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Survey for Functional Kleptoplasty Among West Atlantic Ascoglossa (=Sacoglossa) (Mollusca: Opisthobranchia)

by

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Abstract. Eighteen species of Florida and New England Ascoglossa were examined for chloroplast retention and photosynthetic function, to more precisely delimit the occurrence and determine the levels of kleptoplasty (=chloroplast symbiosis). Previously unexamined genera with functional plastids include *Mourgona, Caliphylla, Bosellia,* and *Placida.* Short-lived function was also detected in *Alderia. Bosellia mimetica* exhibited high levels of carbon fixation, and is probably equivalent to the best-developed examples of kleptoplasty. Three examples of elysiids without functional plastids were found: *Elysia serca* and *E. catulus,* feeding upon seagrasses, and *E. evelinae,* feeding upon diatoms. Six levels of kleptoplasty, in terms of plastid retention and function, are recognized in this paper.

Some shelled Ascoglossa maintain structurally intact plastids for one to several days, but without detectable photosynthetic function. This capability appears to be precursory to retention of functional kleptoplastids and may initially have simply enhanced cryptic coloration. Retention of functional kleptoplastids is a plesiomorphic character among both elysioid and stiligeroid lines, and loss of function among advanced taxa is due partially to adaptive radiation to unsuitable plastid sources. Determination of whether functional kleptoplasty evolved convergently in elysioid and stiligeroid lines, or within a shared ancestor, cannot presently be answered.

INTRODUCTION

The retention of chloroplasts by ascoglossan mollusks was first noted by BRÜEL (1904) in *Caliphylla mediterranea* Costa, 1867, and was subsequently rediscovered by KAWAGUTI & YAMASU (1965) in *Elysia atroviridis* Baba, 1955. This phenomenon has been described as "chloroplast symbiosis." However, various authors have sought a more appropriate term (TAYLOR, 1968; BLACKBOURN *et al.,* 1973; TRENCH, 1980), and we support use of the term "kleptoplasty" (GILYAROV, 1983; WAUGH & CLARK, 1986).

Views on the extent of chloroplast retention have varied; GREENE (1970a) suggested a broad occurrence of kleptoplasty among the order, while MUSCATINE & GREENE (1973) and TRENCH (1975) indicated a much restricted occurrence, principally to Elysiidae feeding on Siphonales. However, exceptions to this were known. *Hermaea bifida* (Montagu, 1816), feeding on the rhodophyte *Griffithsia* (TAYLOR, 1971; KREMER & SCHMITZ, 1976) retained functional plastids, as did the stiligerid *Limapontia depressa* Alder & Hancock, 1862 (interpreted by TRENCH [1975] as an elysiid), feeding upon *Vaucheria* (HINDE & SMITH, 1974). CLARK & BUSACCA (1978) summarized evidence for a broader occurrence of kleptoplasty. CLARK *et al.* (1981) found that *Costasiella ocellifera* (Simroth, 1895) retained highly functional plastids for a period equivalent to that of *Elysia* (*Tridachia*) *crispata* (Mörch, 1863), previously recognized as the best example of functional plastid retention (TRENCH, 1975).

Determining the occurrence of kleptoplasty within the order should provide important information on evolution of the Ascoglossa. Of approximately 200 described species, only about 10% have been examined for kleptoplasty. Several families (Ascobullidae, Volvatellidae, Caliphyllidae, Boselliidae, and Gascoignellidae) have not been studied. In this paper, we present results of a systematic examination of 18 west Atlantic species representing 5 additional families, 14 genera, and 14 plant species.

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Table 1

Sources of experimental material.

