Solar-powered sea slugs defy evolution and horizontal gene transfer

Jeffrey Tomkins

Horizontal gene transfer (HGT) has been proposed as the major explanation for the large number of genes shared among completely unrelated taxon that share no lineage-specific evolutionary trajectory in the schema of life. However, mechanisms to explain HGT between multicellular eukaryotic life are lacking. Initial reports of HGT in a defined biological relationship between the solar-powered sea slug *E. chlorotica* and its algal prey seemed to provide the key evidence for such a mechanism. However, new data from the germline sequencing of the slug's genome via the use of eggs as a DNA source has shown that no algal DNA is present—definitively negating HGT in *E. chlorotica.* The explanation for the original data that seemed to support HGT is now believed to be that it is due to the presence of extra chromosomal circular DNAs (eccDNAs) excized from the algal genome. The nature of how these eccDNAs are formed and utilized in slug cells to help support the maintenance of its stolen algal chloroplasts remains to be deduced. This amazing phenomenon can only be explained within the context of a highly co-designed and biologically complex system inherent to the model of intelligent design.

Solar-powered sap-sucking sea slugs (sacoglossan molluscs) (figure 1) are amazing creatures that feed on various types of algae, sucking out the contents of the large filamentous cells through highly specialized mouthparts.[1–3] While feeding on the algae, the slugs somehow selectively capture (as opposed to digesting them) small photosynthetic intracellular organelles called chloroplasts and use them for solar energy. Amazingly, the chloroplasts are actually internalized and maintained in a functional state inside the epithelial cells that line the sea slug's pervasive intestinal tract (figure 1). Because the slugs obtain these chloroplasts from another organism, they are called kleptoplastic and their stolen chloroplasts are referred to as kleptoplasts.

The study of kleptoplasty in sea slugs is no easy task given that different types of plastid retention and physiological models for it exist between different species of sacoglossans, the only known metazoans to exhibit the unusual trait. In 2009, researchers studied two major groups of sacoglosans (Oxynoacea and Plakobranchacea) to determine the preponderance of chloroplast retention.[4] The only slugs showing chloroplast retention and photosynthetic activity were in the Plakobranchoidea, a taxonomic superfamily within the clade Sacoglossa, of which 13 species out of 17 showed the trait.

The ability to retain and maintain functional chloroplasts is variable and has been divided into two general groups demarcated as short-term and long-term.[4] The short-term retention is characterized by the disintegration of functional chloroplasts after about 7 to 14 days and is the most common type of kleptoplasty observed in sea slugs. The long-term retention group is characterized by the intact persistence of light-harvesting functional chloroplasts after more than 20 days (up to a year in one species) inside the cells that line the slug's digestive tract. The long-term–plastid-retaining sea slugs that have garnered the most interest among biologists are characterized by five main species—*Elysia chlorotica, Elysia clarki, Elysia crispata, Elysia timida*, and *Plakobranchus ocellatus*. Collectively, these animals have a wide geographical distribution and are found in the majority of shallow tropical and temperate marine environments.[2]

Some evolutionary scientists like to imagine that sea slug kleptoplasty is a type of symbiosis, but it does not fit the definition. Nevertheless, it is often referred to as 'chloroplast symbioses' or 'endosymbiosis'. In an authentic symbiotic (from Ancient Greek *σύν* 'together' and *βίωσις* 'living') relationship, two unique organisms are interacting with each other in what is typically a long-term relationship that may be mutualistic, commensalistic, or parasitic. The chloroplast is only an organelle, an isolated subcomponent of the main organism, the algae. Thus, there is no actual organismal symbiotic relationship occurring in kleptoplasty. In contrast, there are instances where algae and cyanobacteria form real symbiotic relationships with corals, sponges, sea anemones, etc. in which the complete photosynthetic organism remains intact.[5]

The types of algae fed on by the various sea slug species are diverse and variable not only between slug species but also during the slug's life cycle. Curtis *et al.* showed that *E. clarki* obtained and sequestered chloroplasts from four different species of algae and that newly metamorphozed juvenile slugs utilized chloroplasts from algal species different than the adults.[6,7] The slug *P. ocellatus* has been shown to feed on chloroplasts from a wide variety of species

representing the members of four completely different genera of algae.[8,9] One of the best studied slugs, *E. chlorotica*, is limited to two species of algae.[10] Amazingly, the *E. chlorotica* relationship with its two algal species is so specific that it is obligate, meaning that the sea slug will not complete its metamorphosis and develop into an adult when no algae are present for plastid uptake.[6,11] Thus, there exists quite a bit of variability in specificity and complexity in the species-species interactions between slug and algae. And no one specific kleptoplasty model defines all types of kleptoplastic sea slugs. For a graphical depiction of the complex interplay of factors affecting kleptoplast longevity, see figure 2.

