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FISH Labeling Reveals a Horizontally Transferred Algal (*Vaucheria litorea*) Nuclear Gene on a Sea Slug (*Elysia chlorotica*) Chromosome

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Abstract. The horizontal transfer of functional nuclear genes, coding for both chloroplast proteins and chlorophyll synthesis, from the food alga *Vaucheria litorea* to the sea slug *Elysia chlorotica* has been demonstrated by pharmacological, polymerase chain reaction (PCR), real time PCR (qRT-PCR), and transcriptome sequencing experiments. However, partial genomic sequencing of *E. chlorotica* larvae failed to find evidence for gene transfer. Here, we have used fluorescent *in situ* hybridization to localize an algal nuclear gene, *prk*, found in both larval and adult slug DNA by PCR and in adult RNA by transcriptome sequencing and RT-PCR. The *prk* probe hybridized with a metaphase chromosome in slug larvae, confirming gene transfer between alga and slug.

Introduction

Kleptoplasty, the process of phagocytosis, sequestration, and utilization of algal chloroplasts by certain digestive cells in some species of herbivorous sea slugs (Sacoglossa), is a well-known phenomenon that has been studied by many investigators for 50 years or more (reviewed by Pierce and Curtis, 2012). The symbiotic plastids, kleptoplasts, can photosynthesize inside the slug cell for varying periods, from hours to months, depending upon the slug species, providing at least some source of energy for the animal. The longer-lived associations between the captured plastids and the slug involve various behavioral, morphological, and biochemical adaptations to maintain the chloroplasts in the absence of the mechanisms of organelle renewal that are present in the algal cell (Pierce and Curtis, 2012).

Of particular interest is the growing evidence that *Elysia chlorotica* (Gould 1870), a slug species with one of the longest (9 mon or more) maintained associations with chloroplasts from its algal food, *Vaucheria litorea* (C. Agardh 1823), has somehow acquired functional algal nuclear genes. The products of these transferred genes help with the long-term maintenance of plastid function within the slug cell. Chloroplast reproduction has never been found in *E. chlorotica* (or any other sacoglossan) (Pierce and Curtis, 2012), and the larval stages feed on unicellular algae (West *et al*., 1984). So, once metamorphosis to the adult form occurs, each generation of adult slugs must take up chloroplasts anew. Inside the adult *E. chlorotica* digestive cells, chloroplast proteins are synthesized for months after plastid uptake (Mujer *et al*., 1996; Pierce *et al*., 1996; Green *et al*., 2000), and many of those are nuclear-encoded in the slug cell (Hanten and Pierce, 2001; Rumpho *et al*., 2009). Evidence for the presence of algal nuclear genes coding for chloroplast proteins in DNA from both *E. chlorotica* adults and larvae has been reported from polymerase chain reaction (PCR) experiments (Pierce *et al*., 2007; Rumpho *et al*., 2008, 2009; Schwartz *et al*., 2010) as well as real time PCR (qRT-PCR) and transcriptome sequencing of RNA from *E. chlorotica* adults (Soule and Rumpho, 2009; Pierce *et al*., 2012). Therefore, all of these data agree that several dozen algal nuclear genes have been transferred from the alga to slug cells, have been integrated into the slug cell biology, are transmitted to the next generation of slugs, and then are actively transcribed and translated in the animals. However, genome sequencing of *E. chlorotica* "egg ribbons," which

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are actually unhatched larvae, failed to find any algal sequences (Bhattacharya *et al*., 2013). This negative result was used to dismiss all of the previous positive results, including those with larvae, that demonstrated horizontal gene transfer between *V. litorea* and *E. chlorotica*, although Bhattacharya *et al*. (2013) "stress[ed]" that their results did not "prove" the absence of algal genes in the slug genome. Instead, alternative hypotheses were proposed to explain the mechanism of long-term chloroplast maintenance in *E. chlorotica* without a gene transfer event incorporated into the germ line, including the possibility of extrachromosomal DNA fragments taken up during feeding and missed in the adult sequencing efforts (Bhattacharya *et al*., 2013). However, the unhatched embryos do not have chloroplasts and have not fed, so the source of the PCR-identified algal sequences in the larval DNA found in earlier studies cannot be from feeding. Furthermore, if extrachromosomal DNA were the source of the transferred algal genes in the adults, it would require the uptake of the correct DNA fragments by every cell in every slug in every generation.

Since a negative result from genomic data will always be subject at least to "not enough data" and "not in the database" criticisms, we have used a different approach to test the *E. chlorotica* genome for the presence of algal genes. Instead of just more genome sequencing, we used fluorescence *in situ* hybridization (FISH) and fluorescent microscopy to test chromosomes from pre-hatched *E. chlorotica* larvae (which have never been exposed to any algae) for the presence of algal sequences. We found that a gene probe made with the coding sequence of a *V. litorea* nuclear gene, *prk*, bound to one of the chromosomes in the unhatched larva of *E. chlorotica,* confirming the positive gene transfer results found by most of the previous studies.

Materials and Methods

Sources and culture of slugs and algae

Specimens of *Elysia chlorotica* were collected from a salt marsh near Menemsha on Martha's Vineyard, Massachusetts, and shipped overnight to our laboratory in Tampa, Florida. The sea slugs were kept at 10 °C in aerated aquaria containing sterilized, 1000 mosm, artificial seawater (ASW; Instant Ocean) on a 14:10-h light/dark cycle. The slugs were starved for at least one month before use under these conditions. Sterile laboratory cultures of *Vaucheria litorea* were maintained in 250 mosm ASW containing a modified F/2 medium (Bidwell and Spotte, 1985) and transferred into fresh medium bi-monthly. The algal culture was grown in an incubator at 20 °C on a 14:10-h light/dark cycle.

Localization of transferred algal nuclear genes on Elysia chlorotica *chromosomes*

Metaphase chromosomes in cells of developing *E. chlorotica* embryos were examined for the presence of algal genes by using FISH labeling and microscopy. Gastrula-stage embryos were tested since many more dividing cells are present in them than in later developmental stages or adults. The slugs were induced to produce egg masses by moving them into aquaria at room temperature (RT), still under the 14:10 light/dark cycle (Harrigan and Alkon, 1978). The aquaria were monitored daily for egg masses, which were collected, rinsed with ASW, and placed into small culture dishes containing ASW. After the embryos had developed to the gastrula stage at RT, the egg masses were gently passed through a 22-ga needle several times to remove the jelly coat and free the egg capsules. The capsule suspension was centrifuged at $1500 \times g$ for 5 min at RT, the supernatant was decanted, and the dissociated jelly coat layer was removed from the top of the pellet using a pipet tip. The egg capsules were transferred to ASW containing 1.0 mmol l^{-1} colchicine (Sigma-Aldrich, St. Louis, MO) and incubated for 1 h at RT in the dark, with slight agitation, to arrest all cell division at metaphase. The suspension was then centrifuged at $1500 \times g$ for 5 min and the supernatant was decanted. The egg capsules were resuspended in 250 mosm ASW and incubated for 20 min at RT to swell the chromosomes, then centrifuged again at $1500 \times g$; the pellet containing the egg capsules was fixed with methanol/acetic acid (3:1) (fixation solution) and incubated for 20 min at RT. This fixation step was repeated two additional times. Finally, the fixed gastrulae were stored in the fixation solution overnight at −20 °C prior to microscope slide preparation (modified from Rønne, 1989).