Ascoglossan species	Locality	Diet/substrate
Conchoidea		
Ascobulla ulla Marcus, 1970	Fort Pierce, FL	*Caulerpa racemosa* (Forsskål) J. Agardh
Lobiger souverbiei Fischer, 1856	Sebastian Inlet, FL	*Caulerpa racemosa*
Oxynoe azuropunctata Jensen, 1980	Key Largo, FL	*Caulerpa paspaloides* (Bory) Greville
Berthelinia caribbea Edmunds, 1963	Deepwater Cay, Bahamas	*Caulerpa verticillata* J. Agardh
Stiligeroidea		
Caliphylla mediterranea Costa, 1867	Fort Pierce, FL	*Bryopsis plumosa* (Hudson) C. Agardh
Mourgona germaineae Marcus & Marcus, 1969	Geiger Key, FL	*Cymopolia barbata* (Linnaeus) Lamouroux
Cyerce antillensis Engel, 1927	Fort Pierce, FL	*Cladophora prolifera* (Roth) Kützing
Aplysiopsis zebra Clark, 1982	Key Largo, FL	*Penicillus dumetosus* (Lamouroux) Blainville
Hermaea cruciata Gould, 1870	Key Largo, FL	*Griffithsia* sp.
Placida dendritica (Alder & Hancock, 1843)	Noank, CT	*Codium fragile* (Suringar) Hariot
Placida kingstoni Thompson, 1977	Fort Pierce, FL	*Bryopsis plumosa*
Ercolania fuscata (Gould, 1870)	Sebastian Inlet, FL	*Cladophora gracilis* (Griffiths *ex* Hårvey) Kützing
Ercolania coerulea Trinchese, 1893	Long Key, FL	*Dictyosphaeria cavernosa* (Forsskål) Børgesen
Alderia modesta (Lovén, 1844)	Gloucester Pt., VA	*Vaucheria* sp.
Elysioidea		
Elysia serca Marcus, 1955	Banana River, FL	*Halophila engelmannii* Ascherson in Neumayer
Elysia catulus (Gould, 1870)	Noank, CT	*Zostera marina* (Linnaeus)
Elysia evelinae Marcus, 1957	Key Largo, FL	*Biddulphia* sp.
Bosellia mimetica Trinchese, 1891	Fort Pierce, FL	*Halimeda tuna* (Ellis & Solander) Lamouroux

MATERIALS AND METHODS

Collection sites and food species for animals used in this study are shown in Table 1. Animals were collected from 1979 to 1980 at sites in Bermuda, the Bahamas, Florida, Connecticut, and Maryland. A voucher collection for species used in this study was previously deposited with the National Museum of Natural History (JENSEN, 1980). Specimens of *Oxynoe azuropunctata* and *Berthelinia caribbea* were laboratory-cultured from stocks collected at the listed sites. Animals were kept in the laboratory in 40-L aquaria with natural seawater and excess food until used for experiments. Aquarium temperature was approximately 25°C, and the aquaria were illuminated by a bank of fluorescent bulbs at an intensity of approximately 110 $\mu Ei \cdot m^{-2} \cdot sec^{-1}$ and a photoperiod of 18 hr light : 6 hr dark.

Ultraviolet epifluorescence was used to determine presence and persistence of intact chlorophylls in freshly fed slugs and in animals starved for various intervals. Bright red fluorescence, confined to plastids in digestive diverticula, suggested the possibility of photosynthetic activity, and these species were further examined by radiocarbon incubation.

Experimental animals were incubated individually for 1 hr in 2- or 4-mL vials containing membrane-filtered seawater (MFSW) and labelled $NaH^{14}CO_3$ at an activity of 2 μCi/mL. Most animals were incubated at a light intensity of 350 $\mu Ei \cdot m^{-2} \cdot sec^{-1}$ and a temperature of 25°C after several days of laboratory maintenance. However, some species in which we suspected plastid activity might be short-lived or subject to effects such as toxin inhibition were incubated *in situ* immediately after collection by placing the incubation apparatus at the site of collection. Thus, *Alderia modesta* was tested *in situ* at 1650 $\mu Ei \cdot m^{-2} \cdot sec^{-1}$ and 24°C. *Placida dendritica* and *Elysia catulus* were incubated *in situ* at 400 and 650 $\mu Ei \cdot m^{-2} \cdot sec^{-1}$, respectively, and 16°C. Dark controls were wrapped in aluminum foil and run simultaneously.