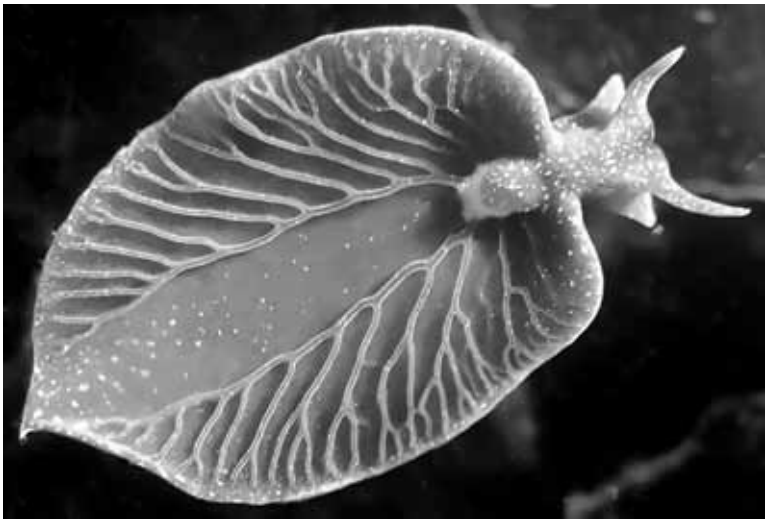

Figure 1. Picture of *E. chlorotica* displaying parapodia (wing like flaps) with finely branched diverticula of the digestive tract.

Biocomplexity of kleptoplasty

The complexity of both acquiring and maintaining foreign chloroplasts by the sea slugs poses multiple challenges that can only be explained by highly designed and engineered processes in the slug, which include 1) selective sequestration of the chloroplasts in the digestive tract from the normal processes that degrade all other ingested algal components, 2) specific subcellular localization of the chloroplasts to adult (differentiated tissues) in the lining of the sea slug's digestive tract, 3) regulation of the cellular and biochemical environment to favour active sustained periods of photosynthesis in the stolen chloroplasts, 4) transfer of photosynthate from the chloroplast for metabolic exploitation in the slug cell, 5) possible provision and replenishment of proteins for turnover in the chloroplast machinery associated with long-term retention, 6) suppression of the slug immune system from rejecting and attacking the chloroplasts for internalization, and 7) the unique morphology and design of the slug's pervasive digestive tract integrated into its maneuverable wing-like flaps called parapodia (figure 1), which allows for the maximized pervasive dispersal of the photosynthetic process.

The first design obstacle to overcome for kleptoplasty to occur is the selective targeting of chloroplasts from the ingested milieu of algal material after and during feeding. What little is known about this process has only recently been discovered in *E. timida*, with the use of recent advanced improvements in electron microscopy.[12] The use of this technique has shown that most of the cellular contents of the ingested alga sap are preferentially degraded very rapidly except for chloroplasts and chloroplast components. Thus, the slug's digestive system and its biochemistry somehow preferentially favours the preservation of chloroplasts and chloroplast components, while digesting other intracellular algal debris.

