Identification of photosynthetic sacoglossans from Japan

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Abstract

Some sacoglossan molluscs, including several species of Elysiidae, are known to incorporate algal chloroplasts, and the incorporated chloroplasts are functional for days to months, depending on species. This incorporation and maintenance of foreign chloroplasts are known as kleptoplasty. In this article, we surveyed photosynthetic activity of several sacoglossans collected in Japan, by analysis of *in vivo* chlorophyll fluorescence. Our survey found 8 new species that have active chlorophylls, judged by the parameter Fv/Fm, an indicative of the functionality of photosystem II. Identified sacoglossans with active chlorophylls belong to the *Bosella*, *Costasiella*, *Elysia*, *Julia*, *Placida*, *Stiliger*, and *Thuridilla* genera. Our results strongly suggest that these species possess kleptoplasts. Possession of chloroplasts was confirmed for one of the identified sacoglossans, *Elysia trisinuata,* by transmission electron microscopy.

Introduction

Some sacoglossans are known to have ability to photosynthesize using chloroplasts present in their cells. These chloroplasts are not inherited from the parents, but "stolen" from their food algae, such as *Codium* or *Caulerpa.* Acquired algal chloroplasts are maintained in cells of digestive glands of sacoglossans for days to months, depending on species. This incorporation and maintenance is called as kleptoplasty (TRENCH and OHLHORST 1976; RUMPHO et al. 2007).

After the first report of kleptoplasty by *Elysia astroviridis* (KAWAGUTI and YAMASU 1965), several species have been shown to have kleptoplasts by transmission electron microscopic (TEM) analysis (TRENCH et al. 1969). Functionality of kleptoplasts was demonstrated by light-driven CO_2-incorporation using radioisotope (^{14}C) (HINDE and SMITH 1974), light-dependent O_2-emission detected by oxygen electrode (RUMPHO et al. 2000), and *in vivo* chlorophyll fluorescence analysis for detection of functional chlorophylls (EVERTSEN et al. 2007). Now, kleptoplasts have been found in more than 20 species of sacoglossans that belong to genera *Alderia, Bosellia, Caliphylla, Elysia, Hermaea, Limapontia, Mourgona, Oxynoe, Plakobranchus, Tridachia,* and *Thuridilla* (EVERTSEN et al. 2007).

In this report, we screened for Japanese sacoglossans containing functional chloroplasts, judged by possession of active chlorophylls that can be detected by *in vivo* chlorophyll fluorescence analysis. We first report possession of active chlorophylls in eight species, including ones belonging to *Costasiella*, *Julia*, *Placida*, and *Stiliger* genera. Our results, together with previous studies in this field, suggest that kleptoplasty occur in a wide range of sacoglossans.

Materials and Methods

Sea slugs were collected in 2007 and 2008 at Kominato (Kamogawa, Chiba, Japan), Misaki (Miura, Kanagawa, Japan), Shirahama (Nishimuro, Wakayama, Japan), Sesoko (Motobu, Okinawa (Ryukyu), Japan), and Zanpa (Yomitani-son, Okinawa, Japan). Collected sea slugs were sent to Nagoya University and subjected to *in vivo* chlorophyll fluorescence analysis using FluorCam (Photon Systems Instruments, Brno, Czech Republic) based on the pump and probe system with a CCD camera detector (NEDBAL and WHITMARSH 2004). Fv/Fm (BAKER 2008) was measured per individual using the camera system, and the average and standard deviation were calculated, if possible. Assays were done within a week after collection except for *Costasiella cf. kuroshimae* and *Elysia ornata.* These two species were assayed in a month after collection. Until assays, *E. ornata*, *Placida* sp., and *Costasiella* cf. *kuroshimae* were kept together with their food algae that are *Codium*, *Codium*, and *Avrainvillea*, respectively. Because we could not prepare food algae for the other sea slugs, it was impossible to adjust days before assay after start of starvation. This problem caused some difficulty in strict comparison of Fv/Fm among species.