Following incubation, animals were quickly rinsed in three changes of MFSW to remove residual isotope and homogenized in 0°C methanol; chlorophyll was extracted by phase separation in diethyl ether and distilled water. Chlorophyll content was determined spectrophotometrically after the following equation (STRAIN & SVEC, 1966):

$$\mu g \text{ chl/mL} = 7.12(Ab_{660}) + 16.8(Ab_{642.5}),$$

where Ab is the absorption at the indicated wavelength (nm).

The alcohol/aqueous (A/A) phase was centrifuged at 15,000 rpm for 20 min. A 100-μL aliquot of the supernatant was mixed with 10 mL Aquasol and counted in a liquid scintillation counter (LSC). The volume of the A/A phase was measured. The centrifuged pellet was solubilized in 1 mL tissue solubilizer and neutralized with acetic acid, and a 100-μL aliquot was used for liquid scintillation counting. Total CPM was corrected for counting efficiency, quenching, and background, and counts were calculated as $DPM \cdot \mu g \text{ chlorophyll}^{-1} \cdot hr^{-1}$ (A/A and tissue-solubil-

Table 2

Summary of carbon fixation experiments in west Atlantic Ascoglossa. Chlorophyll retention: 0, no chlorophyll recoverable from freshly fed animals; 1, less than 12 hr; 2, 12 hr to 3 days; 3, 3 days to 1 wk; 4, longer than 1 wk. D.P.M. = disintegrations per minute; d.f. = degrees of freedom; t = calculated value of Student's "t"; P = significance level; L:D = ratio of light to dark fixation.

Ascoglossan species	Light fixation (D.P.M.)	Dark fixation (D.P.M.)	Chlor. reten. times	d.f.	t	P	L:D
Conchoidea							
Ascobulla ulla	(no pigment)		0	—	—	—	—
Lobiger souverbiei	6090 ± 4500	2810 ± 3270	2	6	1.18	n.s.	2.16
Oxynoe azuropunctata	4050 ± 3310	3150 ± 1710	2	20	0.78	n.s.	1.30
Berthelinia caribbea	1660 ± 1520	1150 ± 700	2	19	0.97	n.s.	1.45
Stiligeroidea							
Caliphylla mediterranea	1100 ± 880	107 ± 58.5	3	5	2.55	<0.05	2.88
Mourgona germaineae	5070 ± 2090	3170 ± 1260	3	13	2.17	<0.05	1.59
Cyerce antillensis	670 ± 82	543	1	1	n.a.	—	1.23
Aplysiopsis zebra	1860 ± 1020	1420 ± 362	1	10	0.99	n.s.	1.31
Hermaea cruciata	2550 ± 1280	2040 ± 939	2	6	1.01	n.s.	1.25
Placida dendritica	296 ± 115	290 ± 163	2	9	0.07	n.s.	1.02
Placida kingstoni	1230 ± 254	769 ± 261	2	12	3.31	<0.01	1.60
Ercolania fuscata	4820 ± 1780	6080 ± 3780	0	9	−0.73	n.s.	0.79
Ercolania coerulea†	618 ± 493	3493 ± 5193	1	11	1.60	n.s.	0.18
Ercolania coerulea‡	1898 ± 594	2133 ± 9.11	1	11	0.57	n.s.	0.89
Alderia modesta	15,800 ± 8930	5490 ± 5160	1	8	2.35	<0.05	2.88
Elysioidea							
Elysia serca‡	1150 ± 1040	959 ± 708	1	10	0.38	n.s.	1.20
Elysia catulus‡	1110 ± 491	1750 ± 980	1	14	−1.66	n.s.	0.63
Elysia evelinae	(pigment traces)		0/1	—	—	—	—
Bosellia mimetica	20,500 ± 4880	702 ± 146	4	3	5.44	<0.02	29.2

† Chlorophyll-specific rate.
‡ Rate per animal (non-chlorophyll-specific).

izer counts were summed). The ether phase contained negligible activity.