The process whereby the chloroplasts are then internalized from the digestive tract is only just beginning to be understood and varies between the different slug species analyzed. Using electron microscopy, researchers have recently documented different stages of phagocytosis (cellular engulfing) of chloroplast components from ingested algal food by the digestive gland cells in *E. timida*; this occurred within 37 to 60 min after feeding.[12] Actual progressive images of the finger-like extensions (processes) of the slug's digestive gland cells could be visualized as they reached out, grabbed, and then internalized the chloroplasts. Amazingly, the chloroplasts were not internalized whole as has been documented in other slug species, but the thylakoid stacks and stroma (internal plastid photosynthetic machinery) had their double-layered membrane removed and, after phagocytosis, were surrounded by a single membrane generated by the slug cell. In contrast, an earlier study in the slug *E. viridis* showed that the entire double-membrane of the chloroplast was engulfed completely intact and then surrounded by a phagocytic membrane.[13] Yet another variation of this theme has been observed in the well-studied slug *E. chlorotica*, which feeds on an algae species that contains four chloroplast membranes of which apparently the outer two are stripped while the inner two remain intact after phagocytosis.[11] When chloroplasts are sequestered, they are restricted to the epithelial cells lining the slug's finely branching diverticula of the digestive tract to achieve an optimal surface area-to-volume ratio for photosynthesis.[11]

One of the most amazing enigmas of kleptoplasty is how the chloroplasts are retained in a functional state and exploited for their photosynthate over extended periods of time. Evolutionists find this aspect of the phenomena the

most intriguing because they believe that understanding the retention process would answer "important evolutionary questions surrounding endosymbiotic events and the emergence and spread of photosynthesis in the eukaryotes."[14]

The most simple model to explain chloroplast retention, especially in the short-term scenarios, is the 'chloroplast stability model'.[14] In this model, the chloroplast maintenance proteins encoded by algal nuclear genes are not required. One aspect of this model is that the internal biochemistry of the slug's digestive cells assists in maintaining the temporal functional state of the kleptoplast despite the absence of algal nuclear genes that would normally produce chloroplast maintenance proteins. The photosynthetic capabilities and retention times of the sequestered chloroplasts can also be modulated through the slug's folding of its parapodia (lateral wing-like body flaps) in response to light intensity, which also has been proven to provide a significant longevity effect on chloroplast retention time.[2] Other research has shown that the slugs can control overall photosynthesis and kleptoplast turnover rates by other behavioural traits, such as shade seeking, water depth, and algal prey species selection, which are specific to different species.[15] Both light intensity modulation and the algal species from which the kleptoplasts are taken play major roles in retention times and turnover rates.

Horizontal gene transfer negated

The second idea of kleptoplast longevity to explain how long-term kleptoplasty might occur involves the use of proteins possibly imported into the chloroplast to facilitate the turnover of photosystem proteins, although this presents several serious challenges. Fully functioning chloroplasts in both plants and algae require specialized transfer portals (translocons) in their outer and inner membranes, termed TOC and TIC, respectively.[16] The TOC/TIC translocons import thousands of specifically synthesized preproteins produced in the cytoplasm from nuclear-encoded genes, which are then processed and utilized in the chloroplast. Amazingly, well over 500 nuclear-encoded genes are required to make and support a functional plastid—none of which are expressed by sea slugs.[9,17] As noted above, the chloroplast membranes in *E. timida* are stripped during phagocytosis and effectively eliminate this key translocon import system. In other slug species, the chloroplast membranes appear to be engulfed intact, but are still covered within a phagoyctic membrane, although this remains to be fully confirmed. Thus, the whole paradigm of how this would effectively occur raises difficult issues for explaining genetic and cellular mechanisms in long-term kleptoplasty.

Despite the apparent insurmountable difficulties associated with protein import, it is postulated that some level of it must occur for at least some of the algal nuclear-encoded proteins. This is largely based on the idea that, as noted previously, some slug species involved in long-term kleptoplasty can maintain captured chloroplasts in a non-replicative functional state for several months to a year.[1–3] Interestingly, this is also about the length of the slug's life cycle. In a recent review, Pierce and Curtis noted,

> "So it is not certain that the decline in photosynthesis of the aging *E. chlorotica* is due to the failure of the chloroplasts per se, which seems to be the case in the shorter, *E. viridis, E. timida*-type 3 associations, or the aging of the entire animal."[1]

Short-term kleptoplasty (hours to weeks) can easily be explained in most cases by photosynthetic maintenance via whatever molecular resources were acquired along with the capture of the chloroplast. However, cases of long-term maintenance, as in the case of *E. chlorotica,* appear to be much more complicated. To do so, it is believed that the slug needs the help of genes found in the nucleus of the algae which code not only for photosystem plastid proteins, but key regulatory factors targeted to the plastid genome.[18–20] Potential sources for these transcribed genes could be long-lived algal transcripts, horizontally transferred genes from the algal genome to the slug genome via horizontal gene transfer (HGT), or the presence of algal genes in extrachromosomal DNAs. The most appealing of these options

