species, representing roughly 10% of those known (Händeler *et al.*, 2009; Yamamoto *et al.*, 2009; Klochkova *et al.*, 2010, 2013). Of these, only 11 limapontioidean species have been examined regarding their ability to incorporate functional plastids. Further investigations are needed to conclude whether other sacoglossans—besides some species of *Costasiella*—perform kleptoplast-mediated photosynthesis.

Confirming previous molecular phylogenetic analyses of Sacoglossa (Händeler *et al.*, 2009; Maeda *et al.*, 2010), our

analysis places *Costasiella* at the base of the Plakobranchacea and not close to the Plakobranchoidea—the group known to exhibit functional kleptoplasty (for at least a few days) in nearly all studied members. It is interesting that the clade of *Costasiella* species with functional kleptoplasts are sister to a clade of *Costasiella* species that do not retain plastids (Händeler *et al*., 2009; Maeda *et al*., 2010; Wägele *et al*., 2011). According to the results of this and previous phylogenetic analyses (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 most limapontioidean lineages (Platyhedylidae and within *Costasiella;* Maeda *et al*., 2010), or (2) functional kleptoplasty evolved independently within *Costasiella* and the Plakobranchoidea (Händeler *et al*., 2009). Until kleptoplasty is investigated in more limapontioidean taxa and phylogenetic relationships are better resolved, these competing hypotheses cannot be tested.

Starvation of C. ocellifera *with blocked photosynthesis*

Survival of starvation for 28 d by the NR species *C. nonatoi* shows that in Sacoglossa the ability to survive food depletion can be independent of the ability to retain functional kleptoplasts. The LtR species *C. ocellifera* survived 38 d of starvation, irrespective of whether photosynthesis was blocked by keeping the animals in the dark or by adding the photosynthesis blocker Monolinuron. Notably, we have not yet compared ultimate survival of *C. ocellifera* under conditions with blocked and nonblocked photosynthesis with that of NR forms. We assume that blocking photosynthesis reduces the life expectancy, but only marginally. Chemical blocking of photosynthesis is likely incomplete, i.e. not every PSII reaction centre of the plastids is blocked. Some plastids may still be able to fix carbon to a small extent, contributing energy-rich polymers that are available to the slugs (Christa *et al*., 2014b). Hence these slugs might survive a little longer than those that are not able to photosynthesize at all, although not so long as those that photosynthesize normally. Similar to our results on *Costasiella*, Christa *et al*. (2014b) and Klochkova *et al*. (2013) observed a high survival of slugs without performing photosynthesis. This contradicts former results (Trench, 1969; Hinde & Smith, 1972, 1975; Hawes, 1979; Giménez-Casalduero & Muniain, 2008), but conditions of cultivation were not always clear in these studies, or were mentioned as not optimal (Yamamoto *et al*., 2012). Klochkova *et al*. (2013) observed that the StR species *Elysia nigrocapitata* could survive starvation for up to 5 months independent of photosynthetically active kleptoplasts. They stated that factors besides photoautotrophic CO_2 fixation are important for surviving starvation periods. Evertsen & Johnson (2009) provided a key observation: starch grains in the plastids of starved *E. viridis* were larger than in freshly collected animals. Vettermann (1973) showed that the thylakoid membranes in the plastids of *Acetabularia* are transformed into starch when this ulvophyte was kept in the dark, under nonphotosynthetic light, or when enucleated. We do not know whether this happens in plastids of all chlorophyte algae, 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 over time seems feasible. We suggest that this starch could provide an energy source for the slug during long-term starvation. By not digesting plastids at once, the slugs may benefit from the prolonged 'life' of the plastids, especially under conditions in which photosynthesis is reduced. The plastids accumulate starch before they degrade, which can subsequently be metabolized by the slugs.

Food of Costasiella

Costasiella species that show functional retention feed upon *Avrainvillea*, *Rhipilia* and *Pseudochlorodesmis*. This is in contrast to LtR plakobranchoidean species that feed mainly on *Halimeda*, *Acetabularia*, *Caulerpa* or *Vaucheria* (Christa *et al*., 2013, 2014a). Feeding experiments with algae found in the natural habitat did not reveal any additional food sources, but supported our barcoding results showing *Avrainvillea* as the main source of kleptoplasts. Interestingly, all NR species of *Costasiella* consumed other algal species; *Avrainvillea* was never detected by barcoding, although it has been mentioned as a food source for *C. nonatoi* in the literature (Jensen, 1993). This provides evidence that functional kleptoplasty within *Costasiella* relies on plastids from *Avrainvillea*. This raises the question whether *Avrainvillea* plastids have attributes relating to robustness, similar to those from *Vaucheria* and *Acetabularia*, which undergo long-term maintenance in *E. chlorotica* and *E. timida*, respectively.

CONCLUSION

The results presented here broaden our knowledge of kleptoplasty among sacoglossans. Based on the diversity of functional kleptoplasty found within *Costasiella*, we suggest that more species within other limapontioidean taxa need to be investigated. Any general deductions for limapontioidean genera based on a few observations on selected species should be treated with caution. Our experiments demonstrate that even if retained kleptoplasts are blocked in their photosynthetic activity, they may still function as a 'larder' that helps the slugs to survive starvation periods. In addition we show that kleptoplasts from the chlorophyte *Avrainvillea* remain functional in the cytosol of an animal cell for a long period; we suggest that the attributes of these and other plastids known to survive for long periods should in future be analysed in more detail.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *Journal of Molluscan Studies* online.

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