Preliminary examination of *Ascobulla ulla* and *Elysia evelinae* showed that chlorophyll was absent in freshly fed animals, so animals were not assayed for carbon fixation. Values for *Elysia catulus* and *Elysia serca* were based on fixation per animal, because most chlorophyll values were so low that meaningful pigment-specific data could not be calculated. Both rates were calculated for *Ercolania coerulea* because chlorophyll values for dark-incubated animals were significantly lower than those for light-incubated animals ($t = 2.73$, d.f. = 11, $P < 0.02$).

RESULTS

Carbon fixation data are summarized in Table 2. No net fixation occurred in the shelled species examined, though chlorophylls were retained up to several days in these species (see also Clark & Busacca, 1978). *Ascobulla ulla* and *Elysia evelinae* apparently degrade chlorophylls immediately upon ingestion, and hence were not examined for radiocarbon fixation.

Among the stiligeroids, four species (*Caliphylla mediterranea, Mourgona germaineae, Placida kingstoni,* and *Alderia modesta*) fixed significantly more carbon in light than in darkness. Of these species, *C. mediterranea* has the highest fixation (verifying Brüel's 1904 report), with photosynthetic activity probably lasting as long as a week. Unfortunately, a shortage of experimental material prevented more precise determination of the duration of photosynthetic activity. *Mourgona germaineae* appears to have similar functional ability, but this species is difficult to study because autotoxicity of stored cymopols (Jensen, 1984) requires large incubation volumes and consequently large quantities of isotope. Fixation ability of *A. modesta* is short-lived, as chlorophylls are retained in diverticula less than 12 hr. This may explain prior reports of non-functionality (Hinde & Smith, 1974; Graves *et al.*, 1979). The remaining stiligeroid species did not exhibit significantly higher fixation in light than in dark.

Among the elysioid species, neither *Elysia catulus* nor *E. serca* possessed functional plastids, and chlorophyll retention was brief. Although traces of chlorophyll occur in freshly fed *E. evelinae,* plastids fluoresce weakly, and the

presence of diffuse plastid margins immediately after feeding indicates rapid digestion. Therefore, we assume this species has non-functional retention. *Bosellia mimetica,* however, fixes large amounts of carbon (L:D ratio of 30), and based on chlorophyll retention, probably retains highly functional plastids for periods equivalent to those of other pronounced examples of kleptoplasty such as *Elysia (Tridachia) crispata* (Trench & Ohlhorst, 1976) and *Costasiella ocellifera* (Clark *et al.,* 1981).

DISCUSSION

Trench (1975) proposed a restrictive criterion for recognition of kleptoplasty: high light fixation rates for more than a week. We feel that the exclusion of less pronounced activity discourages scrutiny of the coevolution of ascoglossans and their algal foods. Based on present results and prior studies, we recognize a gradient between the extremes of non-retention of plastids and long-term retention, and propose the following six stepped levels of kleptoplasty and their criteria:

Level 1. Non-retention: Animal feeds on algal food that has potential as a plastid donor, but plastids are digested prior to, or immediately after, phagocytosis. The digestive diverticula lack algal pigments. Only *Ascobulla,* and perhaps the burrowing species of *Volvatella* (*e.g., V. laguncula* Thompson, 1979) seem to have non-retention.