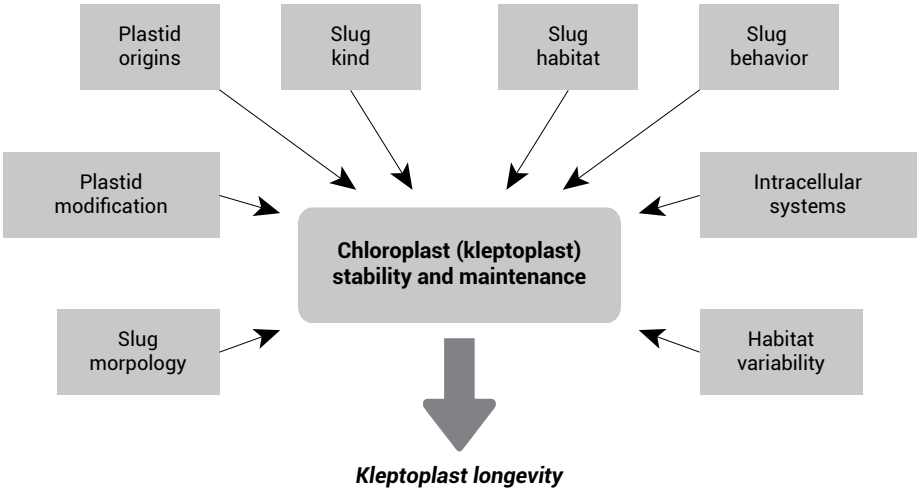

Figure 2. Graphical depiction of the complex interplay of factors affecting affecting kleptoplast longevity.

to many evolutionists is the idea of HGT because it would help explain why the genes of many organisms arise suddenly in lineages with no apparent ancestral precursors—recently reviewed by Tomkins and Bergman.[21] If, in fact, HGT could be proven in sea slug kleptoplasty, it would provide the first instance of such an event occurring between two multicellular eukaryotes.

Much of the idea surrounding HGT in multicellular animals is based on the evidence that eukaryote hosts can be the recipient of foreign genes in their genome from an intracellular bacteria (primarily wolbachia) as proven in a variety of studies, including one in which this author participated.[22,23] In sea slug kleptoplasty, the whole idea surrounding HGT postulates that the seemingly required algal nuclear genes were somehow stolen from the ingested algal milieu and integrated into the genome of a sea slug through a process yet to be postulated, much less identified. Given the increasing importance that HGT is thought to play in the grand schema of evolution, some proponents of it in the kleptoplastic sea slug research community have pushed the idea in the face of strong scientific opposition from even their own colleagues. As noted in a recent review written by nine different secular evolutionists that oppose the idea of HGT in sacoglossans, they state, "But gene transfer stories are hard to stop once they get going."[17]

In several studies in kleptoplastic sea slugs, scientists originally thought they had found evidence of HGT. Most of this information has come from studies of the slug *E. chlorotica*, where the kleptoplasts derive from the filamentous alga *Vaucheria litorea*. After the slug sequesters the chloroplasts, it can continue to photosynthesize for 10–12 months in the absence of any algal food.[11]

The idea of HGT is supported by a number of studies using adult and larval *E. chlorotica* that had been starved and supposedly free of algal contamination, although, as we shall see later, this was probably not the case and the data actually supports a different conclusion. Originally, evidence of about 11 different translated algal nuclear-encoded proteins used in chloroplast maintenance was reported in five different studies published between 2007 and 2010, recently reviewed by Pierce and Curtis.[1] However, the first large-scale alleged evidence of algal transcripts in *E. chlorotica* was published in 2012, when researchers reported the presence of 111 transcripts matching 52 different algal nuclear-encoded sequences.[24] These results were in contrast to another study in which the transcriptomes of two other slug species were sequenced (*E. timida and P. ocellatus*) in which no expressed algal nuclear genes were found.[9] However, the authors of the *E. chlorotica* study believed that the algal transcripts they discovered at nearly single copy level, were due to algal nuclear genes integrated in the slug genome and expressed at very low levels. They based this idea on the assumption that the starved slugs had no other explainable source for the production of transcripts. Thus, the idea that HGT might have actually been proven between two eukaryotes seemed plausible—at the time.