Chromosome preparation for karyotype analysis and FISH labeling

The microscope slide preparations of chromosomes used for karyotyping or FISH analysis required about 100–150 fixed gastrulae per slide to obtain at least 10 isolated metaphase chromosome spreads for analysis. The gastrula-containing *E. chlorotica* egg capsules described above were resuspended in fresh fixation solution, incubated at RT for 5 min, and then centrifuged at $12{,}000 \times g$ for 5 min at RT. The egg capsules were broken open, releasing the metaphase chromosomes, by resuspending them in acetic acid/water (1:1), incubated for 5 min at RT, then, using a pipet tip that was angled ~45° to the slide surface, the suspension was spread onto pre-warmed (42 °C) glass microscope slides and dried for 1 h at 42 °C. Slides were brought to RT and used immediately for karyotyping or aged for 24 h prior to their use for FISH analysis.

Karyotype analysis: Elysia chlorotica

Elysia chlorotica chromosome number and morphology were determined using fluorescence microscopy. The chromosomes were visualized by staining their DNA with 4′,6-diamidino-2-phenylindole (DAPI) antifade mounting buffer

(Invitrogen, Carlsbad, CA) and viewed using an inverted fluorescent microscope (Axiovert 200, Zeiss, Oberkochen, Germany) equipped with a DAPI optical filter and an 100×/1.40 Plan-Apochromat oil objective. Chromosomes were counted, classified, and paired, based on their size and morphology, from about 50 well-isolated, metaphase chromosome spreads acquired from images of five different chromosome preparations captured using a digital camera (Hamamatsu IEEE1394 Orca-Era) and Velocity 6.1.1 software. Images shown in the Results section were cropped and sharpened using Photoshop Elements (ver. 9).

FISH localization of transferred algal genes

Elysia chlorotica metaphase chromosomes were tested with FISH labeling utilizing a gene probe designed to label the *V. litorea* nuclear gene *prk*, which encodes the Calvin cycle enzyme phosphoribulokinase. *Prk* was chosen from among the variety of possible genes because several previous studies have found evidence for its horizontal transfer between *V. litorea* and *E. chlorotica* using both polymerase chain reaction (PCR) experiments in adult and larval slug DNA (Rumpho *et al*., 2009; Schwartz *et al*., 2010) and adult slug transcriptome sequence data analysis (Pierce *et al*., 2012). Also, *prk* encodes a protein used exclusively in the Calvin cycle and has no known homolog in non-photosynthetic organisms.

A 1218-bp, *V. litorea prk*-targeting probe was synthesized using native sequence (GENBANK accession #AF3366986) (Schwartz *et al*., 2010) and labeled with the hapten digoxigenin (DIG) so that it could be detected with, and amplified by, FISH labeling and fluorescent microscopy. Genomic DNA (gDNA) and RNA were purified from *V. litorea,* and cDNA was synthesized using previously described techniques (Schwartz *et al*., 2010). Amplicons of *prk* (1.2 kb using *V. litorea* cDNA) were obtained by using PCR, which used a reaction mixture containg 100 ng of gDNA or cDNA, 12.5 pmol of primer, 200 μmol l^{-1} dNTP mix (Roche Applied Science, Indianapolis, IN), and 1.25 units of IDProof DNA polymerase (ID Labs, London, Ontario, Canada). The reactions were initially denatured for 2 min at 94 °C, then underwent 35 cycles of 30-s denaturation at 94 °C, 30-s annealing at a temperature 5 °C below the melting temperature of the primers, and then a 1-min extension at 72 °C. The reaction products were electrophoresed on a 1% agarose gel; the amplicons were extracted from the gel and then purified (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA). The purified amplicons were cloned (TOPO TA cloning kit, Invitrogen, Grand Island, NY) as per manufacturer's instructions. Individual clones were sequenced (Eurofins MWG Operon, Huntsville, AL) to verify sequence integrity. The bacterial colonies were grown at 37 °C overnight in LB media containing 50 μg/ml kanamycin. The insert-containing plasmid vectors were purified (Wizard Plus SV Minipreps DNA Purification Kit, Promega, Madison, WI) and used as the templates for FISH probe synthesis. FISH probes were synthesized and simultaneously labeled with digoxigenin (DIG) (PCR DIG Probe Synthesis Kit, Roche Applied Science, Indianapolis, IN) as per manufacturer's instructions.

prk primers:
Forward: 5′ATGCTCGTACATAGTCTAATTC 3′
Reverse: 5′TTATGCAGCCTCGGCTTGG 3′

Hybridization of prk *probe with slug chromosomes*

The chromosome spreads described above were pretreated to increase assay sensitivity and specificity using methods modified from Henegariu *et al*. (2001) and Vitturi *et al*. (2000), determined in preliminary experiments. The dried chromosomes on the microscope slides were rehydrated in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at 37 °C. Then the slides were incubated for 7 min in 0.005% pepsin/0.01N HCl (pH 2.0) that was pre-warmed to 37 °C to digest any cytoplasmic proteins that could cause non-specific probe binding. The pepsin digestion was stopped by immediately raising the pH back to 7.4 by immersing the samples in PBS and incubating them for 5 min at RT. The slides were incubated for 10 min in PBS/50 mmol l^{-1} $MgCl_2$ at RT, then post-fixed in PBS containing 50 mmol l^{-1} $MgCl_2$ and 1% formaldehyde to maintain chromosome morphology. They were then washed in PBS for 5 min with gentle agitation, dehydrated through an ethanol series (70%, 95%, 100%, 5 min each concentration), then dried for 15 min at RT. The DNA in the chromosomes was denatured by incubation with 200 μl of 2× saline-sodium citrate buffer (300 mmol l^{-1} NaCl and 30 mmol l^{-1} $Na_3C_6H_5O_7$, pH 7.0; 2× SSC) containing 70% deionized formamide for 5 min at 70 °C. The slides were briefly rinsed in 2× SSC and then dehydrated through an ethanol series.

The DIG-labeled *prk* probe was then bound to the chromosomes and viewed with a fluorescent microscope. A hybridization solution containing the *prk* probe was prepared (2× SSC, 45% deionized formamide, 5% dextran sulfate, 1.75 μg of mouse cot-1 DNA [Invitrogen, Grand Island, NY] and 1.7× Denhardt's solution containing ~20 ng of DIG-labeled *prk* probe), denatured for 7 min at 76 °C in a thermocycler, then snap-cooled in an ice bath for 2 min. Then a 15-μl drop was pipetted onto the denatured chromosomes on the microscope slide, covered by a 20 mm × 20 mm cover slip, and the edges sealed with rubber cement (modified from Vitturi *et al*., 2000). Probe hybridization with the chromosomes was achieved by incubation in a humidified chamber for 17–20 h at 35 °C. This temperature was chosen as the result of preliminary experiments performed to determine an incubation temperature that minimized non-specific probe binding. Then the coverslips were removed and unbound *prk* probe was removed from the

slides by washing for 5 min with agitation: once in 2× SSC (37 °C), three times in 2× SCC containing 45% formamide (37 °C) and then two times in 0.5× SCC (65 °C). All wash steps in the series were performed in a Coplin jar containing 40 ml of each washing solution.

