The position of the ophiuroidea within the phylum Echinodermata

Mary C. Harmon

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The Position of the Ophiuroidea within the Phylum Echinodermata

by

Mary C. Harmon

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
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Keywords: molecular phylogeny, evolution, echinoderm classes, ribosomal DNA, ophiuroid

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Dedication

For my parents, who have instilled in me the desire to succeed, and given me the tools necessary to do so.

For Holly and my friends and family. Thank you for believing in me and for providing enjoyable breaks from my scholarly chores when I needed them.

And when I didn’t.

For Ailey Marie.


She taught me many important things…none of which were related to echinoderms.
Acknowledgments

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Mary Harmon

ABSTRACT

Cladistic analyses of the interclass relationships of the phylum Echinodermata have not provided a phylogeny that is separately supported by both larval and adult characters. Similar to the reported incongruence with cladistic analyses, molecular analyses of ribosomal RNA (rRNA) genes have also given ambiguous results, which could be due to a number of factors. The use of short sequences, systematic errors such as long branch attraction, and mis-alignments of the data that are introduced by programs which are unsuitable for non-protein coding genes, have resulted in a controversy as to the true nature of echinoderm relationships. Historically, it is the position of the ophiuroids among the five extant classes of echinoderms that has been the most poorly understood, and the most recently published proposal is that there are three plausible relationships, albeit none of these are sufficiently supported. Re-analysis of 28S and 18S rRNA gene sequence data, with the addition of more phylogenetically informative sites as well as new taxa, the use of an alignment procedure that is based on rRNA secondary structure, and the testing of a myriad of evolutionary models have resulted in some new findings of ancestry. Interestingly, it is the phylogenetic position of the ophiuroids that proves to be among the more solid results from this analysis, while the historically
supported sister group relationship between the echinoid and holothuroid classes are not greatly corroborated.
Introduction

The phylum Echinodermata is broken into two subphyla; pelmatazoa, in which all living members of the class Crinoidea fall, and eleutherozoa, to which all other members of the phylum belong. These subphyla are further divided into the five classes of extant echinoderm animals; Asteroidea, Crinoidea, Echinoidea, Holothuroidea, and Ophiuroidea. It is clear from the fossil record that the Crinoidea emerged first, but there is disagreement concerning the divergence of the other four classes (see Hyman 1955, Paul and Smith, 1984, Smith 1984, Raff et al. 1988, Smiley 1988, Smith 1988, Strathmann 1988, Ratto and Christen 1990, Marshall 1994, Pearse and Pearse 1994, Wada and Satoh 1994, Littlewood 1995, Littlewood et al. 1997, Scouras and Smith 2001, Janies 2001 for a review of proposed phylogenetic relationships). In this study, I will re-analyze 28S and 18S rRNA gene sequence data, and add more phylogenetically informative sites as well as new taxa to the matrix. In addition, I will use a sequence alignment procedure that is based on rRNA secondary structure, and test numerous evolutionary models prior to an extensive search of tree space.

The phylum Echinodermata

Echinoderms, the spiny skin, true coelomate invertebrates that derived from sessile ancestors (reviewed in Hyman 1955), are defined by three main characteristics that are shared among every species in the phylum. The most defining character is that they all possess a calcium based endoskeleton which is derived from mesenchymal cells
and laid down at different times in development, but ultimately ends up in the dermis of all adults. This skeleton may take the form of a fused, immovable shield as in the test of echinoids and theca of stalked crinoids, or may be composed of numerous separated ossicles which can articulate with one another. The projecting spines (and tubercles which hold the spines) that are found on many species in different classes are also an extension of the skeleton. The second character that defines Echinodermata is the tube feet covering all or certain parts of their body, in differing numbers and patterns, which are used for feeding, locomotion, or sensory receptors. And, lastly, all adult echinoderms are constructed with pentameric symmetry to one degree or another (reviewed in Hyman 1955). The larval echinoderm is bilaterally symmetrical, may be free swimming, and may also possess skeletal spicules; the features of the larvae of each class will be discussed with more detail later. Table 1 summarizes the key adult features and larval patterns of the 5 extant echinoderm classes.

The Crinoidea are the most primitive of the animals, and belong to the subphylum pelmatazoa, which contains mostly extinct, stalked (attached to the sea floor) crinoids that flourished in the Paleozoic era (reviewed in Hyman 1955). About 5000 species of crinoid have existed prior to the current geologic age (reviewed in Hyman 1955). All extant crinoids belong to the fourth and last order of Crinoidea, Articulata. This group of animals appeared in the Triassic (Nichols 1969) and contains about 700 species of feather stars (comatulids) and sea lilies, of which only 100 species have retained the ancient ancestral stalked form (reviewed in Hyman 1955, reviewed in Ruppert et al. 2004). The highest density of crinoids can be found in the shallow waters of the Indo-Pacific region.
Table 1. Summary of the 5 extant echinoderm classes. Key characteristics are adult characters unless specified as larval. At least one species in each class exhibits direct development (brooding or otherwise) from a fertilized egg to the juvenile form. All larval types listed in this table are indirectly developing types, as the morphology and patterns differ amongst classes. Indirectly developing feeding larvae are planktotrophic (p) and indirectly developing non-feeding larvae are lecithotrophic (l).

<table>
<thead>
<tr>
<th>Echinoderm Class</th>
<th>Common Names</th>
<th>Approx. # of Living Species</th>
<th>Key Characteristics</th>
<th>Developmental Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crinoidea</td>
<td>sea lily; feather star</td>
<td>700</td>
<td>the only attached echinoderm; resembles a plant</td>
<td>auricularia (l) to doliolaria (l); doliolaria only (l)</td>
</tr>
<tr>
<td>Holothuroidea</td>
<td>sea cucumber</td>
<td>1200</td>
<td>microscopic skeleton, sluggish</td>
<td>auricularia (p) to doliolaria (l), doliolaria (l) only, vitellaria (l) to doliolaria (l), vitellaria only (l)</td>
</tr>
<tr>
<td>Asteroidea</td>
<td>sea star (starfish)</td>
<td>1500</td>
<td>perfect symmetry, robust skeleton, splendid colors, larvae lack skeleton</td>
<td>bipinnaria only (p), bipinnaria (p) to brachiolaria (p), yolky brachiolaria (l), barrel-shaped larvae (l)</td>
</tr>
<tr>
<td>Echinoidea</td>
<td>sea urchin; sand dollar; sea biscuit</td>
<td>950</td>
<td>hollow test with attached spines, Aristotle’s lantern, echinopluteus larvae</td>
<td>pluteus larvae (p), reduced pluteus (l), yolky ovoid larvae (l)</td>
</tr>
<tr>
<td>Ophiuroidea</td>
<td>brittle star; basket star (serpent star)</td>
<td>2000</td>
<td>graceful, flexible, brittle, great phenotypic diversity, ophiopluteus larvae</td>
<td>pluteus larvae (p), pluteus (p) to doliolaria (l), reduced pluteus (l), doliolaria only (l), vitellaria only (l)</td>
</tr>
</tbody>
</table>

In contrast to the other classes of echinoderms, there is a dearth of crinoids in the Atlantic Ocean and eastern Pacific (reviewed in Hyman 1955), nor are they as rich in the fauna of the Caribbean as the other echinoderm classes (Meyer et al. 1978). The members of this group, especially the feather stars, resemble ornamental plants more than animals, and are passive suspension feeders that flow with and are dependent upon currents in the water to bring them food (Meyer 1982).
Class Holothuroidea contains about 1200 extant species of orally-aborally extended echinoderms, sea cucumbers, which creep about on their differentiated ventral surface using locomotory podia (pedicels), burrow into the ocean floor, climb amongst algae, attach to or live in the crevices of rocks or coral, or even live pelagically (reviewed in Hyman 1955, reviewed in Ruppert et al. 2004). The most striking characteristic of the holothurians, in comparison with the other classes, is the microscopic ossicles which make up the body wall endoskeleton (reviewed in Hyman 1955). These ornate spicules come in many forms, such as rods, crosses, anchors, wheels, and hooks, but to the naked eye, only serve to make the skin of these animals warty and tough (Nichols 1969). Because of their reduced skeleton, the fossil record of holothurians is not as rich as it is for the other classes, and perhaps these modern ossicles represent an ancient form, or even a paedomorphic character (reviewed in Hyman 1955). Sea cucumbers only have one gonad, in contrast to the pentamerous arrangement characteristic of the other living members of the phylum, and every species has tentacles surrounding their oral end which they use to gather food (Birkeland 1988, reviewed in Hyman 1955). Holothurians vary greatly in size and have adapted to become residents of nearly every niche in every sea on the globe (Nichols 1969, reviewed in Hyman 1955).

There are approximately 1500 living species (reviewed in Ruppert et al. 2004) of the class Asteroidea, in which some orders contain species that show such wide variation that they often seem to assort into geographic species variations or subspecies (reviewed in Hyman 1955). They typically have 5 pointed arms symmetrically surrounding the central body, which usually harbor 2 gonads each. There are several species that deviate from the characteristic 5 pointed star, such as Florida native *Luidia senegalensis* which has 9 rays, or *Solaster sp.* that can have 7-14, and the range of sizes within the class is
considerably diverse (reviewed in Hyman 1955). The adult asteroid skeleton is made up of discrete, often overlapping ossicles, bound together with connective tissue (reviewed in Hyman 1955) and attached to skeletal muscles, such that the shape of the body is flexible and can be adjusted (Nichols 1969) to facilitate feeding and movement. Within the phylum, the sea stars are the most voracious predators of other animals, and one will even extrude its stomach to externally digest immobile or encrusting invertebrates (Nichols 1969, Birkeland 1988). As expected, this class of echinoderms is found in oceans worldwide, but the center of greatest concentration of asteroids can be found around Alaska, and other regions of the northern Pacific realm (reviewed in Hyman 1955).

Sea urchins, sand dollars, sea biscuits (clypeastroids), and heart urchins (spatangoids) collectively make up the class Echinoidea, which has 950 extant species (reviewed in Hyman 1955, reviewed in Ruppert et al. 2004). Sea biscuits are in fact a dorsally arched sand dollar; together with the heart urchins they are irregular urchins, while the typical globular shaped sea urchin is termed a regular urchin (reviewed in Hyman 1955). A regular adult echinoid is composed mostly of skeleton, coelomic fluid, and 5 gonads which are radially attached to the interior side of the test, and swell big enough in season to nearly fill the coelom (Nichols 1969, reviewed in Hyman 1955). Echinoids have an endoskeletal shield that is composed of 5 tightly fitted skeletal plates, cumulatively called the test (reviewed in Hyman 1955). There are several extensions of the endoskeleton, all of which are composed of the same calcareous material, such as the symmetrically arranged tubercles around the test, and the spines that project from each tubercle, creating an array of quasi-moveable appendages. It is assumed that all of the spines, pedicellaria, and podium act as sensory receptors for the echinoid (reviewed in
A major skeletal component of echinoids is their feeding (rasping) apparatus, Aristotle’s lantern (possessed by only some irregular urchins), and the 5 strong and persistently growing teeth associated with the lantern, which they use to scrape both plants and sessile animals from a substrate (Nichols 1969, Birkeland 1988). Urchins are not likely to be found in the deepest ocean abysses, but are prominent inhabitants of all seas from the high tide line to about 5000 meters (reviewed in Hyman 1955).

The symmetrically stellate brittle stars and basket stars, whose class Ophiuroidea contains about 2000 living species, are the most cryptic, diverse, and delicate group of echinoderms (reviewed in Hyman 1955, reviewed in Ruppert et al. 2004). The ophiuroids, in contrast to all other echinoderm classes, lack exterior ambulacral grooves, or anything resembling ambulacra on the body surface. In addition, pedicellaria and respiratory extensions are deficient or completely missing, leaving spines and podia as the most noticeable appendages. The spines are moveable in a semi-circular plane, as each one is mounted upon an endoskeletal tubercle, as in echinoids, but many species do not possess spines at all, while others exhibit spines to differing degrees (reviewed in Hyman 1955). The podium of the brittle star lack the ampullae and suckers associated with these structures in the other classes, as they do not rely on the hydraulic action of the tube feet for locomotion. With little or no assistance from their diminutive ambulacral system, ophiuroids move about in the horizontal plane by waving their arms; each of which is stacked with articulating vertebral ossicles (Nichols 1969, reviewed in Hyman 1955). Compared to the movement of the sea urchins and sea stars, both of which utilize tube feet for movement, the brittle stars are incredibly agile and can quickly escape when threatened (personal observation). The gonads reveal a pentamerous arrangement, surrounding the bursal slits at the base of each arm, but the euryalous forms (ophiuroids
that can coil their arms around things; all basket stars and some brittle stars) do not show congruence in the number or arrangement of gonads. Ophiuroids tend to conceal themselves under objects on the sea floor or in seaweed during the day to hide from sunlight, so they may not be as obvious as the echinoid or asteroid groups, but they are indeed among the most prevalent animals from the intertidal zone to the great depths of all seas, in every latitude, and every type of sea floor (reviewed in Hyman 1955).

*Echinoderm larval morphologies*

Adult body plans among the echinoderm classes are distinct, while the larval stages of each class show a great degree of convergence in both development and morphology (Strathmann 1978, 1988, 1993, Smith 1997, Wray 1996, reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). Indirectly developing species, which go through a free-swimming larval stage prior to metamorphosis, and directly developing species, in which an extended metamorphosis leads to an adult directly from the embryo, have been described for all of the eleutherozoan classes (Smith 1997, reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). In addition to the contrasting types of development, indirectly developing larvae exhibit further contrasts of both feeding and non-feeding forms (see Hyman 1955, Nichols 1969, Mladenov 1985, Smith 1997, Wray 1996, McEdward and Miner 2001). Pelmatazoan development lacks a feeding larval stage all together, but it has recently been shown that stalked crinoids have two successive stages of non-feeding larvae (Nakano et al. 2003).

The life history cycles for the eleutherozoans are as diverse within classes as they are between classes, such that similar larval morphologies can exist between classes that
may not even share a sister group relationship, while species within the same class (or even the same genus) may exhibit immense larval diversity (Smith 1997).