In 2013, scientists extracted decidedly uncontaminated DNA from *E. chlorotica* sea slug eggs that had never come in contact with algae.[10] They then sequenced the genome to a 100-fold level of redundancy and evaluated the gene content. The researchers also sequenced the expressed genes from the algal host (*V. litorea*) of the sea slug and then compared those gene sequences to the slug genome data to further test for the presence of HGT. The scientists concluded that "Taken together, the results of our analysis indicate the absence of algal-derived genes in the germ line of *E. chlorotica*" (p. 1844), and "Most importantly, our analysis does not return any genes or DNA regions shared by *V. litorea* [the algae] and the sea slug that are involved in photosynthesis" (p. 1845). It is also noteworthy that the researchers detected no algal plastid genes, indicating that no transfer of chloroplast genes to the slug genome had occurred either. The problem with previous studies that seemed to indicate the presence of HGT was that researchers were always using slug tissue to extract DNA that contained algal contamination. By using DNA extracted from eggs that had never been exposed to algae, the question had been settled.

Despite the fact that no evidence of HGT was found in this recent study, when the researchers also analyzed a number of individual slugs that had been feeding on algae—both immediately and after starving them—they found the presence of 11 different algal gene fragments reported to exist via HGT in earlier studies. Instead of pooling slugs as in previous studies, they tested individual slugs and found a great deal of inconsistency and variability between individuals—showing that pooling would indicate that "all 11 of these genes would be 'present' in the DNA leaving the false impression that HGT is present in all members of the sea slug population" (p. 1847). This also explains why only 14–20% of the samples showed the presence of algal genes.

Are extrachromosomal circular DNAs the answer?

So what is going on in this scenario where HGT is clearly negated by the lack of algal genes in the germline of *E. chlorotica*, but genes sporadically appear later, after the slugs feed on algae—even after prolonged starvation? It appears that the only explanation for this phenomenon is that of extrachromosomal circular DNAs (eccDNA)—also called small polydispersed circular DNA (spcDNA), which have been found in eukaryotes from yeast to humans, including plants.[25–29] In fact, Bhattacharya *et al*. proposed this idea as the best explanation for their recent results in *E. chlorotica*,

but did not elaborate on the possibilities for a model or significantly cover research related to the idea.[10]

EccDNAs are formed from chromosomal DNA in both plants and animals and have been found to vary between 400 and 20,000 bases in length.[26,27] Their stability is based on the fact that they are circularized, apparently after being spliced out of the genome—a fact verified by their distinct signatures in two-dimensional gel electrophoresis.[26,27,29] The sequencing of eccDNAs has revealed a broad range of DNA features, including protein-coding genes, ribosomal genes, transposable elements, tandem repeats, and other features.[25–27,29] However, despite the high representation of tandem repeats in eccDNA, some repeat categories that are also common in the genome are markedly absent in eccDNAs, indicating that the process is somewhat selective and not completely random.[26] Furthermore, because the eccDNAs appear to form distinct ladders on two-dimensional gels, it is thought that they correspond to monomers and higher order repeats that form discrete units of demarcating sequence in the genome, particularly in plants.[26,29]

Even more startling is that eccDNAs also appear to have the property of being copied through a process called 'rolling circle replication'. The rolling circle replication of eccDNA has been demonstrated in *Drosophila* without any apparent correlation to the expression of the replicated genes.[30] In mung beans, eccDNA molecules exhibiting a sigmoid form (circles with hanging tails) were characterized by electron microscopy, indicating that rolling circle replication for these molecules also occurs in plants.[31]

Despite the ubiquitous occurrence of eccDNAs across the complex spectrum of eukaryotic life, their specific mechanism of origin or purpose in the cell largely remains a mystery. Nevertheless, multiple studies have shown that DNA-damaging mechanisms promoted by chemical stress and other agents will stimulate the formation of eccDNAs.[26,32–34]