The chromosomes were prepared for probe detection by a series of RT equilibration washes performed once each in the following: 2× SSC, PBS, and finally in PBS/0.1% Tween 20. DNA-free areas on the slide preparation were blocked in blocking solution (Roche Applied Science, Indianapolis, IN) for 1 h at 37 °C, then rinsed in PBS/0.1% Tween 20. Finally, the *prk* probe was labeled for fluorescence detection by first incubating the chromosomes with blocking solution containing 250 ng/ml mouse monoclonal anti-DIG (Roche Applied Science, Indianapolis, IN) for 1 h at 37 °C, then washing three times for 5 min at RT in PBS/0.1% Tween 20 with agitation. Next, the slides were incubated with blocking solution containing 2.5 μg/ml anti-mouse AlexaFluor488 or AlexaFluor594 (Invitrogen, Grand Island, NY) for 45 min at 37 °C and washed three times in a foil-wrapped Coplin jar for 5 min at RT in PBS/0.1% Tween 20 with agitation. The slides were dehydrated through an ethanol series and allowed to dry for 20 min in the dark. DAPI antifade mounting buffer (Invitrogen, Grand Island, NY) was pipetted onto each slide to counterstain the chromosomes (blue) and provide contrast to the probe (green or red) and covered with a 30 mm × 30 mm coverslip. The chromosomes were then viewed with a confocal microscope (UltraVIEW ERS Spinning Disk, PerkinElmer, Waltham, MA) or an inverted fluorescent microscope equipped with standard optical DAPI, FITC, and rhodamine filters, using a 100×/1.40 Plan-Apochromat oil objective. Once the labeled probe was detected, the chromosomes were viewed using gas lasers (Krypton 561 and/or Argon 488/515) using the following parameters: DAPI—excitation 405-nm, laser emission 485 nm (W60), 705 nm filter (W90); Alexa Fluor488—excitation 488 nm, laser emission 527 nm filter (W55); and Alexa Fluor594—excitation 640 nm, laser emission 485 nm (W60), 705 nm filter (W90). Images were acquired, analyzed, and processed using the same camera and software used for *E. chlorotica* karyotype analysis described above. This procedure was repeated on preparations from 13 different groups of larvae from different egg masses (See Table 1).

Controls to verify prk *chromosome labeling specificity*

Several control procedures were performed to confirm reagent and chromosome quality as well as the specificity of *prk* labeling of the *E. chlorotica* chromosome.

Reagent and chromosome quality in the various chromosome slide preparations was verified by using the FISH procedures described above and substituting a 960-bp probe designed to target the actin gene. The *actin* probe was synthesized from *E. chlorotica* gDNA, as described above for the *prk* probe, using the following primers:

actin primers:
Forward: 5′AGGGTGTCATGGTTGGTA 3′
Reverse: 5′GATCCACATCTGCTGGAA 3′

Actin was chosen because of its ubiquity, sequence conservation, and presence in multiple, similar isoforms in most species (Zappula *et al.,* 2005).

Prk binding specificity was tested in several ways. First, the FISH procedure was performed on chromosome slide preparations with all components except the *prk* probe to ensure that the antibody labeling system was not non-specifically binding to the chromosomes. Second, a gene probe for an algal sequence that should not bind to the slug DNA was tested. The gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) was used for this experiment as it is a chloroplast-encoded gene in *V. litorea* (Curtis *et al*., 2006, 2007). A 631-bp *rbcL* probe was synthesized from *V. litorea* gDNA, using the methods described above and the following primers, and substituted for the *prk* probe in the FISH procedure described above. Both of these controls were tested a minimum of seven times on seven different metaphase chromosome preparations and along with each *prk* labeling run (See Table 1).

rbcL primers: Forward: 5′AATGGATAAATTTGGACG 3′
Reverse: 5′ACGTAATGCTGCCCAATCC 3′

Prk binding specificity for *E. chlorotica* chromosomes was also tested using chromosomes from *Aplysia californica* (Cooper 1863), a non-kleptoplastic, herbivorous slug, relatively phylogenetically close to *Elysia*. Although the chromosome number has been reported as $n = 17$ for several aplysiidaean species including *Aplysia benedicti* (Elliot 1899) (= *Aplysia dactylomela* Rang 1828) (Patterson, 1969), we were unable to locate a karyotype for *A. californica* in the literature, so to be sure all chromosomes were accounted for in our experiments, a karyotype analysis was done before the *prk* binding tests.

Karyotype: Aplysia

Aplysia californica egg strands were kindly provided by Thomas Capo from the National *Aplysia* Resource Facility at the Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, Florida. Freshly deposited egg strands were shipped to Tampa overnight and, upon arrival, were rinsed in 1000 mosm ASW; embryos were allowed to develop to the gastrula phase. The egg strands were transferred to 1000 mosm ASW containing 1.0 mmol l^{-1} colchicine and incubated for 6 h at RT in the dark with slight agitation. After the incubation, gastrulae were liberated from the jelly coat and egg capsules by finely mincing the egg strands with a razor blade. The liberated gastrulae

were centrifuged at 1500 × *g* for 5 min at RT. Chromosomes were swelled by resuspending the gastrulae in 500 mosm ASW and incubating them at RT for 20 min. The *A. californica* gastrulae were collected by centrifugation, then fixed, stored, and karyotyped using the same procedures described above for the *E. chlorotica* preparations. Once the karyotype was established, the chromosomes were tested for *prk* binding as described below.

Nuclear prk *binding:* Vaucheria

A final control experiment was to ensure that the *prk* probe would label *V. litorea* DNA. There is almost no information in the literature about *Vaucheria* chromosomes in general, and *V. litorea* in particular. The filaments of *V. litorea* are coenocytic with nuclei scattered along the length, but nuclear division occurs at the growing tip, usually in the dark. The nuclei are small, and the chromosomes are tiny and may not condense during nuclear division. The older literature indicates possibly 5 chromosomes in some of the freshwater *Vaucheria* species, but other chromosome numbers have been reported also (Gross, 1937). Electron microscopy of nuclear division in *V. litorea* found spindles but no recognizable chromosomes (Ott and Brown, 1972). As a result, we tested *prk* labeling using *V. litorea* nuclei as described below.