Previous knowledge about crinoid larvae was based on developmental morphologies of feather stars, the stalkless members of the class, but recently, Nakano et al. (2003) have provided a look into the larval stages of a sea lily, the stalked crinoid echinoderm. The stalked crinoid, *Metacrinus rotundus*, develops through successive, non-feeding larval stages, the auricularia and the doliolaria. The auricularia has ciliary bands in a partially longitudinal orientation, and metamorphoses into the doliolaria which has ciliary bands in a more transverse pattern (Nakano et al. 2003). The stalkless crinoids begin development with the doliolaria stage, and metamorphose directly into the juvenile from this larval type (Nakano et al. 2003, reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). The doliolaria is short lived, usually existing pelagically outside of the egg membrane for only a few hours to a couple of days before attaching to the substrate, at which time the ciliated bands and apical tuft become epithelium, and endoskeletal ossicles have begun to appear. The doliolaria subsequently undergoes a prolonged metamorphosis into the feeding pentacrinoïd juvenile (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). In addition to free-spawning development, the crinoids exhibit several modes of brooding development, such as a species with a brooded vitellaria larva that lacks surface ciliation, species that brood eggs and embryos that get released as fully formed doliolaria, and species that release larvae that have already begun the process of metamorphosis (reviewed in McEdward and Miner 2001). The stalkless pelmatazoans are derived from the stalked pelmatazoans (Smith 1984, reviewed in Hyman 1955), so the skeleton forms in a similar manner in the two types. Prior to doliolaria attachment, the skeletal spicules are secreted by
mesenchyme cells. The spicules branch and fuse to form a fenestrated plate that grows with the production and fusion of more branches, to eventually become the three dimensional plates and columns of the juvenile and adult skeleton (reviewed in Hyman 1955).

Holothurians develop through the means of at least three bilaterally symmetrical larval morphologies: auricularia, doliolaria, and vitellaria, (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001) while some brooding species may omit the larval stages and develop through direct means via a mesogen, although, according to McEdward and Miner (2001), none of these types are sufficiently well described. The auricularia is a complex, pelagic, planktotrophic larva with lobes that never develop into arms, but which can become quite ornate and numerous, as in the giant larvae of some apodid holothurians. The auricularia larva has a characteristic single ciliated band that is used for feeding and locomotion, which outlines an intricate loop around the ovoid body (reviewed in McEdward and Miner 2001). Prior to metamorphosis, the auricularia morphs into a simple, nonfeeding doliolaria, which includes the loss of the arm-like lobes, breakup of the convoluted ciliated band and larval gut, formation of crosswise ciliary rings, and formation of the vestibule from the oral cavity (Lacalli 1988, 1993, Smiley 1986, reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). The doliolaria is considered to be a transitional, metamorphic stage in these species, as it only exists between the auricularia and the post-metamorphic pentactula juvenile, which bears the 5 buccal podia that are characteristic of the adults of the class (Smiley 1986, reviewed in McEdward and Miner 2001). Alternatively, some species of holothuroid omit the auricularia larva and develop directly through the simple, lecithotrophic doliolaria, which possess transverse ciliated bands around the ovoid body, and metamorphose into the
pentactula (reviewed in McEdward and Miner 2001). Much like the adult holothurians, larval holothurians possess microscopic calcareous ossicles in characteristic shapes that are secreted by mesenchyme cells beginning in the auricularia stage, or in the doliolaria in species that omit the auricularia larvae (reviewed in Hyman 1955). Another simple form of lecithotrophic development exhibited in this class is the vitellaria larvae, which is uniformly ciliated, and can remain in this form until metamorphosis. However, in some species the cilia become organized into bands around the body; forming the doliolaria larva prior to metamorphosis. Both the doliolaria and the vitellaria larvae can be pelagic or benthic, and are known to exist in a variety of holothurian taxa (reviewed in McEdward and Miner 2001).

The larval asteroid is set apart from the other classes by its complete lack of a skeleton. The skeletal spicules are absent in the larval body and in fact ossicles do not even begin to appear until post-metamorphosis, at which time the skeleton starts developing with a triradiate spicule that branches out to grow and form the characteristic fenestrated plate of the adult skeleton (reviewed in Hyman 1955). Sea star larvae are characterized by 5 bilaterally symmetrical, but morphologically distinct types: bipinnaria, brachiolaria, yolky brachiolaria and yolky non-brachiolaria, and barrel-shaped (reviewed in McEdward and Miner 2001). The bipinnaria, which looks very similar to the holothurian auricularia, is a complex larva with ciliated structures for feeding and swimming in the water column. It has small arms, which are an extension of the body wall, but there is no skeletal rod support for these arms (reviewed in Hyman 1955). In some species of asteroid, the bipinnaria changes to a brachiolaria prior to settlement and metamorphosis. The brachiolaria is the developmental successor to the bipinnaria in these groups, as the brachiolaria has three additional arms that not only enable the larvae
to probe the substratum, but also to adhere to it (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). The remaining types of asteroid larvae are non-feeding, and are subsequently simpler in structure as a result of this functional loss. The reason they can survive this lecithotrophic life style is because they have a food reserve (yolk) within the egg that the planktotrophic types do not have. The sharpest contrast to an indirectly developing, planktotrophic, larval morphology lies with the few species that develop directly through the mesogen, wherein a pelagic, non-larval stage leads directly to the juvenile form (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001).

Classical objects of embryological experiments, the echinoid larvae are characterized by only two larval types: the pluteus larva and a non-feeding larva (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). Similar to the larval holothuroid lobes and the asteroid larval arms, the echinopluteus arms are an extension of the body wall, but the ornate pluteus has a calcareous larval skeleton, which supports both the body and the numerous arms. The arms are always associated with the ciliated feeding structures, although the number and size of arms, and subsequently, the body form, varies greatly in the pluteus larval type (Wray 1992, reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). A reduced pluteus with a reduced skeleton, that lacks ciliated feeding structures and a functional gut, is the most complex larval morphology of the simple, non-feeding larval types. The other end of the non-feeding larval spectrum is a very simple form that possesses only a vestigial skeleton and lacks not only ciliated bands and a gut to feed, but also lacks arms, yielding an irregular ovoid shaped larva (reviewed in McEdward and Miner 2001). Regardless of complex or simple structure, all of the larvae are bilaterally symmetrical, and share common features pertaining to organization within the larvae and during metamorphosis to the juvenile
form, which requires substantial rearrangement of larval axes and structures (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). Echinoid larval development differs from the holothuroid, asteroid, and ophiuroid larval development in that there is never a sequential change of larval forms, but rather one larval stage prior to, and ending with, metamorphosis. In contrast to the feeding and non-feeding types of indirect development observed in echinoids, there is also a non-larval mesogen that leads directly to the juvenile form. This type of development has only been observed in *Abatus cordatus*, which is a sub Antarctic brooding species (reviewed in McEdward and Miner 2001).

Ophiuroids are the only other class of echinoderm that contains species that develop through a pluteus larva, termed an ophiopluteus, to distinguish it from the echinopluteus. There are two other forms of indirect larval development in this class, the doliolaria and the vitellaria. In addition, and characteristic of all eleutherozoan classes, there exists a non-larval (directly developing) mesogen. The ophioplutei have less variation in form than do the echinoplutei, but have pairs of arms (usually 4 pairs) that are supported by calcareous skeletal rods and display the ciliated feeding-band structure (reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). The larval skeleton includes the arm rods and, even prior to the pluteus stage, a box-like network of calcium carbonate rods that anchor the larval body bilaterally and from the posterior (personal observation, Byrne and Selvakumaraswamy 2002). Although the echinoid and ophiuroid are the only two echinoderm classes that possess a pluteus larva with a well developed skeleton, there are several differences that exist between the two types. Echinoids have unpigmented eggs but form pigment cells in larval development, while ophiuroids have pigmented eggs that derive larvae that lack pigment cells; thus rendering the ophioplutei
more translucent than echinoplutei, which makes the spicules and digestive system quite visible under low magnification (personal observation, Byrne and Selvakumaraswamy 2002). A fully developed ophiopluteus has a pair of anterolateral, posterodorsal, post-oral, and posterolateral arms, with the posterolateral being the longest and extending laterally from the anterior/posterior axis of the larva. However, the longest echinopluteus arms are the post-orals and posterodorsals, which extend ventrally and dorsally from the anterior/posterior axis of the larva (Byrne and Selvakumaraswamy 2002). The ophiopluteus spicules are solid, whereas the echinopluteus spicules can be solid, or form fenestrated structures (Emlet et al. 2002, reviewed in Hyman 1955, reviewed in McEdward and Miner 2001). In both pluteus types, the spicules are formed from mesenchyme cells; defined by a developmental gene regulatory network (GRN) that differentiates the skeletogenetic cells. The echinoid GRN that gives rise to the spicules from primary mesenchyme cells is well characterized (see Oliveri et al. 2002, Ettensohn et al. 2003), but the homologous GRN pathway in the ophiuroid is only just being examined for genes that may be conserved between the classes.

The wide array of larval types and modes of development characteristic of the phylum introduces a daunting task in trying to piece together a phylogenetic pattern between the echinoderm classes based on ontonogenic data. The question of greatest interest here concerns the mode of development among the echinoids and ophiuroids, in which a free-swimming pluteus larva with well developed skeletal spicules develops from the embryo. No other class exhibits this pluteus type with its extensive skeleton. Some species of both the holothurian and asteroid classes do have an indirect mode of development in which a feeding larva is present, but the skeleton is either greatly reduced or absent in these larval classes. Is the larval skeleton underlying the pluteus larva a
result of convergent evolution (Strathmann 1978, 1988, Strathmann and Eernisse 1994, Littlewood et al. 1997), or is it a homologous character derived from a common ancestor that echinoids and ophiuroids share? Alternatively, perhaps an indirectly developing planktotrophic ancestor gave rise to all four classes of the eleutherozoan clade, but the pluteus form with its well formed skeleton has simply been lost in the holothuroid and asteroid classes (Strathmann 1978, Peterson et al. 2000, reviewed in McEdward and Miner 2001).

_Echinoderm class relationships_

Although a sea-dwelling invertebrate animal that possesses the defining characteristics of the phylum Echinodermata is easily assigned into the phylum, paleontologists, embryologists, zoologists, and cladists have been at odds with the assignment of subphylum, class, and interclass relationships as long as they have been dissecting those relationships (Forbes 1841, Bather 1900, MacBride 1906, 1914, Hyman 1955, Fell 1948, 1962, 1967, Moore and Teichert 1978, Paul and Smith, 1984, Smith 1984, 1988, Smiley, 1986, 1988, Raff et al. 1988, Strathmann 1988, Ratto and Christen 1990, David 1993, Smith et al. 1992, 1995, Pearse and Pearse 1994, Wada and Satoh 1994, Lafay et al. 1995, Littlewood 1995, Littlewood and Smith 1995, Littlewood et al. 1997, Janies 2001, Cohen et al. 2004). The main cladistic issue in defining echinoderm class relationships is that the tree representing seemingly informative characters of larval morphology, and the tree that represents no less informative adult morphological characters, do not show an agreement of ancestry between the classes when the tree topologies are compared. Analysis of both larval and adult morphology within the phylum Echinodermata has proven unresolved. Littlewood et al. (1997) reviewed and
reanalyzed much of the prior work on this subject, and report three equally parsimonious
trees based on larval characters with each tree showing the ophiuroids in a different
position. A single most parsimonious tree based on adult characters was reported, but
trees one and two steps longer show the same problem with the revolving position of the
ophiuroids.

The echinoderm classes arose over a short period of geological time, with an
estimated divergence event about every 20-30 million years; delineating the entire
phylum over a period of 100 million years, about 550-450 million years ago (Smith
1988). The fossil record shows that crinoids appear first, followed by asteroids (530-490
mya), ophiuroids (530-490 mya), and echinoids (480-450 mya), which all emerged at
about the same time, geologically speaking. The emergence of the holothurians,
however, is unclear, as their mostly soft bodies have left an almost non-existent fossil
record (Smith 1988, David 1993).

Molecular data should be helpful in resolving the discrepancies between the
echinoderm phylogenies based on the fossil record and extant adult and larval features. It
is highly possible to misinterpret character data (Smith 1984), and in fact, different
authors have come up with a different class relationships using the same character set
simply because of fundamental differences of opinion in homology (Littlewood 1995)
and polarity of characters. However, it seems harder to confuse and/or be biased by
nucleotide base substitutions and evolution of DNA sequence data (Swofford et al. 1996).

**Ribosomal DNA sequences**

It is important in molecular studies to analyze orthologous genes, or groups of
genomes, and appropriate regions within them. Protein synthesis from messenger RNA is
directed by ribosomal units, which consist of several distinct ribosomal RNAs (rRNA) and associated proteins. Protein biosynthesis is a primary cellular requirement for life; therefore, rRNAs are ubiquitous in all living systems (Hillis and Dixon 1991). This fact defines rRNA genes as orthologous genes that can be used to compare a broad range of phyla to determine molecular phylogenetic relationships within and amongst them. According to Giribet (2002), nuclear rRNA loci are by far the most useful molecules in the field of molecular phylogenetics to discern metazoan relationships. Metazoan animals have a large subunit (LSU) rRNA, small subunit (SSU) rRNA, and internal transcribed spacers tandemly encoded in their nuclear genome in an array that is repeated several hundred times throughout (Long and Dawid 1980). The subunits are often differentiated by their respective units of sedimentation velocity of 28S and 5.8S for the LSU and 18S for the SSU (Hillis and Dixon 1991). There is a rigid secondary structure associated with ribosomal DNA sequences that has been used extensively to discern metazoan phylogenies (see Field et al. 1988, Turbeville et al. 1994, Winnepenninckx et al. 1995, Mallatt and Sullivan 1998, Cameron et al. 2000, Winchell et al. 2002, Mallatt et al. 2004). The 18S rRNA gene is amongst the most intimately evaluated for deep metazoan relationships as it is considered to be a slowly evolving sequence (Hillis and Dixon 1991), and has many regions that are relatively conserved over a wide range of metazoan taxa. The 28S rRNA gene, on the other hand, shows much more variation in evolutionary rates between taxa, and many more divergent domains that potentially hold informative nucleotide data, but could also be so divergent across taxa that the region can not be unambiguously aligned. The 28S rRNA gene is therefore not as useful for discerning the earliest of metazoan divergences, but is potentially useful in the inference
of relationships among more closely related taxa (Hillis and Dixon 1991) where the 18S would not be beneficial.