While many problems exist with attempting to form a model of eccDNA in sea slug kleptoplasty, if this is what is contributing to the recent results, then several scenarios could be possible (figure 3). The first is that algal eccDNAs could be formed and exist in the natural state of the algae and then be ingested by the slug. Or the formation of eccDNAs may be stimulated in the algae in response to the stress of slug feeding. In either case, the ingested eccDNAs would then be exploited by the slug. The second scenario would entail the disintegration of the algal nuclei in the ingested milieu, which would then form the substrate for eccDNA formation in the slug, by its cell machinery. Several hurdles for these scenarios exist, the first being that of transport—how are the eccDNAs or the algal chromosomal fragments imported into the slug's digestive cells? Secondly, if they are successfully imported, where is transcription of the eccDNAs occurring? Would the eccDNAs also be imported into the cell nucleus after being ingested? In any case, the presence of eccDNA would explain the persistence and low-level expression of algal genes in slugs that have fed on algae in contrast to their complete absence in slug germline DNA—negating HGT.

Sacoglossan phylogenetic quagmire

Needless to say, the wide variability in how the whole kleptoplastic process occurs and is maintained among sacoglossan slugs makes phylogenetics extremely difficult for evolutionary studies among the varying taxon.[35] In fact, because of the diversity of mechanisms, Rumpho *et al*. state that "at least four species of sacoglossans independently evolved the ability for long-term plastid retention" (p. 304).[11] Maeda *et al.* also stated that "the evolution to kleptoplasty from non-kleptoplasty would have occurred multiple times".[35] In addition, the various algal targets of the slugs themselves are also sources of origins mystery, as Chan *et al*. reported in a recent publication: "Algae are defined by their photosynthetic organelles (plastids) that have had multiple independent origins in different phyla."[36]

The process of kleptoplasty is separated into functional and non-functional forms, which are largely determined by the retention time of chloroplasts.[35] However, the mechanisms for both non-functional and functional kleptoplasty remain unclear and show great variety among slug species. Although evolutionists have proposed that non-functional kleptoplasty was a precursor evolutionary state that led to functional kleptoplasty, phylogenetic analyses performed in a recent study using a variety of concatenated gene sequences produced a discordant outcome.[35] In the modified phylogenetic model, non-functional kleptoplasts were acquired somewhere in a basal position of the Sacoglossa (including *Cylindrobulla*) in some mystical unidentified ancestral slug, then gained in the Plakobranchea and then selectively lost in subsequent lineages. And this is just a simplified evolutionary generalization of the data, which is replete with incomplete lineage sorting—a pervasive evolution-negating problem with phylogenies constructed across the spectrum of life.[21] The fact is that no contiguous phylogeny for kleptoplastic sea slugs can be constructed that makes any evolutionary sense. The real story is that sea slug kleptoplasty appears suddenly in the schema of life in a diversity of slug taxon, with a diversity of mechanisms, and diversity of algal preferences and biochemical relationships.

In complete contradiction to evolutionary predictions, each of these slug species group within their own clades (which include non-kleptoplast species), based on multiple alignments of concatenated gene fragments. Furthermore, these different species have varying levels of algae host

interactions in addition to plastid retention times. There is no coherent pattern of evolution discernable.

Summary and conclusion

Horizontal gene transfer is considered somewhat common among various types of bacteria because they are known to exchange segments of DNA between each other, although mechanisms of action beyond plasmids and phage still remain to be elucidated.[37,38] In other spheres of life that are more complicated, HGT can also occur between a bacteria and a multicellular host that it interacts with during its life cycle.[22,23] In this case, the genes of the bacteria are transferred to the genome of the eukaryotic host.

In the case of the kleptoplastic sea slug *E. chlorotica*, researchers originally thought that they had detected the first case of solid evidence for HGT between multicellular eukaryotes based on a known and defined biological relationship. This was based on the finding of algal genes expressed at low levels in the slug.[19,24] However, even in the cases of using starved slugs as an RNA/DNA source, the creatures had still been exposed to algae during their life cycle. A new study analyzing the germline genomes of slug eggs has shown that HGT has not actually occurred—the algal genes are simply not present in the slug's genome.[10] Because the scientists also tested slugs exposed to algae, and found the presence of algal DNA in their system, they concluded that the genes most likely resided on extra chromosomal DNAs that are circular and called eccDNA. Surprisingly, eccDNAs that are spliced out of the genome and formed into stable circles are found across the spectrum of life—even humans.[6] How these algal eccDNAs are formed and utilized is still not well understood, although it is known that they are transcribed.