Preparation of Vaucheria litorea *nuclei*

Vaucheria litorea filaments were rinsed in algal culture medium and blotted to remove excess liquid. The filaments were placed in cold (4 °C) homogenization buffer (250 mosm ASW containing 250 mmol l^{-1} sucrose, 10 mmol l^{-1} $MgCl_2$ and 1 mmol l^{-1} DTT), minced with a razor blade, then homogenized in a glass homogenizer. The algal suspension was filtered through six layers of cheesecloth to remove large clumps, then the filtrate was centrifuged at 500 × *g* for 5 min at RT to pellet the nuclei but leave other cellular components in the supernatant. The supernatant was discarded and the pellet was resuspended in 250 mmol l^{-1} sucrose containing 10 mmol l^{-1} $MgCl_2$. The suspension was applied to a two-step sucrose gradient (equal volumes of 1 mol l^{-1} and 0.5 mol l^{-1} sucrose) and centrifuged at 1000 × *g* for 5 min at RT to concentrate the nuclei (modified from Luthe and Quatrano, 1980). The upper, slightly green layer at the top of the 0.5 mol l^{-1} sucrose gradient step was collected, washed two times in 250 mosm ASW, then resuspended in fixation solution and incubated for 20 min at RT. This fixation step was repeated two additional times and the nuclei stored in the fixation solution overnight at −20 °C prior to slide preparation.

Prk *binding on* Vaucheria litorea *nuclei and* Aplysia californica *metaphase chromosomes*

One hundred *V. litorea* nuclei or 100–150 fixed *A. californica* gastrulae per slide were needed to obtain at least 10 single nuclei not associated with any cellular debris or at least 10 well-spaced metaphase chromosome spreads. *V. litorea* nuclei and liberated *A. californica* gastrulae were resuspended in fresh fixation solution and incubated at RT for 5 min. The suspensions were transferred to microfuge tubes and centrifuged at 12,000 × *g* for 5 min at RT. The pellets were resuspended in fixation solution and dropped onto a RT glass microscope slide by using a pipet tip; the fixation solution was allowed to evaporate. The *A. californica* chromosomes were spread by adding 20 μl of 1:1 acetic acid/water. Then both the nuclei and chromosome slides were transferred to a 42 °C incubator and dried for 1 h. They were brought to RT and aged for 24 h prior to their use for FISH analysis.

The *A. californica* chromosome pre-treatment, *in situ* hybridization, detection, and microscopy experiments were performed using the same procedures as described above, except that an *actin* probe synthesized using *A. californica* gDNA was substituted for the *E. chlorotica actin* probe to verify chromosome and reagent quality.

Results

An ideal result from these experiments would be for the various gene probes to bind at a similar location on each sister chromatid of two morphologically similar—therefore, homologous—chromosomes in the middle of a well-isolated chromosome spread that accounted for all of the diploid number of chromosomes, all of which were easily photographed. In practice, that almost never happened in a single preparation, as the result of degree of chromosome coiling, physical position of the chromosome, intermingling of chromosomes from neighboring nuclei, loss of chromosomes, length of probe sequence, working distance, and field of view, among other reasons. Many preliminary experiments were performed to maximize the encounters with the fluorescent signal, but an ideal result was rare. Instead, the results described below are a compilation of hundreds of examinations of many dozens of preparations from many egg masses on multiple occasions (Table 1).

Although there are a few large, easily recognized chromosomes in the *E. chlorotica* cell nucleus, more than half of them are quite small (less than 2 or 3 μm), metacentric, and have arms of very similar length (Fig. 1). These smaller chromosomes are very difficult to distinguish from each other. The chromosomes in Figure 1 were assembled showing potential homologous pairs, but possibly members of the smaller pairs should be interchanged. Nevertheless, counts of chromosome numbers from 50 isolated metaphase smears on slides from 10 different preparations routinely

Table 1

Summary of the results of prk *FISH labeling of* Elysia chlorotica *chromosome preparations*

Date of egg mass[1]	Reagent Control	Negative Control *rbcL* probe	Positive Control *actin* probe	*prk* Probe
5/14/13	Negative	Negative[2]	Positive[2]	Positive, 100%[2]
5/25/13	Negative	Negative	Positive	Positive[3], 100%
5/18/13	Negative	Negative	Positive	Positive, 100%
4/7/13	Negative	Negative	Positive	Positive, 100%
5/30/13	Negative	Negative	Positive	Positive, 100%
6/11/13	Negative	Negative	Positive	Positive, 100%
6/12/13	Negative	Negative	Positive	Positive, 100%
3/4/13	Negative	Negative	Positive	Positive, 100%
6/13/13	Negative	Negative	Positive	Positive, 100%
2/16/13	Negative	Negative	Negative	Negative[4]
2/16/13	Negative	Negative	Negative	Negative[4]
3/8/13	Negative	Negative	Positive	Positive[5], 100%
3/4/13	Negative	Negative	Positive	Positive, 100%

[1] Each egg mass was treated separately on a different microscope slide.
[2] Result (positive labeling/negative), % of labeled chromosomes that were the same morphology.
[3] Fig. 3A.
[4] The positive control (*actin* column) failed in these experiments also, indicating a protocol issue. These were not included in the results.
[5] Fig. 3B.

produced a haploid number of 15 ($2n = 30$). In comparison, among a variety of opisthobranch species, chromosome numbers range from 13 to 17 (Burch and Natarajan, 1967; Thiriot-Quievreux, 2003). Two other species of *Elysia* (*E. amakusana* [Inaba, 1959] and *E. viridis* [Mancino and Sordi, 1964]) each had $n = 17$.

The FISH *actin* probe was always localized on the arms of two, probably homologous, intermediate-sized *E. chlorotica* chromosomes in the metaphase spreads (Fig. 2). It was unusual for all probe binding sites on the chromosomes to be labeled in every preparation (Fig. 2), but the actin label was not found anywhere else among the chromosomes. In all our experiments, the *prk* probe labeled only the arms of two of the tiny *E. chlorotica* chromosomes in the metaphase spreads (Table 1, Fig. 3). Because there are so many of these tiny chromosomes that are not distinguishable from each other with the techniques used here, we cannot say for sure that the same chromosome was labeled in every preparation, but the label never appeared on more than two chromosomes in each nuclear group. So, importantly, a FISH probe for an algal nuclear gene binds to a slug chromosome.

The reagent control experiments were always negative in preparations from all species (Table 1). No chromosome in any preparation was ever labeled without a gene probe present. Similarly, the *rbcL* probe was never observed bound to an *E. chlorotica* chromosome (Table 1). So neither the reagents nor the probes caused non-specific signals under the conditions we used.

The *A. californica* karyotype analysis consistently found 17 chromosomes ($2n = 34$) in metaphase smears from seven different egg masses (Fig. 4). As was the case with *E. chlorotica*, several of the larger chromosomes were easily distinguished by size and centromere position, but some of the smaller chromosomes are quite similar in morphology and require more than DAPI staining to distinguish them adequately. The *actin* probe routinely bound only to two, likely homologous, intermediate-sized *A. californica* chromosomes in the metaphase spreads (Fig. 5). As with the *E. chlorotica* results, it was unusual for all the actin sites to be labeled in every *A. californica* preparation, but no more than two chromosomes were ever labeled. These control experiments ensure that we were looking at all the chromosomes present in *A. californica*, and that the chromosomal DNA was capable of binding the FISH probes. Binding of the *prk* probe was never observed in any of the *A. californica* preparations.