*Mitochondrial sequences*

The circular animal mitochondrial genome has a gene arrangement that seems to be relatively stable in the higher metazoan taxa (Smith et al. 1993). All animals possess mitochondria in their cells and genes within mitochondrial genomes are orthologous across a wide range of animals. Smith et al. (1993) analyzed the first almost complete complement of mitochondrial genes within echinoderms, in hopes that mitochondrial molecular data could shed light upon the relationships within the phylum. Their study was a look into mitochondrial gene arrangement patterns of representative taxa from all classes except Crinoidea. Although the study did not entail a direct phylogenetic analysis, they concluded that the holothurians and echinoids group together based on gene placement patterns, while the asteroids and ophiuroids group together based on a different gene arrangement that is inverted with respect to the echinoid condition. Without a crinoid sequence, it was unknown which of the contrasting gene assemblies was the derived pattern, but nonetheless their study of gene order resulted in a sister grouping of echinoids and holothurians that is distinct from the grouping of ophiuroids and asteroids. Scouras and Smith (2001), obtained the mitochondrial gene order/sequence of the crinoid *Florometra serratissima*, and subsequently performed several phylogenetic analyses with the complete complement of echinoderm mitochondrial data. Scouras and Smith (2001) found that while the gene content is maintained, the order of genes in crinoid mitochondria do not follow the same pattern of either the echinoid or the asteroid, but show a novel arrangement. Within the genome,
from the cytochrome c oxidase (CO) I gene region through to the cytochrome b region, the echinoid and holothuroid patterns show one gene order, the asteroid shows a major inversion with respect to this order, while the crinoid shows a third arrangement as well as fragmentation in the region. Further investigation into the ophiuroid gene order revealed multiple inversion events and alterations (Scouras and Smith 2001) of the gene order that had previously revealed a similarity to the asteroid pattern (Smith et al. 1993). Since the first investigation into echinoderm mitochondrial sequences, a tRNA duplication event that is unique to one genus within the holothuroid class has also been discovered (Arndt and Smith 1998). All of the echinoderm mitochondrial sequences show a G+T nucleotide composition bias, and Scouras and Smith (2001) warn that extreme caution must be exercised in choosing a model that is unaffected by bias in nucleotide replacements when trying to ascertain the proper molecular phylogeny of echinoderm classes. Not only does the ophiuroid arrangement exhibit a G+T nucleotide bias, but also an evolutionary rate that is faster than the rate of sequence evolution in the other classes. Upon maximum likelihood and LogDet paralinear distance analyses of the amino acid and nucleotide alignments, respectively, of the CO I, II, and III genes, the long branch leading to the ophiuroid class causes a phylogenetic anomaly that places the ophiuroid as basal to the crinoid. Figure 1 shows the topology of the most likely tree
[(out(O(C(H(A,E))))), which places the asteroids as sister group to the echinoids, the holothurians as sister taxa to the echinoid/asteroid clade, crinoid as sister to the echinoid/asteroid/holothuroid clade, and with the ophiuroid more closely related to the chordate outgroup than to its echinoderm relatives.


The same data set was analyzed without the ophiuroids in the alignment, and the topology of the best tree did not change, namely, the asteroids and echinoids are still sister groups with the holothuroid outside of that, and crinoid as sister taxa to the eleutherozoan classes. However, the next best tree placed the holothurians with the echinoids, and with this topology plus the gene order data, Scouras and Smith (2001) concluded that there was support for an echinoid/holothuroid clade with the asteroids
outside of this, but admit that the variability in echinoderm mitochondrial gene
arrangements has limitations as a phylogenetic tool.

Phylogenetic tree building algorithms using 18S and/or 28S ribosomal molecular
data have also proven unsuccessful at providing a definitive resolution of echinoderm
class relationships (Raff et al. 1988, Ratto and Christen 1990, Marshall 1994, Wada and
pitfalls of molecular phylogenies in general, all of which have relevance in the
echinoderm rRNA data set, and the inconsistencies of previous 28S/18S analyses
justifiably illustrate their existence.

*Molecular phylogenies*

The sequence alignment in a molecular data set is almost certainly the least
understood and most difficult component of the phylogenetic analysis, where incorrect
alignments can be an effect of biases introduced into computerized alignment algorithms
by mathematical models that were designed to ease the tedium of manually aligning by
eye (Swofford and Olsen 1990, Lake 1994). When regions of the data are so divergent
that they cannot be unambiguously aligned by eye, they should be discarded (Swofford
and Olsen 1990), in hopes of avoiding such false alignments. Clustal W (Thompson et al.
1994) is one such algorithm that inserts gaps in sequence data with the goal of
maximization of total nucleotide similarity at any given site. For a molecule like
ribosomal RNA, that has both paired and unpaired regions (Smith 1989) in its helix and
loop secondary structure, an algorithm that would bias towards an alignment of strict
nucleotide similarity rather than secondary structure seems inappropriate because of the
likelihood of generating the wrong alignment.
Sites in unpaired and/or divergent domains of rRNA are under less evolutionary constraint (comparable to the wobble position in protein coding genes) than paired regions, and are able to evolve more quickly. As mutations become fixed in unpaired or divergent regions, there is a high likelihood of multiple mutations accumulating, given enough time to do so (Swofford and Olsen 1990). This site to site variation with respect to rates of evolution within a single gene can complicate a multiple sequence alignment, and needs to be accounted for in order to avoid this downside of sequence analysis (Lake 1994). The rate variation of nucleotide substitution at each site can be approximated, and visualized through a curve, where the invariant sites are at the far left and the most variable sites are represented by a tail at the far right side. This is called the gamma distribution of the data set, or gamma shape parameter, and it follows that the site to site rate variation in the sequence alignment roughly follows a given distribution of rates of substitution per site (Nei and Kumar 2000).

The holothuroid sequences are evolving much more rapidly across the rDNA repeat unit than the other echinoderm lineage; a truth that manifests itself as a long branch leading to the holothurians (Raff et al. 1988, Wada and Satoh 1994, Littlewood et al. 1997). Long branches can be caused not only by rapidly evolving sequences in an alignment, but also incorrect sequence alignments and the inability to account properly for site to site variation (Lake 1994). The only way to combat the pitfalls encountered in the echinoderm 28S/18S rRNA genes is to align the sequences based on secondary structure, without initial regard to nucleotide similarity, investigate models of evolution that best fit the data set, and use a tree making algorithm that can search for a tree while taking into account the important parameters delineated by the appropriate model of evolution.
According to Nei and Kumar (2000), there are three groups of commonly used methods to reconstruct phylogenetic trees from molecular data sets: 1) distance methods, 2) parsimony methods, and 3) likelihood methods, all of which have strengths and weaknesses. Phylogenetic inferences that operate by means of distance methods seek to define evolutionary scenarios through an algorithm that results in a single tree topology, where parsimony and likelihood methods seek to define phylogenetic relationships by systematically comparing alternative phylogenies and deciding which is the most parsimonious, or the most likely tree. In this way, distance methods are purely algorithmic, but the other two (criterion-based methods) use the algorithm not as a direct means to a tree, but as a means to evaluate a model or objective function and search for trees that optimize that function (Swofford et al. 1996).

Distance methods, such as neighbor joining estimates, evaluate differences between sequences in a pairwise approach by evaluating the number of substitutions per sequence position. The result is a matrix, constructed of all evolutionary distance relationships in the data set, such that the tree that results is an analysis of the pairwise distances (p-distances) where sequence similarity is inversely proportional to evolutionary distance. As sequence similarity decreases (divergence increases) between pairs of taxa, the p-distance between them increases, and they will be placed further apart in the tree than nearly identical taxa that are separated by nearly zero distance (Swofford and Olsen 1990). Branch lengths leading to each taxon are evident in the resulting topologies, and these lengths are directly proportional to the number of nucleotide substitutions in each taxon (Nei and Kumar 2000). There are guidelines (Nei and Kumar 2000) to choosing the appropriate distance measure for each data set; a few models which
take into account transition/transversion ratios are available, and a gamma shape parameter can be invoked as well.

Maximum parsimony (MP) analyses generally operate by selecting the tree which best approximates the least number of steps to account for one character state to another (Swofford and Olsen 1990). In other words, the most parsimonious tree is the topology that delineates divergences in the least number of steps. An MP search method may find an incorrect tree if and when the rate of nucleotide substitution at different sites within the gene sequence varies; creating homoplasy that is indistinguishable from regular substitutions. If MP algorithms create incorrect topologies when the rate of evolution varies among sites, then when the overall rate of evolution varies amongst taxa, there may also be incorrect topologies derived from the analyses (Nei and Kumar 2000).

Maximum likelihood (ML) tree estimations are computer intensive searches that first entail the selection of an appropriate model of evolution for the data set being analyzed. The models are expressed as a set of parameters, and can be relatively simple or quite complex. The mathematical expression of any given model is a matrix of nucleotide substitution rate categories (Swofford and Olsen 1990) and additional parameters, if they apply to the model in question, such as the gamma distribution and proportion of invariant sites. In this way, models of evolution are formulas that depict the probability that nucleotide A, at any given site in the sequence, will evolve into nucleotide B over some evolutionary interval (Swofford and Olsen 1990). The ML approach then evaluates the net likelihood that the defined evolutionary model will generate the observed sequences, such that the resultant phylogenies are those carrying the highest likelihood, where the site-specific (local) likelihoods are added together to equate to the overall (global) likelihood of the tree (Swofford and Olsen 1990).
Littlewood et al. (1997) increased sampling density and taxonomic diversity of previous echinoderm rRNA phylogenies, and used partial sequence (313 bases from the 5' end) from the 28S rRNA gene and the complete 18S rRNA gene to revisit the class relationship problem. See Figures 2 and 3 for the Littlewood et al. (1997) trees; redrawn to show the 50% bootstrap collapsed version of the 28S and 18S topologies, respectively.

Figure 2. 50% bootstrap collapsed 28S rRNA gene consensus tree adapted from Littlewood et al. (1997) maximum parsimony analysis.

All nodes at less than 50% are condensed to show polytomies in the most parsimonious tree of the reduced data set, where Holothuroi=Holothuria, Pawsonia, Leptosynapta, Psychropotes, Echinoid=Asthenosoma, Cidaris, Encope, Ophiuroid=Asteronyx, Ophiocomina, Ophiocanops, Asteroid=Asterias, Asterina, Crossaster, Henricia, Crinoid=Antedon, Outgroup=Herdmania.

Their alignments were generated with Clustal W, first with only intraclass taxa uploaded, followed by the pre-aligned intraclass sequences being aligned, and then the pre-aligned echinoderm class sequences being aligned to the outgroup taxa. After being sequentially aligned with the Clustal W algorithm, the sequences were then aligned by eye with reference to chordate and hemichordate secondary structures. Littlewood et al. (1997)
report that their maximum parsimony analysis of 28S rRNA gene sequences of echinoderm classes shows a class topology of \((C(O(A(E,H))))\), where the echinoid/holothuroid clade is a sister group to the asteroid taxa, supported by a bootstrap value of 91%, and the ophiuroids alone are a sister group to the e/h/a clade. The bootstrap value at the node supporting the ophiuroids grouping outside the e/h/a is a 48%, while the node placing the crinoids outside of the other 4 classes is only a 39%. When these two nodes are collapsed in a 50% bootstrap tree, the echinoids and holothurians are still placed as sister taxa, but the resolution of all other classes, including the crinoids, is represented as a polytomy, meaning there is not a visible ancestral pattern between these groups. Alternatively, their analysis of 18S rRNA gene sequences show that ophiuroids are the sister taxa to the asteroids (bootstrap 48%) and the ophiuroid/asteroid clade is a sister to the holothuroid/echinoid clade (bootstrap 70%), with a class topology of \((C(A,O)(E,H))\). Again, if the nodes with a bootstrap support value of less than 50% are collapsed, the echinoids and holothurians are still grouped together as sister taxa, but the ophiuroid/asteroid clade is not supported, so that the emergence of the ophiuroid and asteroid groups is not recovered, and this is viewed as a polytomy. In the Littlewood et al. (1997) 18S topology, the crinoids are grouped outside with a bootstrap support of 72%. A combined 28S/18S consensus data set was also analyzed with the maximum likelihood algorithm and described to have the topology \((\text{out}(C(O(A(E,H))))))\), but no bootstrap values were reported for this tree.
Figure 3. 50% bootstrap collapsed 18S rRNA gene consensus tree adapted from Littlewood et al. (1997) maximum parsimony analysis.

All nodes at less than 50% are condensed to show polytomies in the most parsimonious tree of the reduced data set, where Holothuroid= *Cucumaria*, *Lipotrapeza*, *Stichopus*, *Psychropotes*, Echinoid= *Asthenosoma*, *Cassidulus*, *Diadema*, *Eucidaris*, *Stomopneustes*, Ophiuroid= *Astrobranchion*, *Ophiomyxa*, *Ophiocanops*, *Ophioplocus*, *Ophiopholis*, Asteroid= *Astropecten*, *Asterias*, *Porania*, Crinoid= *Antedon*, *Endoxocrinus*, Outgroup= *Branchiostoma*, *Styela*, *Herdmania*, *Saccoglossus*.

A combined morphological and 28S/18S molecular tree from Janies (2001), shows the same topology reported by Littlewood et al. (1997), but separately his morphological and molecular analyses generate (C(O(A(E,H)))) and (C(A(O(E,H)))) topologies, respectively. In spite of his re-analysis, the echinoderm class relationships were no more defined after Janies (2001) than they were after Littlewood et al. (1997).