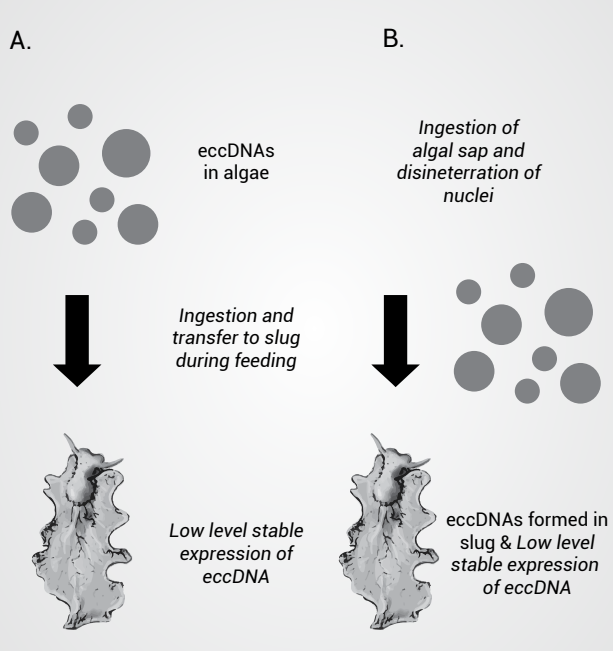

Figure 3. Two different models for the presence of algal eccDNAs in *E. chlorotica*.

The incredible mystery of this whole scenario and its bewildering and confounding complexity was recently expressed by the evolutionist authors that reported the recent germline sequencing of *E. chlorotica* when they assessed the situation and proposed additional research.[10] They stated:

> "This leaves the far more interesting possibility that the animal is able to harvest plastids and carry out photosynthesis using non-chromosomally integrated algal-derived genes, modified animal genes, or some combination of the two. Exploring these mechanisms using a combination of developmentally targeted mRNA-seq approaches, proteomics, and deeper genome sequencing offer potential avenues to elucidate the (nearly abominable) mystery of long-term sea slug photosynthesis" (p. 1848).

Could it be that the creator designed the algae to not only provide chloroplasts for the sea slug, but also the algal eccDNAs that contain the genes to make the whole system work? In this scenario, both the chloroplasts and the eccDNAs would be extracted from the algae at the same time. Alternatively, the eccDNAs could be formed in the slug after feeding.

It is the contention of this author that sea slug kleptoplasty is, in its entirety, a system of divinely created irreducible complexity involving both the slug and algal host. In all of the various sacoglossan slugs, their feeding apparatus, digestive system with its finely branching diverticula (visually resembling a plant's leaf), unique cellular biochemistries that make photosynthesis work in an otherwise seeming foreign environment, the behavioural adaptations of the slug to maximize photosynthesis, etc. must all be in place at the same time along with the unique properties of the algae for the entire system to work. It is truly an all-or-nothing proposition—especially in the slugs that literally depend on the larval feeding of algae to progress in their life cycle. The slug and algal systems are clearly co-designed and engineered to work together.

Adding to the incredible biocomplexity of this phenomenon is the fact that inquisitive and clever humans with all their advanced biotechnology are still baffled by the incredible mystery of this exquisite example of the Creator's wisdom and design in an amazing and beautiful little creature that is no more than a few centimetres in length. It is not a 'nearly abominable mystery', but a perfect example of the scripture in Romans 1:20 which states, "For the invisible things of

him from the creation of the world are clearly seen, being understood by the things that are made, even his eternal power and Godhead; so that they are without excuse."

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Jeffrey P. Tomkins *has a Ph.D. in genetics from Clemson University, an M.S. in plant pcience from the University of Idaho, Moscow, and a B.S. in agriculture ed. from Washington State University. He was on the Faculty in the Dept of Genetics and Biochemistry, Clemson University, for a decade, where he published 58 secular research papers in peer-reviewed scientific journals and seven book chapters in scientific books—in the area of genetics, genomics, and proteomics. For the past five years, Dr Tomkins has been a Research Scientist at ICR where he has published 20 peer-reviewed creation science journal papers, numerous semi-technical articles on the ICR web site and their magazine* Acts & Facts*, and two books.*