Level 2. Short-term, non-functional retention: Animal is pigmented when collected and retains plastids in gut diverticula for at least 2 hr of starvation, but no photosynthate is detectable by isotope tracer techniques. Retention time may vary with illumination. *Elysia catulus, Elysia evelinae,* and *Ercolania coerulea* are examples. The rapid loss of chlorophyll in darkness by *Elysia catulus* suggests that illuminated plastids may somehow inhibit digestion of plastids despite absence of detectable carbon fixation. *Polybranchia viridis* Pease, 1869, rapidly degrades chlorophylls and is pronouncedly photophobic (Clark, personal observation), and thus would also fit this category.

Level 3. Medium-term, non-functional retention: Structurally intact plastids occur at least 24 hr (including one interval of darkness) after ingestion, but no photosynthetic activity can be demonstrated. This category includes most advanced conchoid Ascoglossa (Lobigeridae, Oxynoidae, and Juliidae), and epifaunal *Volvatella* (Stirts, 1980; Clark, 1982a; and present study).

Level 4. Short-term functional retention: Animal exhibits photosynthesis in field environment, but plastids are rapidly digested and function ceases less than one day after removal from field environment. *Alderia modesta* meets this criterion, and some others, such as *Hermaea cruciata,* may fit into this category when more rigorously examined.

Level 5. Medium-term functional retention: Photosynthesis persists for more than 24 hr, including a period of darkness, but photosynthesis ceases or is greatly reduced within a week of starvation, *Hermaea bifida* appears to fit this level (Taylor, 1971).

Level 6. Long-term functional retention: Photosynthesis persists for more than a week in starved animals. *Elysia (Tridachia) crispata, Bosellia mimetica, Limapontia depressa,* and *Costasiella ocellifera* fall in this category.

In the discussion of phylogenetic patterns below, we have followed a consensus of familial relationships based on recent works of several authors. Clark & Busacca (1978) constructed a phylogeny based upon papers by Boettger (1963), Baba (1966), and Kay (1968), and showed that an adaptive radiation in ascoglossan diets has closely paralleled anatomical radiation. In this pattern, primitive ascoglossans feed upon *Caulerpa* (as shown by Kay, 1968), and progressively more advanced taxa feed on other Siphonales, Siphonocladales, Cladophorales, and then a variety of other foods. Following Gascoigne's (1985) revision, we have reduced the number of Conchoidea families to three. Relationships of stiligeroid families were derived by Gascoigne (1976) from reproductive anatomy, and by Clark (1982b) based on other anatomical characteristics. The dietary radiation has been confirmed for *Elysia* species with genetic analysis using starch gel electrophoresis (Nuttall, 1987), with *Caulerpa* as the food of primitive species and other algae as foods of advanced species. Additional support for the phylogeny was provided by Clark & DeFreese (1987) based on habitat characteristics.

When the six levels of kleptoplasty are considered together with familial relationships, a pattern begins to emerge (Figure 1). The first indication of kleptoplasty—the retention of non-functional plastids—occurs in shelled ascoglossans, whereas functional plastids appear in most elysiacean (parapodium-bearing) families and irregularly among species in the stiligeroid (cerata-bearing) families. Functional kleptoplasty appears to be a primitive character among elysiids, with secondary loss among species that have adopted unusual diets (*Elysia serca, E. catulus,* and *E. evelinae*). Among the stiligeroid families, highly functional plastids appear among more primitive families (Caliphyllidae and Costasiellidae) feeding upon Siphonales and Dasycladales. With increasing ecological and dietary specialization, forms of kleptoplasty appear to progressively weaken. Thus, in the Hermaeidae, *Hermaea bifida* shows well-developed functional kleptoplasty (level 5), while *H. cruciata* has level 3 retention, and *Aplysiopsis smithi* (Marcus, 1961) (Greene, 1970b) and *A. zebra* have non-functional retention (level 2). Among the Stiligeridae, most species have non-functional retention (levels 2 and 3), though functionality may appear in species that feed on primitive foods, such as *Placida kingstoni* on *Bryopsis.* However, some other species, utilizing Siphonocladales (*Ercolania coerulea* on *Dictyosphaerium*), do not maintain functional plastids. This suggests that there are taxon-specific factors that need to be identified. Possibly the benefits of kleptoplasty are incongruent with the opportunistic growth strategies characteristic of most stiligerids and hermaeids (Clark, 1975; Clark & DeFreese, 1987), and the physiological demands of functional kleptoplastids (Clark *et al.,* 1979; Hinde & Smith, 1975) may interfere