A definitive phylogeny of echinoderms is desired

Previous analyses of informative morphological characters, mitochondrial gene sequences, and ribosomal RNA gene sequences have proven phylogenetically inconclusive with respect to the position of the ophiuroids within the Echinodermata (Hyman 1955, Paul and Smith, 1984, Smith 1984, Raff et al. 1988, Smiley 1988, Smith

An ultimate set of relationships between the five classes of echinoderms, and more specifically, the definitive position of the ophiuroids within the phylum is desired. A summary of some sequence information and of the methods used in both the alignment and analyses from the studies of Littlewood et al. (1997), Janies (2001), Scouras and Smith (2001), and the current study can be found in Table 2. In this study, I have re-analyzed sequences from previous studies, as well as added a few new taxa to the matrices. With regards to the 28S rRNA gene, I have increased the number of nucleotide sequence in the alignment in order to increase the available sites that may yield phylogenetically important information. The holothurian sequences appear to be evolving much more rapidly across the rDNA repeat unit than the other echinoderm lineages, causing a long branch leading to the holothurians as a result of the unequal rate (Raff et al. 1988, Wada and Satoh 1994, Littlewood et al. 1997). Because the holothurian rRNA gene sequences contain numerous insertions in several divergent domains that the other classes do not have, as well as a higher rate of nucleotide substitution throughout the gene, it became beneficial to remove the holothurians from the data set prior to alignment, which made the divergent regions less ambiguous, and therefore more informative. I used the DOS program DCSE (Dedicated Comparative Sequence Editor, De Rijk 1993) and carried out the multiple alignment by eye, based on rRNA secondary
structure so that biases toward nucleotide sequence composition similarity were avoided. The sequence alignments were analyzed separately and together under three different tree making algorithms; the neighbor joining distance method, a maximum parsimony search, and a maximum likelihood search under defined models of evolution. Prior to this study, echinoderm 18S and 28S phylogenies have not been executed with a strictly manual alignment or an extensive maximum likelihood search, making both procedures unique to this analysis.
Table 2. Summary of the methods used in both the alignment and analyses of echinoderm class relationships.
Sequence and analysis information are reported from the studies of Littlewood et al. (1997), Janies (2001), Scouras and Smith (2001), and the current study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alignment</th>
<th>#Nucleotides</th>
<th>#Variable sites</th>
<th>#P-I Sites</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Littlewood, et al.</td>
<td>28S Clustal W; GDE</td>
<td>313</td>
<td>?</td>
<td>107</td>
<td>Parsimony (PAUP); Maximum Likelihood (PHYLIP)</td>
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<tr>
<td></td>
<td>18S</td>
<td>1723</td>
<td>?</td>
<td>424</td>
<td>&quot;</td>
</tr>
<tr>
<td>Janies</td>
<td>28S Clustal X; GDE; POY</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Parsimony (PAUP)</td>
</tr>
<tr>
<td></td>
<td>18S</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>&quot;</td>
</tr>
<tr>
<td>Harmon, this study without holothurians</td>
<td>28S DCSE</td>
<td>1889</td>
<td>541</td>
<td>303</td>
<td>Parsimony; Maximum Likelihood (PAUP); Neighbor Joining (MEGA);</td>
</tr>
<tr>
<td>with all classes</td>
<td>28S</td>
<td>1301</td>
<td>248</td>
<td>127</td>
<td>&quot;</td>
</tr>
<tr>
<td>without holothurians with all classes</td>
<td>18S</td>
<td>1713</td>
<td>325</td>
<td>194</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>18S</td>
<td>1614</td>
<td>411</td>
<td>205</td>
<td>&quot;</td>
</tr>
<tr>
<td>Scouras and Smith</td>
<td>COI,II,III ESEE</td>
<td>994aa</td>
<td></td>
<td></td>
<td>Maximum Likelihood (MOLPHY),(PUZZLE)</td>
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<tr>
<td>Harmon, this study</td>
<td>COI,II,III</td>
<td>1708aa</td>
<td></td>
<td></td>
<td>Minimum evolution (MEGA)</td>
</tr>
<tr>
<td></td>
<td>Cyt b, NADH I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Methods

28S and 18S rRNA

Echinoderms were obtained live from Florida Bay in the Florida Keys (*Ophiocoma echinata, Ophiocoma wendtii, Astrophyton muricatum, Eucidaris tribuloides, Synaptula hydriformis*), the Gulf of Mexico at Lido Key (*Luidia clathrata, Luidia senegalensis*), or shipped live from Marinus Scientific in California (*Ophioplocus esmarki, Asterina miniata*). Sperm or gonads were harvested through KCl injection or excision, respectively, and used for genomic DNA extraction. In the case of *S. hydriformis*, the whole animal was used for extraction, following a brief period of starvation. Genomic DNA (gDNA) was extracted according to the Qiagen, Inc. (Valencia, CA) gDNA extraction protocol, with the slight modification of at least a 3-hour incubation in Proteinase K.

Polymerase chain reaction was performed on the gDNA in order to separately amplify 28S and 18S genes. Primers, listed in Table 3, were synthesized by Integrated DNA Technologies (Coralville, IA) and shipped in dry form; Taq DNA polymerase was obtained from Eppendorf Brinkmann (Westbury, NY), USA. Numerous reactions with various primer pairs (Table 3) were required for different species, so differing annealing temperatures were used with the PCR amplification reaction, which ran 95° 4 minutes; 95° 1 minute; 48°-59° 45 seconds; 72° 3 minutes, for 30 cycles, followed by 72° 7 minutes.
Table 3. Primers used for PCR and/or sequencing of 28S and 18S rRNA genes. LSU universal primers taken from the European ribosomal RNA database (http://www.psb.ugent.be/rRNA/); SSU primers from Winnepenninckx et al. (1995).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Universal Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>28S rRNA</strong></td>
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<td></td>
</tr>
<tr>
<td>5' region</td>
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<td></td>
</tr>
<tr>
<td>5p1</td>
<td>NLF184/21</td>
<td>ACCCGCTGAAYTTAAGCATAT</td>
</tr>
<tr>
<td>5p2</td>
<td>NLR1126/22</td>
<td>GCTATCCTGAGGGAAACTTCGG</td>
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<tr>
<td>28S5F</td>
<td>NLF1105/22</td>
<td>CCGAAGTTTCCCTCAGGATAGC</td>
</tr>
<tr>
<td>28S5R</td>
<td>NLR1432/23</td>
<td>GTTGTACACACTCCTTAGCGGA</td>
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<td>28S5LRF</td>
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<td>GCTATCCTGAGGGAAACTTCGG</td>
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<tr>
<td>3' region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3p1</td>
<td>NLF1999/18</td>
<td>CCGCAKACAGTCTCCAA</td>
</tr>
<tr>
<td>3p2</td>
<td>NLR3284/21</td>
<td>TTCTGACCTAGGCGGTCAG</td>
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<tr>
<td>28S3R</td>
<td>NLR3535/22</td>
<td>MRGGCTKAATCTCARYRGATCG</td>
</tr>
<tr>
<td>28S3LRF</td>
<td></td>
<td>GGTYAGTCGRTCCTRAG</td>
</tr>
<tr>
<td>MCH 3F</td>
<td></td>
<td>CGCATGAAATGGATACAG</td>
</tr>
<tr>
<td>MCH 3R</td>
<td></td>
<td>TCGTTAATCCATTCATGCG</td>
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<tr>
<td><strong>18S rRNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S2c</td>
<td></td>
<td>TAAATGATCTTCCCGAGGTTCACCT</td>
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<td>18S4c</td>
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<tr>
<td>18S9</td>
<td></td>
<td>CAACTACGAGCTTTTTTAACTGCA</td>
</tr>
</tbody>
</table>
Reactions were cycled in a Mastercycler gradient PCR machine (Eppendorf Brinkmann, Westbury, NY) or Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer, Boston, MA). Regions of the 28S rRNA gene that were difficult to amplify were finally obtained with the addition of “Buffer B” from Invitrogen (Carlsbad, CA), increasing the annealing time to 2 minutes, and increasing the number of PCR cycles to 40.

PCR products were ligated into the pcr4-TOPO vector from Invitrogen (Carlsbad, CA) and transformed into Top10 chemically competent *E. coli* cells following the protocol in the TOPO cloning kit. Plasmid DNA was isolated from liquid cultures with Eppendorf Perfectprep Plasmid Mini kit (Eppendorf Brinkmann, Westbury, NY) and subsequently digested with *Eco*RI (Promega Life Science, Madison, WI) restriction enzyme. Digested plasmid DNA was separated by agarose gel electrophoresis to confirm presence of inserted 28S/18S genes, and positive clones chosen for sequencing.

DNA sequencing reactions were carried out according to the Beckman Coulter (Beckman Coulter, Inc., Fullerton, CA) DTCS Quick Start kit protocol. The reactions were sequenced using a Beckman Coulter CEQ 8000 Genetic Analysis System, and read in the CEQ 8000 Genetic Analysis System Software. Sequence data was exported from the CEQ 8000 system for analysis in Lasergene Expert Sequence Analysis Software Version 5; EditSeq and SeqMan programs (DNASTAR, Inc., Madison, WI). Numerous shorter sequences obtained from external and internal primers (see Table 3 for a list of primers) were analyzed here for overlap to assemble one long concatenated sequence per species, of which the final contigs were exported for alignment.

Pre-aligned sequences based on secondary structure of the 28S and 18S ribosomal RNA genes were obtained from the European database on large and small (respectively) subunit ribosomal RNA at [http://www.psb.ugent.be/rRNA](http://www.psb.ugent.be/rRNA) (Wuyts et al.)
Published 28S and 18S sequences from the GenBank database (28S: AF212170, AF212171, AF212169, AF212168; 18S: L28055, Z80950, D14358, D14357) and newly sequenced taxa were combined to create separate 28S and 18S data sets. All sequences were formatted using Multi-Edit version 9.10 (Multi-Edit software, Inc., © 2002-2004), opened in the Dedicated Comparative Sequence Editor (DCSE) program and aligned and edited by eye based on rRNA secondary structure annotations. Hemichordates Ptychodera flava and bahamensis (28S and 18S, respectively) were chosen as outgroups; as it has been shown Hemichordata is the most closely related phylum to Echinodermata (Cameron et al. 2000, Giribet 2002), and the Ptychodera species showed a shorter branch than other hemichordate taxa that were originally analyzed in this study.

Holothurian sequences were removed from the data set, and the first alignment was carried out with echinoid, asteroid, ophiuroid, crinoid, and outgroup sequences only. The divergent C domain, excepting the slightly conserved C1 and C1’ helices, could not be unambiguously aligned, and were deleted. The 28S gene is missing the middle portion between D7’ and E18’, but all other variable and non-variable regions were aligned by eye. The remaining sequence data were checked for congruence according to hemichordate secondary structure models and RNA pairing rules. Each test of evolution and phylogeny was tested on the data sets independently (total sites including alignment gaps: 2377=28S; 1909=18S) as well as a complete, concatenated data set representing 7 ingroup and 1 outgroup taxa. Holothurian sequences were added back into the alignment, and sequences re-aligned by eye, again removing variable regions that could not be unambiguously aligned. Regions of the genes which were excised from the complete alignment correspond to the eukaryotic helix numbering (HNE) system B10-B10’, B13_1’-B14, B15-B14’, all of the C domain except C1 and C1’, D3’, D4_1-D5_1’, E18-
E20_2’, G4’, G5_2-G5_2’, and H1-H3’in the 28S gene, and 1-1’, a portion between
E10_1-E10_1’, E23_1-E23_4, and 50-50’ in the 18S gene. Sequence data were checked
according to hemichordate secondary structure models and RNA pairing rules. Each test
of evolution and phylogeny was tested on the data sets independently (total sites
including alignment gaps: 1630=28S; 1813=18S) as well as a complete, concatenated
data set representing 9 ingroup and 1 outgroup taxa. Tree topologies were generated with
three different tree making methods; the neighbor joining distance method, a maximum
parsimony search, and a maximum likelihood search under defined models of evolution.
Neighbor joining (NJ) trees were computed with MEGA (Molecular Evolutionary
Genetics Analysis) (Kumar et al. 2001) using the Kimura 2-parameter distance model
plus a gamma shape parameter, as estimated for each data set. The gamma shape
parameter averages that were computed in the search for the best fit model for the
maximum likelihood analyses were the gamma shape estimates used in the NJ formulas.
All NJ trees computed carry bootstrap values that were generated with 1000 replicates.
The PAUP* (Swofford 2002) program was used to search tree space under maximum
parsimony criterion; the analyses were carried out with gaps treated as missing data, and
replicated 1000 times for bootstrap value information. To obtain the models of evolution
for the maximum likelihood (ML) algorithm, all four of the alignments were subjected to
Modeltest v3.6 (http://darwin.uvigo.es/), whereby hierarchical likelihood ratio test
statistics (hLRTs) and Akaike Information Criterion estimates tested 56 models of
evolution, and the best model for each data set calculated (Posada and Crandall 1998).
Each aligned data set and the best estimated parameters were loaded into PAUP*
(Phylogenetic Analysis Using Parsimony *and other methods) where ML trees were
computed under the best fit models of evolution for each data set. In the ML analyses,
the model of sequence evolution that best fit each data was estimated, and while the most likely model was used, so, too, were the next likely models to search for the most likely tree topology for all data sets; each of the ML trees were sampled with 100 bootstrap replicates.

The algorithms used in the computational processes of each tree making program are increasing complex from maximum parsimony through maximum likelihood. The mathematical aspect is beyond the scope of this paper, but the reader can refer to Nei and Kumar (2000) if more information is desired.

A few tests on the sequence alignments and tree topologies were carried out. First, pairwise distances were compared between the ophiuroid taxa and the holothuroid taxa as well as the ophiuroid taxa and the echinoid taxa with the MEGA program nucleotide: pairwise distance (p-distance) computation and the nucleotide: Kimura 2-parameter p-distance computation. As a point of reference, the distances between the ophiuroid taxa were also compared. Next, all data sets were analyzed in PAUP* for nucleotide base composition percentages, and a chi-square test of nucleotide composition homogeneity was subsequently performed. And last, Templeton and Hasegawa tests were performed in PAUP*, with the aid of Treeview (Page 1996), to test the significance of two competing tree topologies.

Mitochondrial genes

Cytochrome c oxidase I, II, and III, cytochrome b, and NADH I protein sequences from the complete genomes of 9 echinoderm taxa, Ophiura lutkeni, Ophiopholis aculeata, Cucumaria miniata, Florometra serratissima, Strongylocentrotus purpuratus, Arbacia lixula, Asterias amurensis, Asterina pectinifera, Paracentrotus lividus, were
obtained from GenBank (accession numbers NC_005930, NC_005334, NC_005929, 
NC_001878, NC_001453, X80396, NC_006665, NC_001627, NC_001572, 
respectively). The Hemichordate *Balanoglossus carnosus* (GenBank NC_001887) was 
chosen as an outgroup taxa, and the individual genes were concatenated to total a 1692 
amino acid sequence. The multiple amino acid sequences were aligned with Clustal X, 
exported to MEGA, and a Neighbor Joining tree with Poisson correction was computed 
to 1000 bootstrap replicates.
Results

28S and 18S ribosomal RNA genes

28S and 18S data were analyzed both separately and together using different algorithms with neighbor joining, maximum parsimony and maximum likelihood estimates.