For transmission electron microscopy (TEM), samples were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 3% NaCl for 2 h at 4˚C. In this fixative, the samples were cut into small fragments. Then they were postfixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) containing 3% NaCl for 2 h at room temperature. They were dehydrated with a graded acetone series and embedded in Spurr's epoxy resin. Thin sections were cut by a diamond knife on a Ultracut (Reichert-Jung, Wien, Austria), mounted on formvar-coated slot grids, stained with 4% uranyl acetate and lead citrate (REYNOLDS 1963), and observed with a JEM-1011 electron microscope (JEOL, Tokyo, Japan).

Results and Discussions

Sacoglossans collected at several shores in Japan were subjected to *in vivo* chlorophyll fluorescence analysis. *Plakobranchus ocellatus* and *Pteraeolidia ianthina* were used as positive controls. *P. ocellatus* has kleptoplasts (GREENE 1970b; HIROSE 2005), whose functionality has been confirmed by detection of light-dependent CO_2-fixation (GREENE 1970a) as well as Fv/Fm determined by *in vivo* chlorophyll fluorescence analysis (EVERTSEN et al. 2007). *P. ianthina* is known to be a host of endosymbiotic zooxanthella, rather than kleptoplasts. *P. ianthina* has also been reported to have photosynthetic activity due to the symbionts, revealed by light-dependent carbon fixation activity (HOEGH-GULDBERG and HINDE 1986).

As shown in Table 1, both *P. ocellatus* and *P. ianthina* showed high Fv/Fm, as expected. These results confirmed possession of active chlorophylls in these organisms. It should be noted that this analysis cannot distinguish between kleptoplasts (*P. ocellatus*) and endosymbiotic algae (*P. ianthina*).

We collected 12 species in sacoglossa in Japan, and subjected them to *in vivo* chlorophyll fluorescence analysis. Among them, 8 species showed measurable Fv/Fm values (Table 1). These results indicate that the positive species, that belong to genera of *Costasiella, Bosellia, Elysia, Thuridilla, Julia, Placida*, and *Stiliger*, possess active chlorophylls and thus photosynthetically functional chloroplasts. Considering that all these species belong to sacoglossa, these results strongly suggest that they have kleptoplasts. Photographs of positive species are shown in Figure 1.

One of the positive species, *Elysia trisinuata*, was subjected to electron microscopic analysis (Figure 2). As expected, cells of digestive glands contained chloroplasts (Ct in the Figure 2B), judged by their ultrastructure. As shown in

Table 1: Sacoglossans with active chlorophylls identified in this study

Species	Sampling place	Sampling date	Number of individuals	Size (mm)	F_v/F_m
SACOGLOSSA					
Costasiellidae					
Costasiella cf. *kuroshimae*	Zanpa	2008 Dec.	5	1-3	0.661 ± 0.0395
Elysiidae					
Bosellia sp.	Sesoko	2008 Apr.	4	7-11	0.716 ± 0.052
Elysia ornata	Shirahama	2007 Sep.	3	20-40	0.815 ± 0.0201
Ibid.	Misaki	2007 Oct.	5	20-40	0.844 ± 0.0190
Elysia trisinuata	Misaki	2007 Aug.	6	15-30	0.853 ± 0.042
Thuridilla vatae	Sesoko	2008 Jun.	1	14	0.725
Juliidae					
Julia zebra	Sesoko	2008 Apr.	1	2	0.616
Limapontiidae					
Placida sp. (*sensu* (BABA 1986)	Sesoko	2008 Apr.	9	4 - 8	0.475 ± 0.075
Stiliger ornatus	Shirahama	2008 Oct.	3	7-10	0.595 ± 0.0795
Plakobranchidae					
Plakobranchus ocellatus	Sesoko	2008 Jun.	1	22	0.811
NUDIBRANCHIA					
Pteraeolidia ianthina[1]	Kominato	2008 Oct.	4	20-50	0.831 ± 0.023