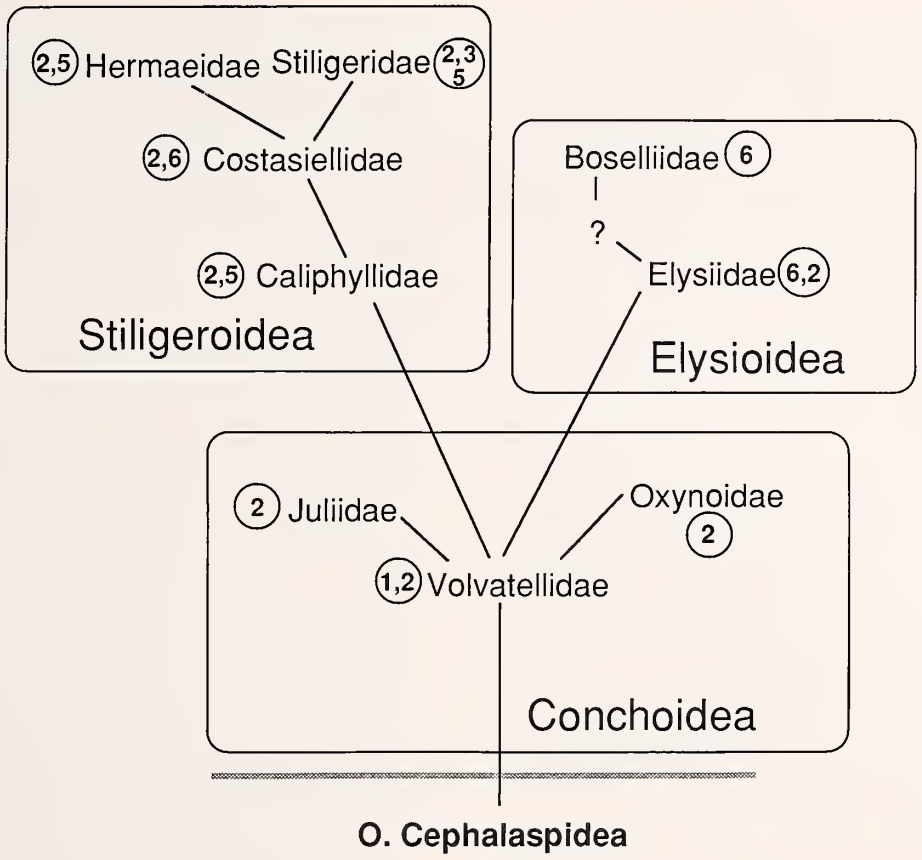

Figure 1

Distribution of the six levels of kleptoplasty in relation to provisional phylogeny of the Ascoglossa. The phylogeny is based on anatomical, dietary, and genetic analyses by Clark & Busacca (1978), Gascoigne (1985), and Nuttall (1987).

with rapid growth. As previously noted (Muscatine & Greene, 1973), the Cladophorales are structurally unsuitable for kleptoplasty, which explains the non-functionality in most Stiligeridae and in *Aplysiopsis,* which feed primarily on this group.