The neighbor joining (NJ) trees were computed by MEGA (Kumar et al. 2001) using the Kimura 2-Parameter (k2p) model of evolution, where transitions and transversions in the data set are handled differently. Various estimates of alpha, or the gamma shape parameter (G), which estimates the distribution of the rate of nucleotide substitutions per site within the data set (Nei and Kumar 2000), were included in the tree calculations, such that the distance method used for neighbor joining trees was k2p+G. For each data set, ModelTest, through PAUP*, calculated average shapes of the gamma distribution, based on 1) all models including a gamma shape as a parameter, and 2), all models including both G and the proportion of invariable sites (I) as a parameter. It is interesting to note that when the data sets do not include the holothurians, NJ tree topologies are identical when calculated with the G calculated in the case of 1, or 2, (above) or any value in between. However, when holothurians are included in the data sets, changes in G associated with different model averaged values changes the tree topology from one calculation to the next (not all trees shown). Each NJ tree was calculated with 1000 bootstrap replicates.
Maximum parsimony (MP) trees were estimated with PAUP*, with gaps treated as missing (complete deletion approach), and calculated with 1000 bootstrap replicates.

Maximum likelihood (ML) trees were calculated through heuristic search methods in PAUP* using general time reversible (GTR), modifications of GTR, and Tamura-Nei (TrN) models of evolution. Table 4 shows the implied models used with each data set, and estimated G at ncat=8, and/or I parameters shown. Each model contains a variety of parameters that must be estimated from the data, according to Swofford et al. (1996), each of which contain the tree topology; the branch-length estimates; the relative rate parameters of the substitution models; the base-frequency parameters, and the parameters used in modeling rate heterogeneity. The GTR model has six rate parameters of substitution: a) A to C, b) A to G, c) A to T(U), d) C to G, e) C to T(U), and f) G to T(U), meaning each transition or transversion has its own category of possibility, such that the substitution matrix represents (a,b,c,d,e,f) where there are 6 substitution rate categories (nst=6), in contrast to a model like Jukes and Cantor where (a=b=c=d=e=f) and each substitution category is equally likely (nst=1) (Posada and Crandall 1998, Swofford et al. 1996). The chosen maximum likelihood models were preferred over other models on the basis of likelihood ratio tests (LRTs) and the Akaike information criterion (AIC), as computed by Modeltest 3.6 (Posada and Crandall 1998).

The Modeltest program is run through PAUP*, tests the data set(s) with 56 different models of evolution, and compares the models for a resultant justification of which one fits the data best. It thus provides a confidence in these methods of phylogenetic inference.
Table 4. Parameters used in the maximum likelihood analyses.

The models of evolution were chosen based on Modeltest’s Akaike information criterion and the parameters for each were subsequently estimated in PAUP*. I = proportion of invariant sites; G = gamma distribution and shape parameter; GTR = general time reversible model of nucleotide substitution where substitution types are (a) A to C, (b) A to G, (c) A to T(U), (d) C to G, (e) C to T(U), (f) G to T(U), and substitution rate is (a,b,c,d,e,f); TIM = Transitional model where (a=f,c=d,b,e); TrN = Tamura-Nei where (a=c, d=f,b,e); TrNef = Tamura-Nei with equal nucleotide base frequencies (as opposed to an empirical estimation); and TIMef = Transitional model with equal nucleotide base frequencies (Posada and Crandall 1998).

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameters</th>
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<th>18S</th>
<th>28S-18S</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>I=0.4546</td>
<td>G=0.5034</td>
<td>I=0.4980</td>
<td>I=0.5145</td>
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<tr>
<td>No holothurians</td>
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<td>G=0.4260</td>
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<tr>
<td></td>
<td>I=0.4260</td>
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</tr>
<tr>
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<td>G=0.4265</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIM+I+G</td>
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<tr>
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<tr>
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<td>TrNef+I+G</td>
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<td>I=0.4124</td>
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<td>GTR+I+G</td>
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<td>G=0.5034</td>
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<td>I=0.4772</td>
<td>G=0.6730</td>
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</table>
Hierarchical LRTs (hLRTs) are a commonly accepted statistic for investigating the reliability of a model to estimate the proper parameters and signal of a data set, such that \( \delta = -2 \log \Lambda \) per data set, where

\[
\Lambda = \frac{\max[L_0 (\text{Null Model})]}{\max[L_1 (\text{Alternative Model})]}. 
\]

The null model is a simple model with few parameters, as compared to a parameter rich, model as the alternative, more complex model of evolution. The value of this statistic is represented as a negative log likelihood (–lnL) value and K value, which represents the number of estimated parameters. AIC is an estimator of the Kullback-Leibler (1951) information quantity that is a quantification of the information that is lost when a mathematical model is implemented as a tool to approximate full reality. We select the model with the lowest AIC, which is to say that the expected Kullback-Leibler distance between the true model and the estimated sample is minimized, such that the model that best fits the data is chosen. The AIC formula is

\[
\text{AIC}_i = -2 \ln L_i + 2 N_i,
\]

where \( N_i \) (represented as K in Table 5) is the number of free parameters in the \( i \)th model and \( L_i \) is the maximum-likelihood value of the data under the \( i \)th sample. While both hLRTs and the AIC take into account the goodness of fit of a model to the data set(s), the AIC also penalizes models with a greater number of parameters, such that the variance of parameter estimates is also taken into account with this estimation (Posada and Crandall 1998). Table 5 delineates the Akaike Weights per model of evolution that were used in the maximum likelihood analyses. These weights were used as a confidence interval to determine the model of evolution that was the best fit (from the original 56 tested) for
each data set. Because of the immense computer time required to search tree space with the ML algorithm, each tree was calculated with 100 bootstrap replicates.

Table 5. Modeltest 3.6 model selection weights for maximum likelihood analyses.

These weights were used to determine the model of evolution that was the best fit for each data set. The Akaike weights represent a measured confidence interval; rated from best to worst. Shown here are only the best models, and therefore the models that were used in the analyses. The \(-\ln\text{Likelihood}\) number reflects the maximum likelihood value of the aligned data set. The delta value (d) shows the best fit model set to zero. Numbers within \(\Delta_i 1-2\) of the \(\Delta 0\) model are considered to have substantial support as viable models, while those having \(\Delta_i\) within 3-7 have much less support, but were considered in several cases. The models which carried a \(\Delta_i\) of greater than 10 have essentially zero support (models and weights not shown). The K value represents how many total parameters exist in each model; this number is used in the AIC calculation, where the higher numbers are more penalized. The AIC is the summation of \((-2)\ln\text{L}\) and \((2)K\) (Posada and Crandall 1998).

<table>
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<th>Data Set</th>
<th>Model</th>
<th>Akaike Weights</th>
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</tr>
</thead>
<tbody>
<tr>
<td>28S No holothurians</td>
<td>GTR+I+G</td>
<td>(-\ln\text{L}=7083.4487) AIC=14186.8975 K=10 d=0.0000</td>
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<tr>
<td></td>
<td>TIM+I+G</td>
<td>(-\ln\text{L}=7087.4927) AIC=14190.9854 K=8  d=4.0879</td>
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<tr>
<td>28S No holothurians</td>
<td>TrN+I+G</td>
<td>(-\ln\text{L}=3841.2468) AIC=7696.4937 K=7   d=0.0000</td>
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<tr>
<td></td>
<td>TIM+I+G</td>
<td>(-\ln\text{L}=3841.1921) AIC=7698.3843 K=8   d=1.8906</td>
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<tr>
<td></td>
<td>TrN+G</td>
<td>(-\ln\text{L}=3843.2788) AIC=7698.5576 K=6   d=2.0640</td>
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<tr>
<td>18S No holothurians</td>
<td>TrN+I+G</td>
<td>(-\ln\text{L}=4956.4883) AIC=9926.9766 K=7   d=0.0000</td>
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<td></td>
<td>GTR+I+G</td>
<td>(-\ln\text{L}=4953.5137) AIC=9927.0273 K=10  d=0.0508</td>
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<tr>
<td></td>
<td>TIM+I+G</td>
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<tr>
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<td>(-\ln\text{L}=4962.4873) AIC=9932.9746 K=4   d=5.9980</td>
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<tr>
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<td>28S-18S</td>
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<td></td>
<td>TIM+I+G</td>
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Ribosomal RNA tree topologies

All trees shown in Figures 4, 5a, and 5b are 50% bootstrap consensus trees, with nodes carrying a less than 50% bootstrap value collapsed, which illustrates an unresolved relationship between classes where a collapsed node (polytomy) is present. Differential sampling of the data set(s) and incorporation of variable assumptions within a variety of algorithms reveals both congruence and divergence in tree topologies.

28S-without holothurians

All analyses and all parameter inputs resulted in the same tree topology for the 28S data set when the holothurians are not included in the alignment. Without the fast evolving holothurian sequences in the data set, it provided the opportunity for an additional 588 variable nucleotides to be analyzed by the different tree making algorithms.

Neighbor joining. The Kimura 2-parameter model with gamma shape parameter inputs, as estimated by PAUP*, results in the tree topology ((((O,E)A)C)out), as shown in Figure 4. The bootstrap value of 89% that links the ophiuroids and echinoids as sister taxa and the asteroids outside of this ophiuroid/echinoid clade, is a highly supported node. The average gamma shape parameter, estimated by PAUP*, of all ModelTest models with G+I parameters is 0.50, while the average of +G only models is 0.19, but the tree topology does not waver with this wide range of alpha inputs. As expected, the crinoids come out as a sister group to the eleutherozoan clade.

Maximum Parsimony. Although the ophiuroid/echinoid clade is only supported by a bootstrap value of 60% in the parsimony analysis, the tree (see Figure 4) still shows
the topology (((((O,E)A)C)out). All classes are monophyletic, and the three eleutherozoan classes group together with a bootstrap value of 100%.

**Maximum Likelihood.** The maximum likelihood tree topology shown in Figure 4 was estimated with four different models of evolution (GTR+I+G and TIM+I+G being the most supported models), as shown in Table 5; each one supported the same topology, (((((O,E)A)C)out). The echinoid/ophiuroid clade is supported here (94% bootstrap value) as it was with both 28S NJ and MP analyses. The asteroids group outside of the ophiuroid/echinoid clade, and the crinoids group outside of the eleutherozoan classes.

**28S-holothurians included**

The complete 28S rRNA data set results show that tree topologies corroborate the results of the data set with holothurians excluded; discussed above. Ophiuroids and echinoids share a sister group relationship in both maximum parsimony and maximum likelihood analyses, and the neighbor joining topology is not inconsistent with this finding.

**Neighbor joining.** The tree in Figure 5a, calculated with G=0.20, illustrates the long branch of the holothurian class is placed outside of the polytomy between the ophiuroid, echinoid, and asteroid classes, with a bootstrap value of 76%. The crinoid class is placed outside of the eleutherozoan clade with a bootstrap value of 96%.

**Maximum Parsimony.** The echinoid/ophiuroid clade, with asteroids as sister taxa, is highly supported by a 91% bootstrap value in this analysis. The holothurians group alone outside of the echinoid/ophiuroid/asteroid clade, with a bootstrap value of 59%. As expected, the crinoids group outside of the eleutherozoan clade with a bootstrap value of 96%, as can be seen in Figure 5b.
**Maximum Likelihood.** Figure 5b shows the maximum likelihood results under the model parameters listed in Table 4, where all models of evolution generated the same topology. This topology substantiates the topology of the same data set calculated in the maximum parsimony search, discussed above, albeit with bootstrap values of 69%, 59%, and 100%, respectively.

**18S-without holothurians**

The 18S data set without holothurians results in two different topologies with maximum parsimony and maximum likelihood analyses, while the neighbor joining tree is not inconsistent with either the MP or ML topologies. This is obviously much less certain than the 28S data set that showed the same relationships with each type of tree making algorithm.

**Neighbor joining.** Figure 4 shows the topology (((O,E,A)C)out) delineating only those nodes which carry greater than 50% bootstrap value. The polytomy represents that there is clearly not a discernable relationship between the three ingroup taxa. Average G with models including the gamma shape=0.16 and average G with models including G+I=0.61; the tree topology is the same throughout.

**Maximum Parsimony.** With the 18S data set and holothurians not included in the alignment, the asteroid and echinoid classes come together as sister groups, with the ophiuroids grouping outside of this; the node is supported by a bootstrap value of 59%. The pelmatazoan species group outside of the ingroup taxa, with a bootstrap support of 98%, and all groups have 100% bootstrap support for monophyletic origins, as shown in Figure 4.
Maximum Likelihood. The relationships between the echinoderm classes with TrN+I+G, GTR+I+G, TIM+I+G and TrNef+I+G models of evolution show the relationship ((((O,E)A)C)out), which corroborates the 28S tree topologies. The ophiuroid/echinoid clade with asteroids grouping outside, carries a bootstrap support value of 58%, and all classes show a monophyletic bootstrap support of 100%. The crinoids group outside of the (O,E,A) clade, with a bootstrap support of 100%. See Figure 4.

18S-holothurians included

The results of the 18S data set with holothurians in the alignment are different from both the 28S and the 28S-18S data sets, in that a sister group relationship between the echinoids and holothurians is supported. The MP and ML tree making methods with this gene result in unresolved relationships of the ophiuroids and asteroids, but the echinoid/holothuroid clade is supported, while the NJ analysis results in a completely unresolved topology with respect to all four of the eleutherozoan classes.

Neighbor Joining. The tree topology (G=0.67) shown in Figure 5a reveals a four part polytomy between the ophiuroid, echinoid, asteroid, and holothuroid classes, with the crinoid taxa outside of the clade, supported by a bootstrap value of 86. Again, the long branch of the holothurians is shown.

Maximum Parsimony. The maximum parsimony search (Figure 5b) results in a sister grouping of the echinoid and holothuroid classes, supported by a bootstrap value of 62%. This tree topology reveals an unknown relationship between the ophiuroid and asteroid classes to each other and to the echinoid/holothuroid clade, with the eleutherozoan classes together supported by a bootstrap value of 84%. The crinoids
group outside the eleutherozoan classes. This topology supports the maximum parsimony finding revealed by Littlewood et al. (1997), as shown in Figure 3.

**Maximum Likelihood.** TrNef+I+G, TrN+I+G, and TIMef+I+G models of evolution (see Table 4 for specific parameters) all result in the topology shown in Figure 5b. This tree is identical to the tree produced with maximum parsimony, but with the echinoid/holothuroid clade even more supported, with a bootstrap value of 72%, and the eleutherozoan clade supported by a bootstrap value of 83%. All classes are monophyletic with 100% bootstrap support.