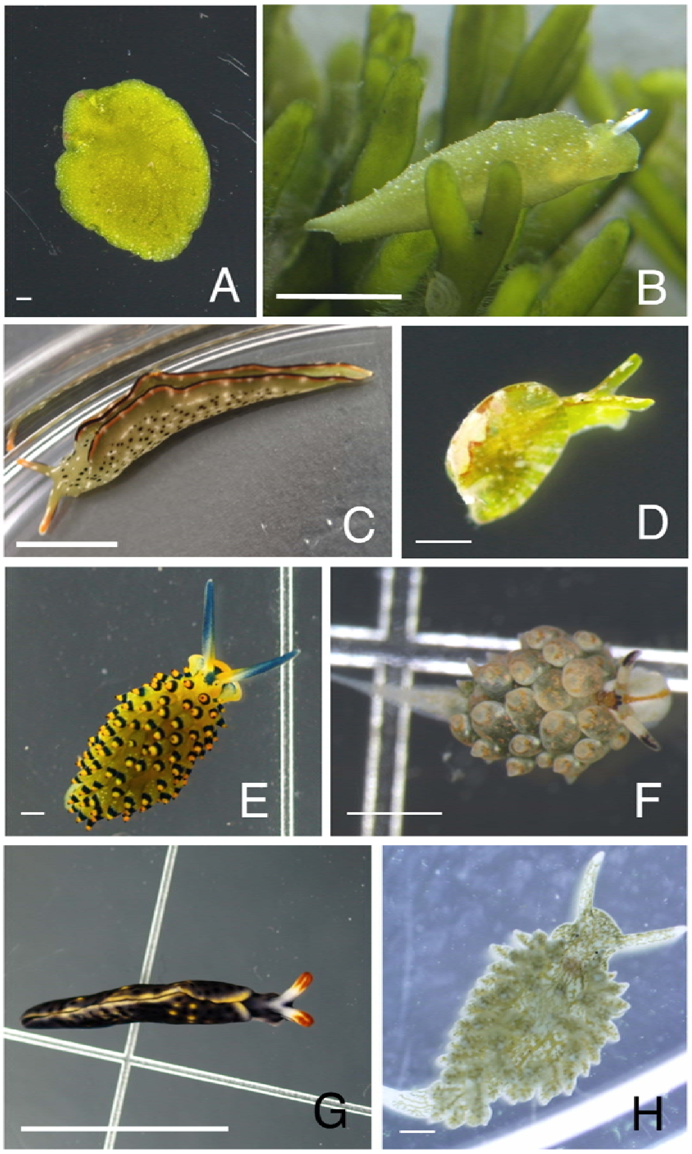
Kominato: Kamogawa, Chiba; Misaki: Miura, Kanagawa; Shirahama: Nishimuro, Wakayama; Sesoko: Motobu, Okinawa; Zanpa: Zanpamisaki, Yomitani-son, Okinawa. [1] Known to retain *Symbiodinium* by endosymbiosis (BURGHARDT et al. 2008).

the figure, some chloroplasts possess starch grains (white ovals, Figure 2B), suggesting functionality of these kleptoplasts.

As shown in Table 1, Fv/Fm varies from 0.85 (*Elysia trisinuata*) to 0.48 (*Placida* sp.) according to species, and variation within a species was much smaller than difference among species. As for *Elysia ornata*, we analyzed two batches, one from Shirahama collected in September and the other from Misaki caught in October. Although these two groups were caught at different places in different seasons, again they showed small difference in Fv/Fm (0.815 ± 0.0201 and 0.844 ± 0.0190, respectively).

Fv/Fm is a parameter that reflects maximum efficiency of photosystem II, and thus it indicates intactness of photosystem II (BAKER 2008). Injury of photosystem II, that can be caused by environmental stresses, results in reduction of Fv/Fm (BAKER 2008). Although degree of injury against environmental stresses can vary among individuals, we found that there is little variation of Fv/Fm among the analyzed individuals of the same species.