Finally, as expected, the *V. litorea* nuclei were small, 3–4 μm in diameter, with a prominent nucleolus (Fig. 6a). Although we failed to visualize *V. litorea* chromosomes, the *prk* probe labeled the algal nuclei (Fig 6b), sometimes showing two probe-binding sites per nucleus.

Discussion

The FISH labeling presented in the results clearly demonstrates that one of the *Elysia chlorotica* chromosomes has a site that binds a probe made up of the nucleotide sequence for the *Vaucheria litorea* nuclear gene, *prk*. This is the third line of evidence that genes from the slug's algal food species have not only been transferred into the slug cell but have been incorporated into the chromosomes and are now vertically transmitted in the slug germ line. This result is supported by earlier work that located *prk*, and other *V. litorea* nuclear genes as well, in the genomic DNA of both

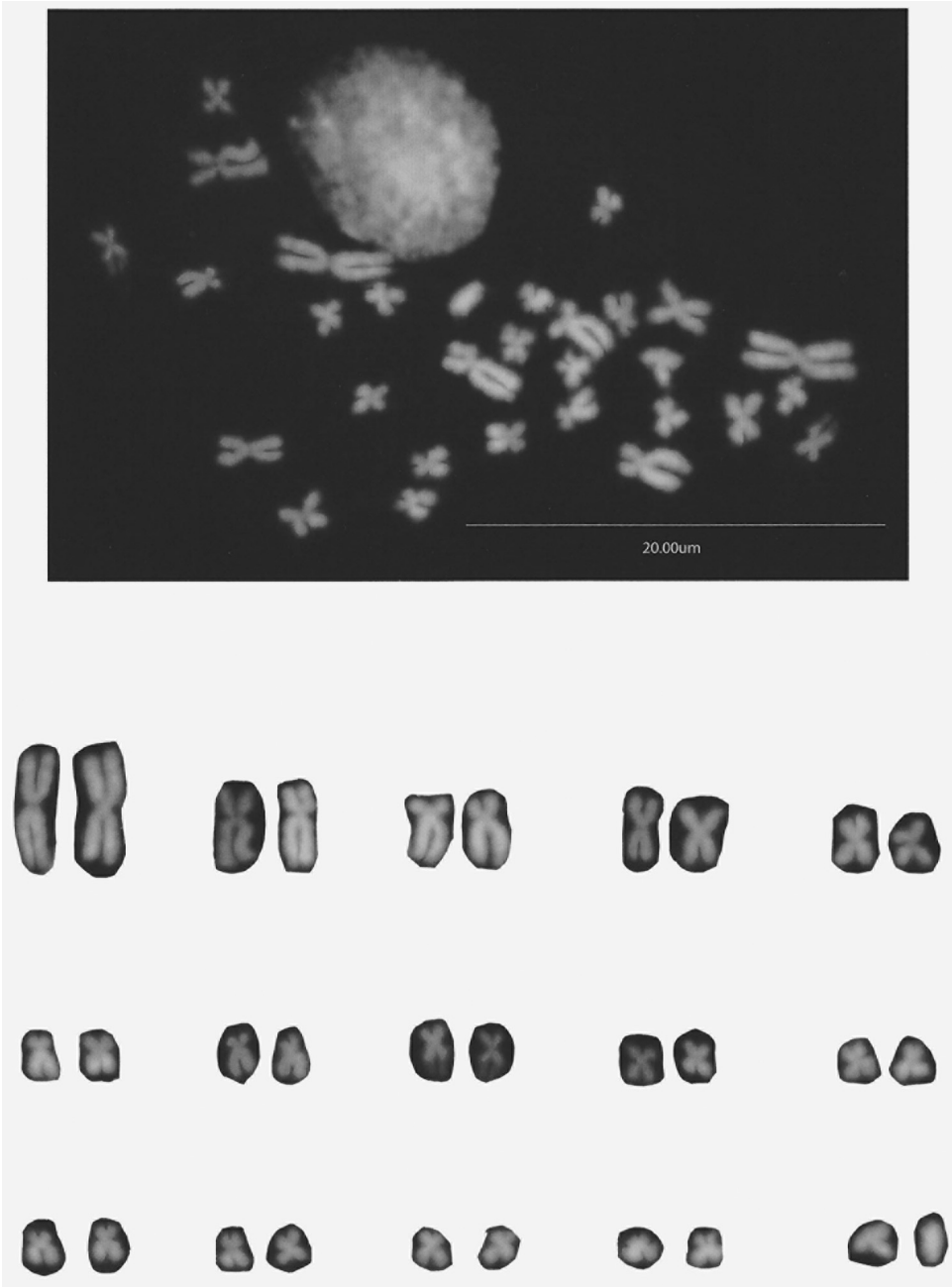

Figure 1. DAPI-stained karyotype of metaphase chromosomes of *Elysia chlorotica*. The chromosomes in top row are readily recognized individually by their size and position of the centromere. The intermediate-sized chromosomes (second row, first four from the left) are recognizable as a group, but not from each other, as is the case with the smallest group (bottom row and right-hand middle row). The round object in the field is a nucleus. This image was taken at 1000× under oil. Scale bar = 20 μm.

E. chlorotica larvae and adults using PCR (Pierce *et al*., 2007; Rumpho *et al*., 2009), in RNA from adult slugs by qRT-PCR (Soule and Rumpho, 2009), as well as in the *E. chlorotica* adult transcriptome (Pierce *et al*., 2012). Two other sequencing efforts failed to find algal sequences in the *E. chlorotica* transcriptome (Pelletreau *et al*., 2011) or the larval ("egg") genome (Battacharya *et al*., 2013). However, those sequencing studies either did not produce a complete genome and used an incomplete *V. litorea* genomic data set (Battacharya *et al*., 2013), or used no *V. litorea* data at all (Pelletreau *et al.,* 2011) for sequence matching purposes. Further, the larval sequencing study (Battacharya *et al*., 2013) pooled DNA from hundreds of individuals, which could have produced assembly issues resulting from