28S/18S-without holothurians

The maximum parsimony analysis of the concatenated data set shows an unresolved relationship, while the neighbor joining and maximum likelihood results show the ophiuroids and echinoids as sister taxa with high bootstrap support. The support for an ophiuroid/echinoid clade is congruent with supported results of all tree making methods with the 28S data set alone, and with the maximum likelihood analysis of the 18S data set.

**Neighbor joining.** The topology shown in Figure 4 demonstrates that the combined 28s/18s data set corroborates the topology shown with the 28S data set alone, with slight deviation from the bootstrap values at only two nodes. Gamma shape input 0.17, but the topology remains unchanged through to G=0.63.

**Maximum Parsimony.** An unresolved topology is introduced in the maximum parsimony analyses in Figure 4 with the concatenated data set. All groups are monophyletic, and the three ingroup taxa are highly supported, but the tree shows a three
part polytomy between the ophiuroids, echinoids, and asteroids. This is not inconsistent with an ophiuroid/echinoid pairing.

Maximum Likelihood. The topology of the combined data set shown in Figure 4 reveals the topology (((((O,E)A)C)out), which corroborates the results of both the 28S and 18S maximum likelihood topologies. The ophiuroid/echinoid clade with the asteroids outside, is supported here by a bootstrap value of 90%. As expected, the crinoids fall outside this eleutherozoan clade with a bootstrap value of 100%.

28S/18S-holothurians included

The complete data set with holothurians included in the alignment shows that the trees estimated with NJ and ML algorithms result in opposite topologies with respect to the ophiuroids, echinoids, and asteroids. The MP topology neither discounts nor is inconsistent with either the NJ or ML trees of this data set.

Neighbor Joining. The complete rRNA data set with all taxa present results in a tree topology of (((((A,E)O)H)C)out) under a k2p+G model assumption with G=0.40. The asteroid and echinoid classes come together as sister groups, with the ophiuroids grouping outside of this; the node is barely supported by a bootstrap value of 52%. The holothurians group outside of the asteroid/echinoid/ophiuroid clade with a bootstrap support of 69%. The pelmatazoan crinoid groups outside of the ingroup taxa, with a bootstrap support of 96%, as shown in Figure 5a.

Maximum Parsimony. An unresolved relationship between the ophiuroids, echinoids, and asteroids, supported by a bootstrap value of 64%, with the holothurians outside is illustrated in Figure 5b. The tree topology ((((O,E,A)H)C)out) shows the crinoids outside of the 4 class clade, with a bootstrap support of 92%.
Maximum Likelihood. Figure 5b shows the maximum likelihood results under three models of evolution (parameters listed in Table 4), where all models generate the same topology. This topology substantiates the topology of the 28S data set with the searches of both MP and ML methods. With the complete data set, the tree topology, ((((((O,E)A)H)C)out), supports the ophiuroids and echinoids as sister taxa with asteroids outside, and holothurians as sister taxa to the ophiuroid/echinoid/asteroid clade, with bootstrap support values of 60% and 52%, respectively. The eleutherozoan clade is supported by a bootstrap value of 99%, with the crinoids basal.

Figure 4. Neighbor joining, maximum parsimony, and maximum likelihood tree topologies; without holothurians in the data sets.

28S, 18S, and the concatenated sequence results are shown on the next page as a 3x3 matrix, in simplified form, and with nodes at less than a bootstrap support of 50, collapsed. For the concatenated data set, Ophiuroid=Ophiocoma echinata and Ophioplocus esmarki, Echinoid=Strongylocentrotus purpuratus and Arbacia sp. (A. punctulata for 28S and A. lixula for 18S), Asteroid=Asterias sp. (A. forbesii for 28S and A. amurensis for 18S) and Asterina sp. (A. miniata for 28S and A. coronata for 18S), Crinoid=Florometra serratissima for 28S and Antedon serrata for 18S, and Outgroup=Ptychodera sp. (P. flava for 28S and P. bahamensis for 18S). The neighbor joining trees were computed through MEGA under the k2p+G model of evolution, with 1000 bootstrap replicates, and are drawn to depict branch lengths. The maximum parsimony analyses were carried out with all columns containing gaps deleted; the bootstrap consensus trees are reported. Specific models of evolution and parameters used in maximum likelihood analyses can be found in Table 4.
Figure 5a. Neighbor joining tree topologies; all echinoderm classes are included in the data sets.

28S, 18S, and the concatenated sequence results are shown here in simplified form and with nodes at less than a bootstrap support of 50, collapsed. For the concatenated data set, Ophiuroid = *Ophiocoma echinata* and *Ophioplocus esmarki*, Echinoid = *Strongylocentrotus purpuratus* and *Arbacia sp.* (*A. punctulata* for 28S and *A. lixula* for 18S), Asteroid = *Asterias sp.* (*A. forbesii* for 28S and *A. amurensis* for 18S) and *Asterina sp.* (*A. miniata* for 28S and *A. coronata* for 18S), Crinoid = *Florometra serratissima* for 28S and *Antedon serrata* for 18S, and Outgroup = *Ptychodera sp.* (*P. flava* for 28S and *P. bahamensis* for 18S). The neighbor joining trees were computed through MEGA under the k2p+G model of evolution, and are drawn to depict branch lengths.
Figure 5b. Maximum parsimony, and maximum likelihood tree topologies; all echinoderm classes are included in the data sets. 28S, 18S, and the concatenated sequence results are shown on the next page, in simplified form, and with nodes at less than a bootstrap support of 50, collapsed. For the concatenated data set, Ophiuroid=Ophiocoma echinata and Ophioplocus esmarki, Echinoid=Strongylocentrotus purpuratus and Arbacia sp. (A. punctulata for 28S and A. lixula for 18S), Asteroid=Asterias sp. (A. forbesii for 28S and A. amurensis for 18S) and Asterina sp. (A. miniata for 28S and A. coronata for 18S), Crinoid=Florometra serratissima for 28S and Antedon serrata for 18S, and Outgroup=Ptychodera sp. (P. flava for 28S and P. bahamensis for 18S). The neighbor joining trees were computed through MEGA under the k2p+G model of evolution, and are drawn to depict branch lengths. The maximum parsimony analyses were carried out with all columns containing gaps deleted; the bootstrap consensus trees are reported. Specific models of evolution and parameters used in maximum likelihood analyses can be found in Table 4.
Pairwise distance analysis

For any given orthologous gene, when a pair of taxa with different DNA sequences, B and C, have diverged from a common ancestor with DNA sequence A, the descendant sequences have differentiated from the A sequence by gradual nucleotide substitutions. The straightforward measure of sequence divergence between pairs of sequences (A to B, A to C, or B to C) can be calculated by the number of nucleotide sites that are different, divided by the total number of nucleotide sites. This difference is then the proportion (p) of sites at which the pair of sequences are divergent from one another, which represents an evolutionary distance between the sequences, and is called the p-distance. Kimura’s two parameter method of p-distance estimation takes into account that the rate of transitional substitutions is usually higher than the rate of transversional substitutions, so that the rate at which transitions and transversions evolve over time are given different values (Nei and Kumar 2000).

Without the holothurians in the alignment, more variable regions (588 nucleotides and 99 nucleotides for 28S and 18S, respectively) of the rRNA genes were unambiguously aligned and used in the final matrices. The data sets without holothurians included more sites and more variability, while the data sets that included the holothurians had many sites removed because they could not be accurately aligned. Therefore, the complete data sets contain fewer nucleotides and a higher proportion of conserved regions than the data sets missing the holothuroid class. I analyzed the distances between the ophiuroid taxa and the holothuroid taxa as well as the ophiuroid taxa with the echinoid taxa; each of these distances is displayed in Table 6 as an overall class average of each taxon per class.
Table 6. Pairwise distances between selected sequences.

These corrected and uncorrected distances illustrate the number of nucleotide differences between species in selected classes. O=Ophiuroid, E=Echinoid, H=Holothuroid. The 28S data set without holothurians has 588 nucleotides more than the complete 28S data set; the 18S data set without holothurians has 99 nucleotides more than the complete 18S data set. P-distance=uncorrected pairwise distances average between all species in given classes, k2p p-distance=see above; corrected for transition/transversion ratios. Without holothurians the alignments were variable nucleotides for the 28S and nucleotides for the 18S, respectively.

<table>
<thead>
<tr>
<th></th>
<th>28S</th>
<th>18S</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>p-distance</td>
<td>k2p p-distance</td>
</tr>
<tr>
<td>Without holothurians</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-E</td>
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</tr>
<tr>
<td>O-O</td>
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<td>0.089</td>
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<tr>
<td>Complete data set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-H</td>
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<td>0.093</td>
</tr>
<tr>
<td>O-E</td>
<td>0.041</td>
<td>0.042</td>
</tr>
<tr>
<td>O-O</td>
<td>0.028</td>
<td>0.029</td>
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</table>

As a point of reference, the distances between the ophiuroid taxa were compared. The pairwise distances of both taxa per class (or in the case of ophiuroid-ophiuroid, the comparison was species to species) were computed by MEGA with both the nucleotide: pairwise distance (p-distance) option and the nucleotide: Kimura 2-parameter p-distance option.

The p-distances between the ophiuroid taxa in the data set without the holothurians vs. the complete data set, shows that the variable regions in the 28S set are, indeed, quite variable. The p-distances between the ophiuroid and echinoid classes, when holothurians are removed, shows that greater distances are present in the 28S set than the 18S set, as a result of the many variable regions present in the 28S rRNA gene.
When the holothurians are added into the alignment, and subsequently, the highly variable regions removed, the p-distances between the ophiuroids/holothurians and ophiuroids/echinoids are greater in the 18S data set than they are in the 28S data set. This shows that to include the fast evolving holothurian 28S rRNA gene into the alignment of the echinoderm 28S data set is to remove the information that the divergent regions of the 28S provide, rendering the 28S alignment no more informative (in distance data) than the 18S.

The distances between the ophiuroid taxa, whether or not the holothurians are in the alignment (highly variable regions present or removed), shows that the 18S distances are less than the 28S distances, which is consistent with the fact that the 18S rRNA gene is a more conserved gene than the 28S rRNA gene.

Nucleotide composition and test of nucleotide composition homogeneity

A PAUP* estimation of the nucleotide base composition and a chi-square test of homogeneity of the nucleotide frequencies was determined for each of the rRNA data sets independently and on the concatenated 28S-18S data sets. The nucleotide frequencies were calculated using all sites in each alignment, and the frequencies of each base are reported as a percentage of total nucleotide composition. The frequencies of uracil, cytosine, adenine, and guanine bases, as well as total number of nucleotides per class, can be found in Tables 7, 8, and 9, for 28S, 18S, and the combined data set, respectively.

The chi-square test of homogeneity tests the degree of inequality in the nucleotide composition, and is important because G-C rich or A-T rich sequences can add systematic error into tree topologies by introducing bias and artificial attraction of
sequences. Rejection of homogeneity is at p<0.05, which would cast doubt upon the phylogenetic analysis because of the pitfall mentioned above. The chi-square test was performed on the composition of nucleotides in each data set in order to discount nucleotide biases in each, and therefore solidify the topologies of the calculated trees. The chi-square ($x^2$) values, p values, and degrees of freedom (dF) for each data set are listed in Tables 7, 8, and 9, for 28S, 18S, and the combined data set, respectively, and reveal that all data sets pass the test, and are homogeneous.
Table 7. Nucleotide composition and chi-square test of nucleotide composition homogeneity for 28S rRNA data sets.

<table>
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<tr>
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<th>Nucleotide composition</th>
<th>Chi-square test</th>
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</thead>
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<tr>
<td></td>
<td>T (U)</td>
<td>C</td>
</tr>
<tr>
<td><strong>28S rRNA without</strong></td>
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</tr>
<tr>
<td><strong>holothurians</strong></td>
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<tr>
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</tr>
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<td><strong>data set</strong></td>
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<tr>
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<td>22.7</td>
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</table>
Table 8. Nucleotide composition and chi-square test of nucleotide composition homogeneity for 18S rRNA data sets.

<table>
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<tr>
<th></th>
<th>Nucleotide composition</th>
<th>Chi-square test</th>
</tr>
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<tr>
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<tr>
<td>18S rRNA complete</td>
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<tr>
<td>data set</td>
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<tr>
<td>Hemichordate</td>
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Table 9. Nucleotide composition and chi-square test of nucleotide composition homogeneity for the concatenated 28S-18S rRNA data sets.

<table>
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<tr>
<th>Nucleotide composition</th>
<th>28S-18S rRNA without holothurians</th>
<th>28S-18S rRNA complete data set</th>
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<td>C</td>
</tr>
<tr>
<td><strong>Asteroid</strong></td>
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<td>22.6</td>
<td>22.9</td>
</tr>
</tbody>
</table>
Mitochondrial gene analysis

Cytochrome c oxidase I, II, and III, cytochrome b, and NADH I amino acid sequences generated the tree topology shown below in Figure 6. These results corroborate the tree topology shown in the Scouras and Smith (2001) analysis (Figure 1) that was generated using only the concatenated amino acid sequences of the Cytochrome c oxidase I, II, and III proteins. The ophiuroids have a long branch that is a result of fast evolving sequences in this class, and they group outside of all other echinoderm classes, including the crinoids. The neighbor joining tree with 1000 bootstrap replicates groups the echinoids and asteroids as sister taxa with a bootstrap value of 64%, with the holothurians as the sister taxa to the echinoid/asteroid clade with a bootstrap value of 99%. The crinoids are aberrantly grouped to the echinoid/asteroid/holothuroid clade with a bootstrap value of 64%.
Figure 6. Mitochondrial gene amino acid sequence tree.

Discussion

The phylogenetic position of the Ophiuroidea within the Echinodermata has been poorly understood. While adult morphology and the fossil record place the ophiuroid class as sister taxa to the asteroid class, investigations into larval morphology pair the ophiuroid class and the echinoid class as sister taxa. Molecular phylogenies have placed the ophiuroids as sister taxa to an echinoid/holothuroid clade, sister taxa to an echinoid/holothuroid/asteroid clade, sister taxa to the asteroids, or even as a sister taxa to all other echinoderms, including crinoids (see Hyman 1955, Paul and Smith, 1984, Smith 1984, 1988, 1992, Smiley 1988, Raff et al. 1988, Strathmann 1988, Ratto and Christen 1990, Wada and Satoh 1994, Pearse and Pearse 1994, Littlewood 1995, Littlewood et al. 1997, and Janies 2001 for a review of morphological, fossil, and molecular data analyses). In this study, the highly variable regions of the 28S rRNA gene, aligned with respect to only secondary structure and not nucleotide similarity, have shed light on this quandary. An extensive maximum likelihood analysis points to the finding that the ophiuroids and the echinoids shared a recent common ancestor in the phylum Echinodermata.