In the case of kleptoplasts, decrease of Fv/Fm was observed during starvation, corresponding to the loss of kleptoplasts (EVERTSEN et al. 2007). Periods until loss of kleptoplasts vary from days to months, depending on spe-



Thin bar: 0.5 mm, thick bar: 10 mm

Figure 1: Sacoglossans with functional chlorophylls
Thin and thick scale bars indicate 0.5 mm and 10 mm, respectively. A: *Bosellia* sp., B: *Elysia trisinuata*, C: *Elysia ornata*, D: *Julia zebra*, E: *Stiliger ornatus*, F: *Costasiella* cf. *kuroshimae*, G: *Thuridilla vatae*, H: *Placida* sp. (sensu BABA, 1986)

cies. Sacoglossans at a higher level of kleptoplasty maintain chloroplasts for a longer period (CLARK et al. 1990). The reduction of Fv/Fm during starvation is related to the loss of kleptoplasts, but it is not due to the reduction of the chloroplast number, because Fv/Fm should not be affected by amount of chloroplasts or chlorophylls in an individual (BAKER 2008). Reason for the reduction of Fv/Fm during starvation is not clear. Theoretically, the reduction can be caused by either increase of injured photosytem II as mentioned above, or saturation of the electron transport system after photosystem II in the dark (BAKER 2008). Anyway, this reduction causes variations of Fv/Fm within a species. In spite of the above tendency, observed Fv/Fm was quite similar within a species (Table 1). This would be in

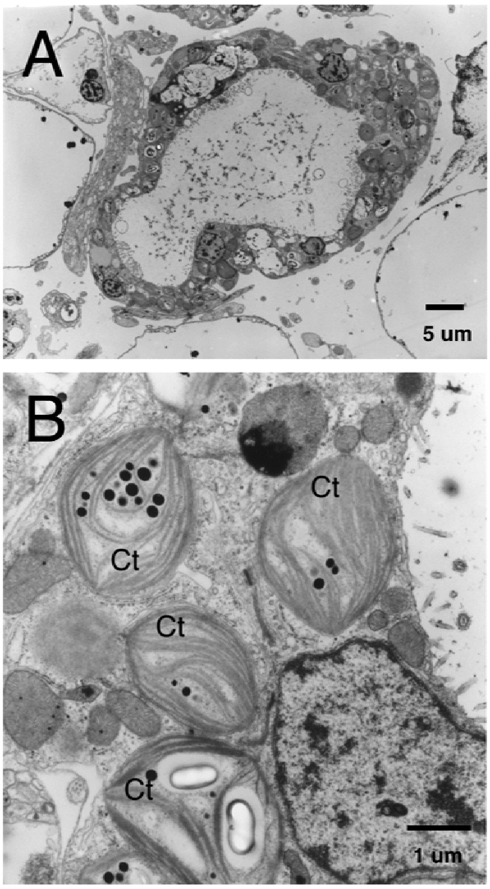


Figure 2: Transmission electron micrographs of *Elysia trisinuata*
A. Section of digestive glands. B. part of a cell in digestive glands. Ct: chloroplast. Electron-dense black circles in chloroplasts are plastoglobules, and electron-sparse ovals in chloroplasts are starch grains.

part due to the same starvation period for a batch of assays.

Among the sacogrossans shown in the table, *Elysia ornata*, *E. trisinuata*, and *Plakobranchus ianthobaptus* showed the highest Fv/Fm values. These species can survive for the longest periods (more than one month) in the list during starvation (Greene 1970b) (data not shown). On the other hand, *Placida* sp. shows the lowest Fv/Fm value among the positive list, and this species survived for the shortest period during starvation among the positive species, that is, less than a week (data not shown). Therefore, there seems to be a rough correlation between Fv/Fm and survival period during starvation.

As for molluscs with photosynthetic activity, there are two possible cytological situations: possession of kleptoplasts or of endosymbiotic algae (Venn et al. 2008). We suggest that the photosynthetic sacoglossans shown in Table 1 have kleptoplasts, because almost all of the known photosynthetic sacoglossans possess kleptoplasts and there is no report on endosymbiosis of algae in adult sacoglossans.