The most primitive ascoglossan, *Ascobulla,* does not retain plastids at all. However, these mollusks normally live below the sediment surface, without light, where kleptoplastids would be useless. During brief periods in which *Ascobulla* crawls on the sediment surface (DeFreese, 1987), retention of pigmented plastids might also increase predation. The remaining conchoidean species are all epialgal, and all exhibit level 2 or 3 (short- or medium-term, nonfunctional) retention. This relationship probably functions in nutritional homochromy, as intact plastids provide cryptic coloration virtually identical to that of the host alga. Molluscan intracellular digestion and the resistant plastids of siphonalean algae (Giles & Sarafis, 1972) are preadaptive characteristics that probably favored early appearance of this level in epifaunal species. This level of kleptoplasty should be considered a plesiomorphic trait, preadaptive to development of functionality among shellless clades. On an anatomical level, the division of plastid diverticular cells into two types, one of which retains plastids, occurs among volvatellids and all higher families (Stirts, 1980).

It is unclear why conchoidean species did not evolve photosynthetically functional kleptoplasty. However, the presence of a shell seems likely involved in this limitation. One possibility is that calcium metabolism and carbonate equilibria are somehow involved. For example, metabolically generated CO_2 is used in molluscan shell deposition (Wilbur, 1964), and metabolism keyed toward shell deposition may limit photosynthetic rate by reducing carbonate availability. Cladohepaty (branching of the digestive gland) seems a necessary feature for photosynthetic function, because this feature occurs in all species with functional plastids, but is not sufficient, because partial cladohepaty occurs in both *Volvatella* and the Juliidae (Clark & Busacca, 1978; Clark, 1982a). The Oxynoidae (including *Lobiger*) are all holohepatic. Cladohepaty is plesiomorphic to both the stiligeroid and elysioid lines

and may also be preadaptive to photosynthetically functional kleptoplasty.

Precise relationships between Volvatellidae, primitive Elysioidea (parapodium-bearing taxa), and primitive Stiligeroidea are presently unclear. However, functional plastid retention appeared early in both the elysioid and stiligeroid lines, occurring in caulerpivorous elysiids and in caliphyllids feeding on siphonocladalean algae. Both these dietary patterns are apparently plesiomorphic in their respective clades (Clark & Busacca, 1978; Clark & DeFreese, 1987; Nuttall, 1987). However, this does not solve the problem of the origin of functional kleptoplasty, for we do not know whether the Stiligeroidea and Elysioidea had a common shell-less ancestor, in which function first appeared, or whether these clades were derived separately from shelled, cladohepatic forms (probably volvatelloid), with convergent evolution of functional kleptoplasty in each line. Because the two major preadaptive changes, cladohepaty and supportive diverticular cells, appear precursorily in two families of Conchoidea, the change between non-functional and functional kleptoplasty may have involved a very small genetic change, such as partial suppression of immune recognition, or translocation of a few genes from plastid/plant to animal genome. Clark & DeFreese (1987) suggested that functional plastid retention may have increased fitness among early shell-less forms by compensating for difficulty in feeding on calcified Siphonales.

Kleptoplastic abilities of two families remain uninvestigated: Platyhedylidae and Gascoignellidae. These highly modified shell-less forms have uncertain relationships with other families, and knowledge of their diets and kleptoplastid retention capabilities might clarify these. *Gascoignella aprica* Jensen, 1985, the only known gascoignellid, has dark green diverticula, but these are shielded by melanic pigment, a character usually associated with non-functional plastids, as in the black form of *Limapontia depressa* (Hinde & Smith, 1974), and in *Ercolania fuscata* and *Elysia catulus* (present study).

Considered at the generic level, and excepting the primitive shelled species, Greene's (1970b) perception of widespread distribution of functional kleptoplastids is probably the most appropriate view. The ability to maintain functional kleptoplastids occurs in most shell-less genera (though it may be absent in some species of a genus and among ecotypes). Its absence may be related to inappropriate plastid structure, and such other factors as light, temperature (Stirts & Clark, 1980), and life-history strategies (Waugh & Clark, 1986; Clark & DeFreese, 1987). The widespread occurrence of functional kleptoplasty among Elysiidae should be considered primarily the result of retention of an evolutionarily conservative diet of siphonalean algae, and not an evolutionarily advanced condition.

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