Tree topologies

The Holothuroidea rRNA genes are evolving at a faster rate than the other echinoderm classes (Wada and Satoh 1994, Littlewood et al. 1997). If the holothurian taxa are removed from the data sets for an initial alignment, an additional 588
informative sites for the 28S gene and 99 informative sites for the 18S gene prove to be alignable with less ambiguity than when the aberrant holothurians remain. The 28S, 18S, and concatenated data sets that include these variable regions and are lacking holothurian taxa will subsequently be called the variable data sets. The analyses of the variable 28S DNA alignment showed high support (bootstrap values of 89%, 60%, and 94% for NJ, MP, and ML trees, respectively) for an ophiuroid/echinoid clade. The variable 18S DNA alignment showed support for an ophiuroid/echinoid clade in maximum likelihood (bootstrap 58%) analyses, while the neighbor joining tree was not inconsistent with that finding. The concatenated data set showed high support (bootstrap values of 83% and 90% for NJ and ML, respectively) for an ophiuroid/echinoid clade, and was not inconsistent with that finding in the MP search. With the fast evolving holothurian sequences removed from the alignment, the data sets are reasonably stable, as evidenced by the similar results obtained from the three different tree making methods under differing assumptions (see Figure 4). Changes in parameters such as gamma shape values, proportion of invariant sites, and nucleotide frequencies are not as prone to change the overall tree topologies, illustrated by the fact that only one of the nine trees shown in Figure 4 is inconsistent with a sister grouping of the ophiuroid and echinoid classes. It is worthwhile to note that there were between two and four trees computed with NJ and ML (data not shown) algorithms, under varying parameters and models of evolution for each data set, and the tree topologies in each case corroborated the reported topologies, only differing slightly in bootstrap values.

According to Swofford and Olsen (1990), when regions of sequences are so divergent that a reasonable alignment cannot be attained, those regions should be eliminated from the analysis. The holothurian taxa have numerous insertions that the
other taxa do not have; a fact that makes it difficult to align nucleotides into their appropriate position in already highly substituted domains. As such, when the holothurian taxa were added back into the alignment, the aforementioned variable sites had to be deleted from the alignment, creating shorter (less 588 nucleotides from the 28S and less 99 nucleotides from the 18S alignment) data sets with a higher percentage of conserved sites to total sites, and will subsequently be called the complete data set. The complete 28S rRNA gene data still shows support for an ophiuroid/echinoid clade, with bootstrap values of 91% and 69% in the maximum parsimony and maximum likelihood trees, respectively, while the neighbor joining tree is not inconsistent with this result.

The complete 18S rRNA gene data set groups the echinoid and holothuroid classes as sister groups in both the maximum parsimony (bootstrap support 62%) and the maximum likelihood (bootstrap support 72%) trees, which is consistent with support for an echinoid/holothuroid clade as previously shown with 18S DNA analyses (Raff et al. 1988, Ratto and Christen 1990, Marshall 1994, Wada and Satoh 1994, Littlewood 1995, Littlewood et al. 1997, Janies 2001). The neighbor joining tree computed from the distances of the complete 18S data set is not inconsistent with support for an echinoid/holothuroid clade, but is not inconsistent with any finding for the eleutherozoan classes, as the topology is a clade showing a four part polytomy. The complete data set with both 28S and 18S genes represented shows support for an ophiuroid/echinoid clade (bootstrap 60%) under the maximum likelihood algorithm, while the maximum parsimony search for the same data set generated a three part polytomy between the ophiuroids/echinoids/asteroids, so is not inconsistent with an ophiuroid/echinoid grouping. The neighbor joining tree of this data set shows an aberrant sister class
grouping of the asteroids with the echinoids, although it is barely supported with a 52% bootstrap value, with the ophiuroids as a sister group to the echinoid/asteroid clade.

Analyses of echinoderm phylogeny using larval characteristics have previously shown support for a sister grouping of the ophiuroid and echinoid classes (MacBride 1914, Hyman 1955, Smith 1984, Smiley 1988, Littlewood et al. 1997), but molecular data analyses have previously not corroborated support for an ophiuroid/echinoid clade. Specifically, ribosomal RNA gene analyses, both 28S and 18S, have previously shown support for an echinoid/holothuroid clade (Raff et al. 1988, Ratto and Christen 1990, Marshall 1994, Wada and Satoh 1994, Littlewood 1995, Littlewood et al. 1997, and Janies 2001), while the relationship of the ophiuroids to the other classes is largely unknown. The more supported sister grouping in this analysis is the ophiuroid and echinoid clade, as shown with 28S rRNA data alone and with the combined 28S/18S alignment, while the 18S alone shows support for an echinoid/holothuroid clade.

Previous inquiries into echinoderm phylogenies have concluded that each class represents a true monophyletic group, and that Crinoidea groups outside of the other four classes, separating the eleutherozoan classes from the pelmatazoa (Hyman 1955, Paul and Smith 1984, Field et al. 1998, Smiley 1988, Smith 1988, Ratto and Christen 1990, Wada and Satoh 1994, Pearse and Pearse 1994, Littlewood 1995, Littlewood et al. 1997, Janies 2001). The results presented here, with both 28S and 18S rRNA gene alignments, support previous conclusions that there are 5 monophyletic classes in the phylum, and that the pelmatazoa are the most basal of the echinoderm classes. The eleutherozoan classes will subsequently be referred to as the ingroup taxa of the phylum. Littlewood et al. (1997) showed support for an echinoid/holothuroid clade as a result of independent analyses of both the 28S and 18S rRNA data sets. In this study, the 18S tree topology
corroborates the echinoid/holothuroid pairing, as the data sets are not remarkably
different in terms of nucleotide sequence data and available signal from the alignment.
The 28S rRNA gene analysis in this study, however, reveals a tree topology that is
markedly different, showing support for an ophiuroid/echinoid clade. It is assumed that
the greatest reason for this difference is the 4-fold increase in 28S rRNA nucleotide
sequence data. It should be noted that the overall increase in aligned nucleotides in the
28S data set is 1088 nucleotides from the original 313 analyzed by Littlewood et al.
(1997). The number of parsimoniously informative sites (p.i. sites) reported from the
Littlewood et al. (1997) study (107 p.i. sites) was from an original data set that included
41 echinoderm taxa, however, the bootstrapped MP tree was generated with only 16 of
those taxa, but the statistics of the number of p.i. sites for this reduced alignment were not
reported. The comparison between p.i. sites for the 28S DNA listed in Table 2 would
reveal more of a difference if it were a contrast between the Littlewood et al. 16 taxa, 313
nucleotide alignment and the 9 taxa, 1301 nucleotide alignment analyzed in this study.
The method of alignment is also different, in that the Littlewood et al. (1997) data were
aligned with a progressive alignment algorithm, but the sequences in this study were
aligned manually by eye based on known hemichordate rRNA secondary structure. In
addition, the ML tree making method used in this analysis made compensations to
overcome molecular phylogenetic pitfalls that are likely to be encountered when
molecular sequence data is analyzed with MP, as the MP method was originally designed
to compute trees based on binary state morphological characters.
**18S ribosomal RNA**

In spite of the compensations made to the 18S rRNA data set, the results obtained from this study corroborate previous analyses of this gene. Namely, the echinoids and holothurians group together, the crinoids group outside of the eleutherozoan classes, and there remains an unresolved relationship with respect to the ophiuroid and asteroid classes. A careful alignment method that was chosen to avoid biases that may have been previously introduced by progressive alignment algorithms did not make a huge impact on the relatively conserved nature of the 18S rRNA gene. An extensive search for the correct model of evolution, and subsequent ML analyses with the models fully defined did not alter the results of previously reported topologies of the echinoderm 18S rRNA gene using MP or distance methods. The fact that the 18S DNA alignments result in the same topology with and without a manual alignment, and with and without an extensive ML analysis, begs the question of the usefulness of this gene in discerning echinoderm class relationships. Compared to the 28S rRNA gene, the more conserved, slowly evolving 18S can be aligned with ease, but as a result of the nucleotide conservation that makes the alignment relatively easy to work with, the phylogenetic signal is unlikely to be strong enough to record the correct divergence events. It was noted by Smith (1989), just one year after the pioneer (Raff et al. 1988 was the first to use sequence data from all 5 classes) 18S DNA echinoderm phylogeny was published, that there is a lack of signal in echinoderm 18S rRNA sequences, and it is unlikely that distance or parsimony analyses of raw 18S rRNA sequence data will resolve the echinoderm class relationship problem. Philippe et al. (1994) went further to claim that the 18S rRNA sequence data could not be used in confidence to resolve any metazoan divergence event that is separated by a time interval of less than about 40 million years. Based on the fossil record, the echinoderm
classes all evolved and radiated within a very short period of geological time, which makes the relationships between them very hard to discern. The fossil record left by the echinoderm radiation in the Mid-Cambrian and Ordovician ages indicates that all 5 classes differentiated and diverged in about 100 million years, meaning there was a divergence event on the average of every 20 million years (Smith 1988). Yet, the complete 18S rRNA gene has been consistently used (Raff et al. 1988, Marshall 1994, Wada and Satoh 1994, Littlewood 1995, Littlewood et al. 1997, Janies 2001) in studies of the molecular phylogeny of echinoderm classes. In light of the advancements made in this study to compensate for the possible pitfalls of molecular phylogenetic analyses, the 18S DNA tree topology supports the topology of previous studies. In the graphs of the gamma shape distribution curves shown in Figures 7 and 8, the areas to the far left represent invariant sites, while the areas to the far right are the most variable and all categories in the middle are then on a scale from decreasing rate to increasing rates of substitution from left to right. If we look at the shape of the gamma distribution of the 18S rRNA sequence, as represented in Figures 7 and 8, it is clear that there are less variable sites in the 18S alignment as compared to the combined alignment, and especially as compared to the 28S alignment. On the other hand, the gamma shape distribution of the 28S alignment shows many more sites in the categories between about 10 and 20 on the x-axis; representing sites that are not completely without substitution, but which have less substitutions than the most highly variable sites. With most of the sites in the 18S alignment clearly between about 0 and 10 on the x-axis of the gamma shape distribution graph, it is easy to visualize why the 18S echinoderm gene does not carry sufficient signal to discern the echinoderm class relationships, as these are completely invariant to fairly conserved sites.
Figure 7. Plot of gamma shape distribution curves of the echinoderm rRNA data sets used in this study.

The categories of nucleotide substitution have an increasing rate from left to right, such that sites to the far left are invariant, while sites to the far right have multiple substitutions, meaning they are more variable sites. Clearly the 28S alignment is most represented by sites in the mid region of the categories, not too fast, but not invariable. The sites in the 18S alignment are mostly invariant to slightly variable, while the combined data set proves to be a combination of the variable characters of the independent data sets.
Figure 8. Plot of gamma shape distribution curves of the 28S and 18S echinoderm rRNA data sets used in this study.

The categories of nucleotide substitution have an increasing rate from left to right, such that sites to the far left are invariant, while sites to the far right have multiple substitutions, meaning they are more variable sites. Clearly the 28S alignment is most represented by sites in the mid (10-20) region of the categories; not too fast, but not invariable, while the 18S has barely any sites in these important mid-range categories. The sites in the 18S alignment are mostly invariant to slightly variable.
The greatest proportion of sites in the 18S gene are either invariant or highly conserved, as verified in the variability map of the gene (SSU) shown in Appendix 1. The variability map of the eukaryotic 28S (LSU) rRNA gene is shown in Appendix 2 for comparison to the 18S map, where 0 (purple) represents invariant sites in the alignment, < 25% (grey) represents sites in the alignments that were not alignable across taxa or missing altogether in some taxa. Blue, green, yellow, orange, and red (1, 2, 3, 4, 5, respectively) represent sites from highly conserved to highly variable.

Kishino-Hasegawa (K-H), Templeton, and Winning-sites tests were performed on the 18S data set. These tests compared the tree topologies (out(C(A(O(E,H)))))) and (out(C(H(A(O,E)))))) as competing phylogenetic hypothesis for the 18S alignment (the trees can be seen in Figure 9). The results of each test reveals that the topology grouping echinoids and holothurians together has the lowest -lnLikelihood and the shortest tree length (change in -lnLikelihood for the ophiuroid/echinoid topology is -5.01779 and change in length is 4 from the -lnLikelihood value and length of echinoid/holothuroid topology), where the p-values of the comparison between the two topologies are 0.4329, 0.4328, and 0.5572 for K-H, Templeton, and Winning-sites tests, respectively. These values are all well above the 0.05 p-value that would define the two topologies as significantly different, meaning that although the topology grouping the echinoid/holothuroid taxa together has a slightly shorter length and a slightly more positive -lnLikelihood value, neither topology can be declared as optimal over the other one. It does not appear that the echinoderm 18S rRNA gene alignment will ever be able to definitively reconcile the relationships between the classes, so perhaps after sixteen years of knowingly trying to make a mountain (or a tree) out of a mole hill, echinoderm
systematic studies should let the 18S rRNA molecules rest, and move on to something that has the potential to shed light on the class relationship problem.

28S ribosomal RNA

The 28S DNA contains many more regions with evolutionary rates that may be sufficient to discern echinoderm class relationships (Smith 1992). Ratto and Christen (1990) pioneered the first echinoderm 28S rRNA gene phylogeny, using a distance analysis computed from 243 nucleotides of the 5 prime end of the gene, and came up with a tree topology that showed an echinoid/holothuroid clade, with the ophiuroid as sister taxa to the echinoid/holothuroid clade, and the asteroids in between the 3 class clade and the crinoids. However, this study did not include any bootstrap or other support values, so the validity of the grouping in this tree is unknown. Littlewood et al. (1997) expanded the same 5’ region of 28S DNA to a sequence alignment of 313 nucleotides with many more taxa, aligned the multiple set with Clustal W, and computed a parsimony analysis that resulted in the same topology as that obtained by Ratto and Christen (1990), but with nodes at less than 50% bootstrap values collapsed, all resolution in the tree is lost except for the grouping of the echinoid and holothuroid taxa. Janies (2001) re-aligned the 28S rRNA sequences, again using Clustal, but ran the alignment through two more progressive alignment algorithms. The alignment was again analyzed with the MP method, and again the results showed support for an echinoid/holothuroid clade. The re-analysis by Janies (2001) did not provide a definitive answer into the question of echinoderm class relationships.