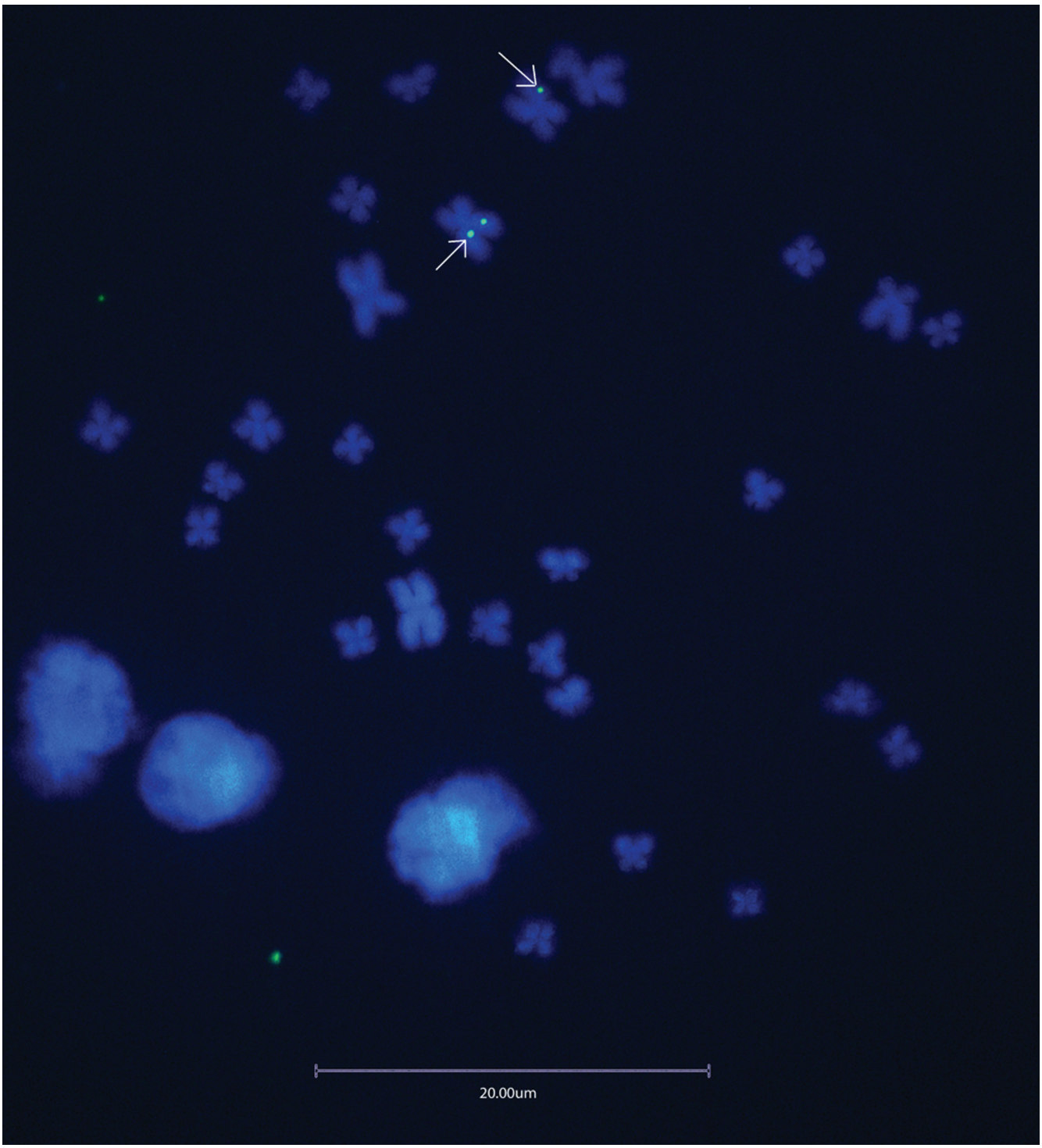

Figure 2. FISH-labeled micrograph of *Elysia chlorotica* chromosomes labeled with the probe for *actin* counter-stained with DAPI. This view contains chromosomes from more than one cell, but does not have a complete set of chromosomes. The probe has labeled both arms of sister chromatids on an intermediate-sized chromosome and one arm of another (arrows), morphologically similar, chromosome. No other chromosome morph was ever labeled with the *actin* probe. The three round objects are intact nuclei. Probes occasionally bound to the microscope slide surface, which is sticky to hold the chromosomes and nuclei in place, and a few are visible here. This image was taken at 1000× under oil. Scale bar = 20 μm.