Sacoglossans with kleptoplasts, including our results, are summarized in Table 2. As shown in this table, around 30 species are now known to have kleptoplasts. The largest group in the table is Elysiidae, and most of the families in Sacoglossa are included in the table, except for two families, Gascoignellidae and Volvatellidae. This demonstrates widespread distribution of kleptoplasty in Sacoglossa.

Table 2: List of sacoglossans reported to possess functional chloroplasts

Species	Method	Reference
Caliphyllidae		
Caliphylla mediterranea	^{14}C	(CLARK et al. 1990)
Mourgona germainea	^{14}C	(CLARK et al. 1990)
Costasiellidae		
Costasiella cf. *kuroshimae*	Fv/Fm	This work
Elysiidae		
Bosellia mimetica	^{14}C	(CLARK et al. 1990)
Bosellia sp.	Fv/Fm	This work
Elysia australis	^{14}C	(HINDE 1980)
Elysia cauze (= *E. subornata*)[1]	Chl[a]	(CLARK and BUSACCA 1978)
Elysia chlorotica	O_2	(RUMPHO et al. 2000)
Elysia crispata (= *Tridachia crispata*)[2]	Chl[a]	(CLARK and BUSACCA 1978)
Elysia hedgpethi	^{14}C, Chl	(GREENE 1970a)
Elysia ornata	Fv/Fm	This work
Elysia pusilla	Fv/Fm	(EVERTSEN et al. 2007)
Elysia sp.	Fv/Fm	(EVERTSEN et al. 2007)
Elysia timida	Fv/Fm	(EVERTSEN et al. 2007)
Elysia tomentosa	Fv/Fm	(EVERTSEN et al. 2007)
Elysia trisinuata	Fv/Fm	This work
ibid.	TEM	This work
Elysia tuca	Chl[a]	(CLARK and BUSACCA 1978)
Elysia viridis	^{14}C	(TRENCH et al. 1973; HINDE and SMITH 1974)
Thuridilla carloni	Fv/Fm	(EVERTSEN et al. 2007)
Thuridilla lineolata	Fv/Fm	(EVERTSEN et al. 2007)
Thuridilla vatae	Fv/Fm	This work
Hermaeidae		
Hermaea cruciata	^{14}C	(CLARK et al. 1990)
Juliidae		
Julia zebra	Fv/Fm	This work
Limapontiidae		
Alderia modesta	^{14}C	(CLARK et al. 1990)
Placida sp. (*sensu* (BABA 1986)	Fv/Fm	This work
Limapontia depressa	^{14}C, Chl[a]	(HINDE and SMITH 1974)
Stiliger ornatus	Fv/Fm	This work
Oxynoidae		
Oxynoe antillabrum	Chl[a]	(CLARK and BUSACCA 1978)
Plakobranchidae		
Plakobranchus ocellatus (=*P. ianthobaptus*)[3]	Chl[a]	(GREENE 1970a)
ibid.	Fv/Fm	(EVERTSEN et al. 2007), this work
ibid.	TEM	(HIROSE 2005)

The table shows sacoglossans containing functional chloroplasts (Level 4 and higher categories (CLARK et al. 1990)). Chl: extraction and measurement of chlorophyll content, Fv/Fm: PSII activity determined by *in vivo* chlorophyll fluorescence measurement (pulse amplitude modulation (PAM) or pump & probe methods), O_2: light-dependent O_2 emission, ^{14}C: light-dependent incorporation of $NaH^{14}CO_3$. [a]Possession of chlorophylls does not directly mean functionality of chloroplasts, but stable maintenance of chlorophylls suggest it (EVERTSEN et al. 2007). References for synonymous species are: [1](CLARK 1984), [2](GOSLINER 1995), [3](JENSEN 1992).

Acknowledgements

We would like to thank Ms. Ayami Sekizawa of Osaka City University for collection of some sacogrossans. This study was supported in part by a grant from the Research Institute for Marine Invertebrates, Tokyo, and by Grants-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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