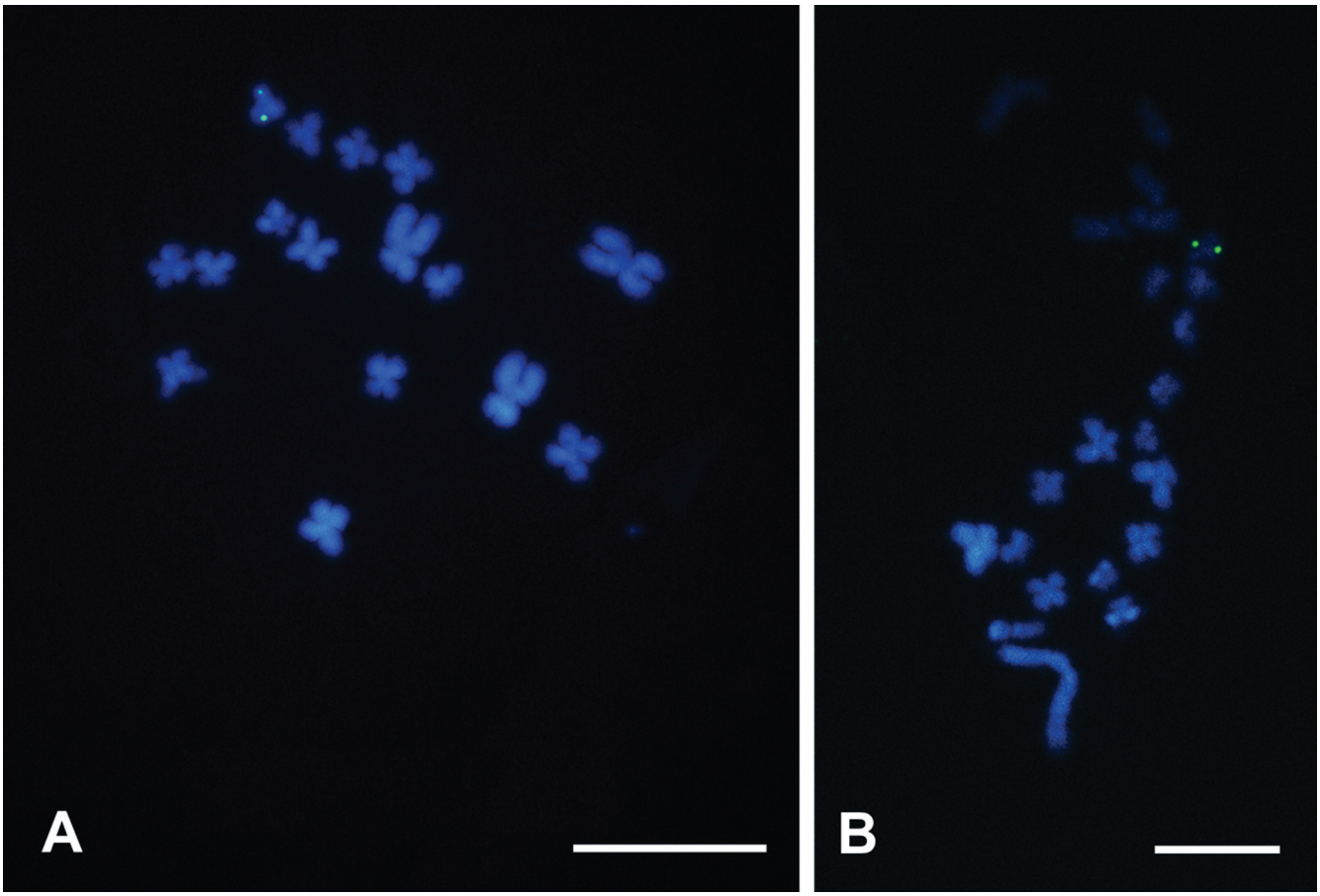

Figure 3. FISH-labeled micrographs of *Elysia chlorotica* chromosomes from two different egg mass preparations (A, B) done on different dates (Table 1), labeled with the *prk* probe counter-stained with DAPI. Several of both the larger and smaller chromosomes are not present in these fields of view. The *prk* probe has labeled the arms of both sister chromatids of one of the smallest chromosomes in both A and B. No other chromosome morph was ever labeled with the *prk* probe. This image was taken at 1000× under oil. Scale bar = 20 μm.

heterozygocity, and analyzed only the unassembled raw reads. Clearly, the FISH technique and the control experiments have produced unambiguous evidence of a transferred algal sequence in the *E. chlorotica* genome.

The mechanism of gene transfer is of interest, but unknown. The phagosomal-based feeding mechanism utilized by gastropods (Owen, 1966) could take up nuclei and other algal cell inclusions, as well as the chloroplasts (Taylor, 1968; Martin *et al*., 2013). Transcriptome sequencing suggests that several dozen algal nuclear genes are expressed in the *E. chlorotica* cell, perhaps indicating that relatively large pieces of algal DNA rather than individual genes were transferred. However, the *E. chlorotica* transcriptome annotation found genes only in the slug RNA associated with photosynthesis proteins and their processing (Pierce *et al*., 2012). A wider variety of algal proteins might be expected to be present if large pieces of algal DNA were involved. Furthermore, although protistan kleptoplasty often involves the presence of algal nuclei or nuclear remnants in addition to the chloroplast within the host cell (Gast *et al*., 2007; Johnson *et al*., 2007; Nowack *et al*., 2011), all attempts to locate algal nuclei or their remnants with molecular or microscopical experiments in several species of *Elysia* have been negative (reviewed in Pierce and Curtis, 2012). So the results so far suggest that pieces of DNA rather than entire chromosomes or individual genes may constitute the transferome. It will require a great deal more FISH testing to learn the chromosomal positions of other algal genes.

The location of algal nuclear genes on the host cell chromosomes further demonstrates the high amount of integration of the chloroplast and the photosynthesis maintenance mechanisms into the *E. chlorotica* cell biology. Although chloroplast reproduction has never been reported in any species of kleptoplastic sacoglossan, a wide array of adaptations for maintaining the chloroplasts are present in the various species, ranging from essentially nothing—chloroplasts are taken up into phagosomes and rapidly digested—to the high level of integration in *E. chlorotica*

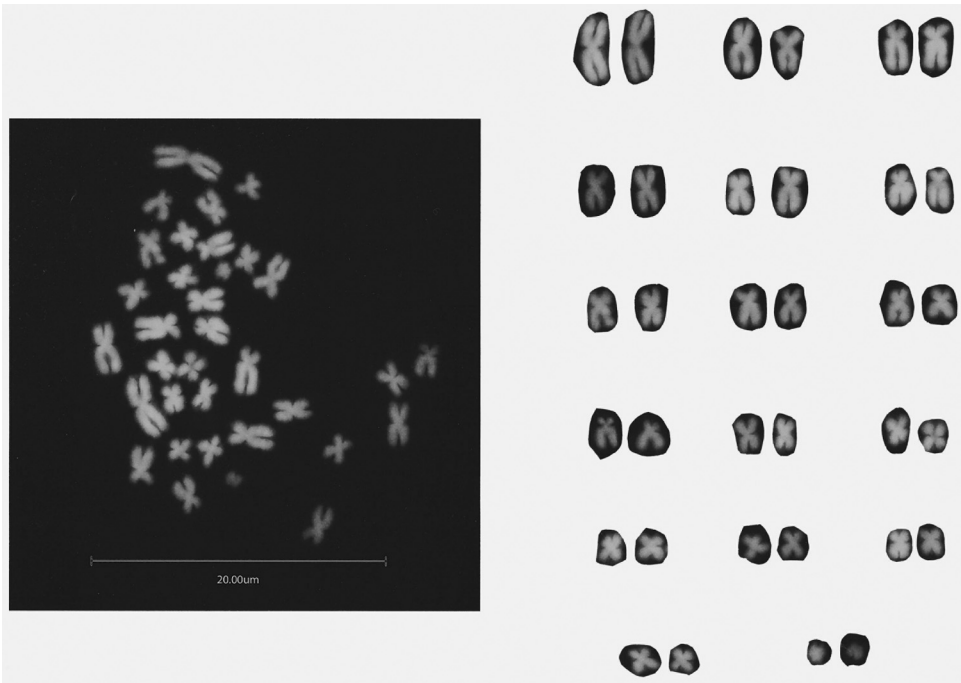

Figure 4. DAPI-stained karyotype of the metaphase chromosomes of *Aplysia californica*. There is less morphological variation between the chromosomes than in *Elysia chlorotica*, but the top two rows are readily identifiable by arm length and centromere position. The smallest chromosome (bottom right) is also easily resolved. Of the remaining, two groups of intermediate-sized chromosomes are evident, which are all morphologically similar by DAPI staining, except for one (fourth row left), which has an acentric centromere. This image was taken at 1000× under oil. Scale bar = 20 μm.

(reviewed in Pierce and Curtis, 2012). Not only do the *V. litorea* chloroplasts persist for 7–8 mon or longer in starved *E. chlorotica*, but they continue to photosynthesize, fix carbon, produce oxygen, synthesize chlorophyll, and be translationally active. Both chloroplast- and nuclear-encoded chloroplast proteins are synthesized, nuclear-encoded transcripts for chloroplast genes are present in the slug cell RNA, and the genes for some of them have been located in genomic DNA of both larvae and adults by PCR (reviewed by Pierce and Curtis, 2012). In addition, while many other species of sacoglossans—including some that are able to keep the chloroplasts for several months, such as *E. timida* (Risso 1818)—are rarely found away from the algal chloroplast source and continually turn over the algal organelles, individual *E. chlorotica* are often found in our field site during seasons-long absences of *V. litorea*. Unlike *E. chlorotica*, *E. timida* seems to have little capacity to sustain photosynthesis for more than a few weeks (Casalduero and Muniain, 2008) although the chloroplasts are present for 2–3 mon, and it also may not have any cellular biochemical support for them. Indeed, transcriptome sequencing of *E. timida* did not find any algal transcripts, although the data set was small and donor algal (*Acetabularia*) genome sequences were only partially available (Wägele *et al*., 2011), so matches might have easily been missed. Other sacoglos-

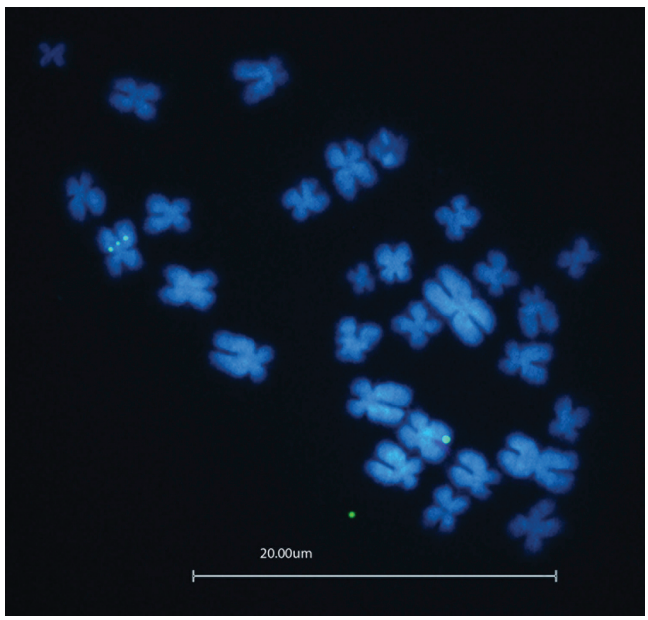

Figure 5. FISH-labeled micrograph of *Aplysia californica* metaphase chromosomes labeled with the *actin* probe and counter-stained with DAPI. Two chromosomes in the field are labeled, one has 3 probes bound to it, perhaps indicating some non-specific binding or binding to another *actin* gene copy. This image was taken at 1000× under oil. Scale bar = 20 μm.

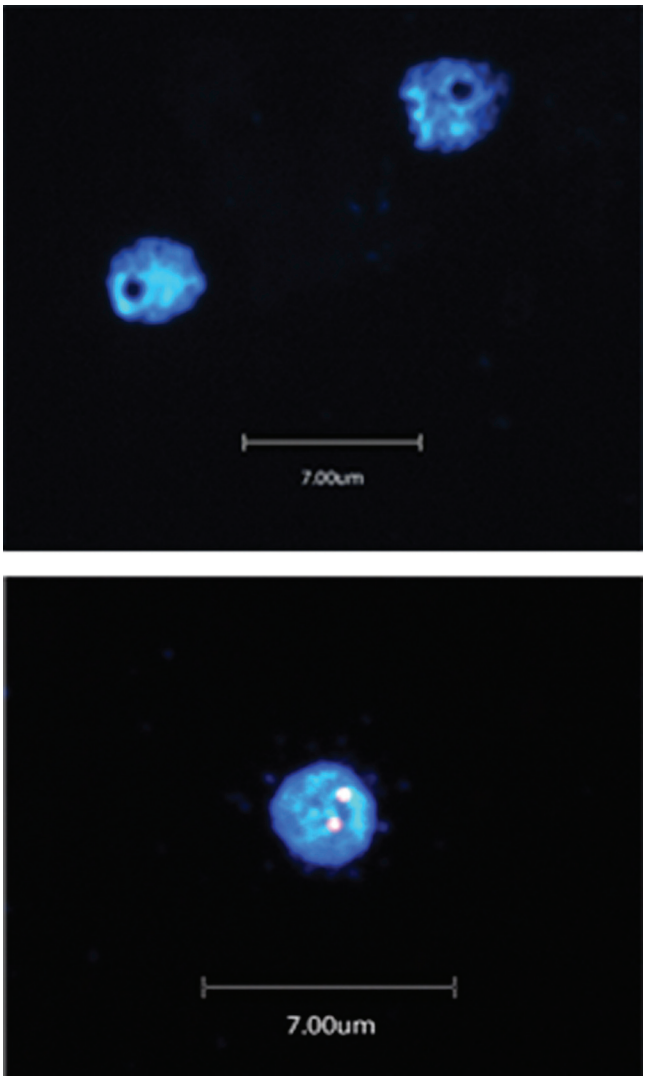

Figure 6. (A, top) DAPI-stained, isolated *Vaucheria litorea* nuclei. The round dark structure, slightly off center in both, is the nucleolus. (B, bottom) FISH-labeled micrograph of an isolated *V. litorea* nucleus stained with *prk* probe. Both images were taken at 1000× under oil and enlarged with the computer. Scale bars = 7 μm.

san species with similar lengths of plastid function longevity as *E. timida* (2–4 mon; namely *E. viridis, E. clarki*) show a variety of specific adaptations in their cell biology to sustain the function of the algal organelle. In some cases, such as *E. clarki*, plastid maintenance mechanisms are sufficient to enable enough photosynthesis to occur for long enough to permit some independence from a food source (Middlebrooks *et al*., 2011, 2012, 2014). Although a few recent accounts based on *E. timida* have discounted the importance of photosynthetic function as well as the longevity and integration of symbiotic plastid function among the sacoglossans as a group (Wägele *et al*., 2011; Christa *et al*., 2013), it is clear that the broad differences in the adaptations among species makes generalizations based on one slug species unwarranted.

All of the research on gene transfer in *E. chlorotica* so far has been descriptive, and another aspect of major significance, about which nothing is presently known, is the underlying cell biology that permits the phenomenon. The transfer of genes between species has been demonstrated in groups other than sea slugs (for example, International Aphid Genomics Consortium, 2010; Nowack *et al.,* 2011; Kent *et al*., 2011), and the integration of foreign organelles into host cells and subsequent gene transfer is the basis of the endosymbiotic theory of the origin of eukaryotic cells. However, the underlying adaptations that permit long-term survival of a foreign organelle and the integration of a functional gene and gene product, as well as its transmission to future generations, are mostly unknown. The foreign gene must not only get to the host nucleus and find its way into the expression process, but the gene product must be appropriately synthesized, folded, transported, targeted, inserted, and unfolded, all in a cytoplasm that should not be biochemically equipped to do all that. Still, those kinds of biochemical adaptations have occurred many times before, most obviously in the cases of the integrations of mitochondria and chloroplasts into the eukaryotic cell complex. The transcriptome analysis of *E. chlorotica* found not only the presence of transcripts for proteins that must be turned over for photosynthesis to persist, but also transcripts for algal cellular enzymes that are involved in targeting and trafficking chloroplast proteins (Pierce *et al*., 2012). In addition, the chloroplast genome is translationally active in *E. chlorotica* (Hanten and Pierce, 2001; Pierce *et al*., 2012) and *E. clarki* (Pierce *et al*., 2003). Possibly, at least some of the chloroplast protein synthesis, trafficking, and modification could be done by hijacking host cell native proteins, but some presence of algal-specific, protein-trafficking molecules would seem to be necessary, and indeed, some of them are present in the *E. chlorotica* transcriptome. A FISH search for these kinds of genes using *V. litorea* gene sequences might be informative.

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