This is the first study that has used 1) a nearly complete 28S rRNA gene, 2) many more variable regions of the gene than the previously analyzed 5’ portion, 3) a
completely manual method of sequence alignment (i.e. not based on a progressive alignment algorithm), and 4) an extensive ML analysis with numerous compensations for the rates at which the data evolve. Field et al. (1988) suggested that branching resolution in a ground breaking 18S DNA study of metazoan phylogeny (including sequence data from all but holothuroid echinoderms) could be solved by acquiring the more extensive 28S rRNA data sets, and developing new techniques to analyze data from regions of rapid sequence evolution. Molecular phylogeny was a field in its infancy in the early 1990’s, and the tree making algorithms in wide use at that time, such as MP, were not designed to handle the different ways and rates at which DNA sequence can evolve. It was thought that perhaps the 28S gene substitutions could be diluted and cause confusion in any original phylogenetic signal, because this more quickly evolving gene (in comparison to the 18S gene) has possibly undergone mutation several times over (Smith 1992). However, we now have the ability to extensively test for the best model of evolution, the ability to compensate for site to site variation and unequal rate effects, and the computer capability to perform an ML bootstrap analysis on a decent sized data set, with a model of evolution fully defined, in record time of two-three days each. With this, I believe that the 28S gene has, in fact, shed some light on the question of echinoderm class relationships. According to Swofford et al. (1996), even in analyses where parsimony is consistent, alternative methods that incorporate models of evolutionary change can make more effective use of the data set in question. In fact, at the time of publication of the 2nd edition of *Molecular Systematics*, using models that require explicit modifications based on evolutionary change over models that do not was viewed as a positive trend that was transforming the face of molecular phylogenetic analyses (Swofford et al. 1996).
In phylogenetic analyses, the parsimony method is a more effective method of achieving the best tree topology than is a distance method (Littlewood 1995, Swofford et al. 1996), while a likelihood approach has clear advantages over both MP and distance methods. Both MP and ML are criterion-based methods that have an immediate advantage over NJ (distance) methods, because a search through tree space (possible groupings) results in topologies that are ranked in order according to chosen criteria.

With the ML method, the user has the capability to define more criteria than in the MP method, and in this way it is a more complex and robust model. However, even though the MP method does not require any explicit model to be defined, the inherent criteria still make assumptions regarding the data, such as all sites evolve at the same rate, or all nucleotide bases are equally represented. The MP method could obviously lead to a violation of the data in the absence of a defined model, whereas in an ML analysis a model and all of its assumptions are fully defined. The purely algorithmic NJ method results in a single topology, so the user could be misled that the one topology was the best tree, when MP or ML methods would reveal that there were 100 or 1000 other topologies that are equally as likely (Swofford et al. 1996). When we consider, however, that both NJ and ML methods can compensate for homoplastic nucleotides and recognize that all sites in the alignment do not evolve at the same rate (Swofford et al. 1996), the NJ method has some theoretical qualities that the MP method doesn’t have. Regardless of whether a parsimony analysis is more suited than a distance analysis, or vice versa, it is clear that the maximum likelihood method not only has the positive qualities and capabilities of both the MP and NJ methods, but it also has qualities and capabilities
above either MP or NJ, making maximum likelihood the best suited tree making method for the 28S rRNA alignment.

**Novel results from a novel analysis**

This study is the first maximum likelihood analysis of the echinoderm 28S or 18S rRNA gene to show bootstrap values or any such type of tree topology validation (see Raff et al. 1988, Ratto and Christen 1990, Wada and Satoh 1994, Marshall 1994, Littlewood 1995, Littlewood et al. 1997, and Janies 2001 for a review of all the methods used). With the site to site variation within different regions of the 28S gene, the unequal rate of evolution of the holothuroid lineage, and the inherent difficulties characteristic of a non-protein coding sequence, a supported ML analysis is more likely to shed light on the echinoderm class relationship issue than any other type of analysis, as it is the method that is least affected by the properties of the alignment. According to Swofford et al. (1996), maximum likelihood recognizes information about branch lengths, and treats long branches and short branches differently with regard to changes along their respective lengths. Maximum likelihood is less likely to be fooled by mis-informative sites that will lead to erroneous results in MP or (to a lesser degree) NJ analyses. In addition, maximum likelihood is frequently the method that is least affected by sampling error or even incorrect notions in the defined model of evolution (Swofford et al. 1996).

Maximum likelihood searches could be misleading when unequal rates of evolution and site to site variation are present in the alignment, as they are in the echinoderm rRNA gene data sets, but unaccounted for in the assumptions. In other words, ML analyses could be misleading if the evolutionary model of the data set is not properly defined (Swofford et al. 1996). In this study, the best fit models of evolution
were chosen out of 56 possible models, ranging from the simplest to the most complex of evolutionary models. Each best fit model chosen for the data sets included two parameters that accommodate for rate heterogeneity across sites. Namely, the (I) parameter, or measure of invariant sites, and the (G), or gamma shape parameter, that distributes the remaining sites (those that aren’t invariant, i.e., those capable of accepting substitutions) into a distribution that models the rates at which the sites evolve. Without the G parameter it would be assumed that the non-invariant sites evolve at the same rate and without the I parameter it would be assumed that all sites are capable of accepting substitutions (Swofford et al. 1996). Defining both parameters in the model of nucleotide substitution (evolution) used to search tree space relinquishes the propensity of the ML search to be affected by unequal rates. In addition, a chi-square test of nucleotide homogeneity was performed to discount any bias that may have been suffered by unequal nucleotide composition. Each data set used in this analysis passed the chi-square test of homogeneity, meaning that the nucleotide composition in each alignment is stationary, so that the tendency to disbelieve the ML results based upon this facet of the alignment can be mollified. It is interesting to note that the chi-square and associated p-values for the 18S data set (Table 8) barely change numerically in a comparison between the “variable” and “non-variable” (without and with holothurians, respectively) alignments. Looking at the same numbers represented by the 28S and combined data sets (Tables 7 and 9, respectively), the values defining the different data sets change drastically. Regardless of the changes between alignments, all data sets pass the chi-square test; I simply point this discrepancy out as a testimony to the more conserved, less informative nature of the 18S rRNA data set.
According to Libbie Hyman (1955), the Holothuroidea probably diverged from the three other eleutherozoan at a very early stage in echinoderm evolution, and the holothurians share characteristics with the Crinoidea, so those two classes probably shared a common ancestor more recently than did the crinoids with the other three classes. Asteroidea, Echinoidea, and Ophiuroidea probably share a common ancestry, but there is evidence of an early divergence of asteroids from this line of evolution, leaving the ophiuroids and echinoids to continue alone down a common path. Hyman notes that it does not seem possible to account for the existence of a pluteus larva with similar skeletal rods in the ophiuroid and echinoid classes except on the basis of common ancestry. The phenomenon of convergent evolution was used to explain the similarities between the fully developed skeleton and other characteristics of the ophiopluteus and echinopluteus (Strathmann 1978, 1988, Strathmann and Eernisse 1994, Littlewood et al. 1997). This explanation had made sense because 1) none of the other echinoderm classes exhibit a pluteus larvae or an extensive larval skeleton and 2) none of the combined (larval + adult) morphological or molecular phylogenies had placed the ophiuroids and echinoids as sister taxa. The 28S DNA tree topology shown here places ophiuroids and echinoids together as sister taxa, and subsequently eliminates the need to explain away larval morphological congruence between the two classes. The ophiuroid/echinoid grouping makes sense now, especially with the knowledge that elements in the gene regulatory network (GRN) leading to the extensive larval skeleton in the echinoid are being discovered in the homologous GRN pathway leading to the skeleton in the ophiuroid (Harmon, unpublished data). This gene conservation points to a common ancestor from which both ophiuroids and echinoids were derived, and supports the notion
that the pluteus form only evolved one time (Hyman 1955, Smiley 1988, Strathmann 1988) rather than independently in each lineage.

Conclusions

The Ophiuroidea and Echinoidea belong together as sister taxa in the phylogenetic tree of the Echinodermata. The one issue with the phylogeny that deserves further resolution is the position of the Holothuroidea, as the extremely long branch leading to this class as a result of the unequal rate of evolution in the rRNA genes could cause an artifactual placement of the holothurians compared to the other classes. 18S rRNA phylogenies will not clear up the placement of the holothurians. The 28S rRNA phylogeny presented here places the holothurians basal to the other eleutherozoan classes, as Hyman (1955) proposed, and as has been corroborated by studies of embryological characteristics of the phylum (Smith 1984, Strathmann 1988, Littlewood et al. 1997).

Figure 9 shows two possible topologies of echinoderm relationships. Based on the 28S and combined 28S/18S rRNA gene data sets that were analyzed in this study, the ophiuroids are the sister group of the echinoids, while the holothurians are basal to the other three eleutherozoan classes. Tree (A) in Figure 9 shows the actual results from the 28S and 28S/18S data, which shows the echinoderm phylogeny where the ophiuroids and echinoids are defined as sister taxa. This portrayal of an ophiuroid/echinoid clade can be described as a definitive relationship, based on the fact that it is the most compensated, defined, and supported tree to date. However, tree (B) in Figure 9 is drawn to commemorate the 18S results, the results of which I am making the claim to finally throw out, but also to illustrate the point that it is not clear based on rRNA genes where the holothurian taxa belong.
It is shown based on 28S and combined 28S/18S rRNA gene data that the ophiuroids are the sister group of the echinoids. In 28S and 28S/18S data the holothurians are basal to the other three eleutherozoan classes. The 18S rRNA gene data supports a sister grouping of holothurians and echinoids. Tree (A) shows actual results from the 28S and 28S/18S data, while tree (B) shows the echinoid/holothuroid clade supported by 18S data, but includes the ophiuroids as the sister group to the echinoid/holothuroid clade. It is not clear based on rRNA genes where the holothurian class belongs, as their sequences are so much more divergent than the other classes.

With respect to rRNA genes, the holothurian sequences are quite different from the other classes, but there are discrepancies regarding holothurian placement in morphological studies, depending upon the characters used in the matrix, and fossil record analyses.
because of the dearth of holothurian fossils. While the ophiuroid/echinoid pairing illustrated in tree (A) in Figure 9 agrees with previous larval morphology studies (Hyman 1955, Smith 1984, Smiley 1988, Strathmann 1988, Littlewood et al. 1997, Janies 2001), tree (B) in Figure 9 copies the echinoid/holothuroid relationship that has been shown in studies of adult morphology (Smith 1984, Pearse and Pearse 1994, Littlewood 1995, Littlewood et al. 1997, Janies 2001). Combinations of larval and adult characters were chosen to supplement the summary tree shown in Figure 10.

Figure 10. Echinoderm phylogeny summary tree, based on results of this study and morphological characters. All characters are adult characters unless specified as larval.

Key:
- Pentameric symmetry derived from bilateral larval symmetry
- Ambulacra present
- Adult calcareous skeleton present
- Larvae with arms or lobes
- Calcified madreporite present in adult water vascular system
- Internal, symmetrically arranged, multiple gonads
- Articulating spines as an extension of the skeleton
Figure 10. Key (continued).
- Larvae remain unattached throughout metamorphosis
- Epineural folds enclose radial water vessel (ambulacra) and nerve
- Epineural sinuses present
- Entomesoderm forms as early as blastula stage in the larvae
- Muscular jaw apparatus
- Hydrostatic larvae with elongated arms that are fully supported by calcareous rods
- Ambulacrum does not develop internally, but is internalized by epineural folding
- Larval mouth lost during development
- Adult symmetry pentamerous and bilateral
- Only one gonad present
- Loss of articulating spines

This topology shows the predicted class relationships within the Echinodermata, based on the results of this study, with morphological characters mapped into the molecular phylogeny. While the 28s rRNA shows strong support for an ophiuroid/echinoid clade, there is a long branch associated with the holothurian sequences, and because there are no other long branches associated with the phylum or Ptychodera hemichordate outgroup, the position of the holothurians may be erroneously placed basal due to the long branches of the holothurian taxa. Consistent results based on morphological analyses of strictly adult characters have placed the holothurians and echinoids as sister taxa (Pearse and Pearse 1994, Littlewood 1995, Littlewood et al. 1997, Janies 2001), hence, my proposal for a new echinoderm phylogeny is depicted in Figure 10. This topology shows both adult and larval morphological characters mapped onto an echinoderm tree that does show an ophiuroid/echinoid/holothuroid clade. Based on the results of the 28s rRNA topology derived from this study, however, it is the ophiuroids and echinoids that share the closest sister pairing within the clade.
The definitive placement of Holothuroidea will require another investigation into echinoderm phylogeny with a different gene, or sets of genes to either solidify the notion that the holothurians are the sister taxa to the ophiuroid/echinoid clade, or are basal to the other eleutherozoan classes. Perhaps through more work with gene network analysis (developmental GRN leading to the skeleton, or other homologous gene networks) of the five echinoderm classes we can resolve the class relationships within this phylum once and for all, or, alternatively, continue the debate.
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Appendices
Appendix 1. Map of 18S rRNA showing the secondary structure and regions of nucleotide variability.

Taken from the European ribosomal RNA database at http://www.psb.ugent.be/rRNA/varmaps/Scer_ssu.html, this map was created from an alignment of 500 eukaryotic sequences.
Appendix 2. Map of 28S rRNA showing the secondary structure and regions of nucleotide variability

Taken from the European ribosomal RNA database at http://www.psb.ugent.be/rRNA/varmaps/Saccere_lsu.html, this map was created from an alignment of 77 eukaryotic sequences.
Appendix 3. Aligned 28S-18S ribosomal RNA data set without holothurians

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<td>-CAGU-ACG</td>
<td>-GGCGAGUGA</td>
<td>AGCGGGAAC</td>
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<td>-CAGU-ACG</td>
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<td>-CAGU-ACG</td>
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<td>GGAC-UCGGC</td>
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<td>-GGCGAGUGA</td>
<td>AGCGGGAAC</td>
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<td>-CAGU-ACG</td>
<td>-GGCGAGUGA</td>
<td>AGCGGGAAC</td>
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<tr>
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<td>GGAC-UCGGC</td>
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<td>ACU-AACC-A</td>
<td>GGAC-UCGGC</td>
<td>-CAGU-ACG</td>
<td>-GGCGAGUGA</td>
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Appendix 3 (Continued).
Ptychodera_sp.  GGCGCACGCC GUCC------ --------------- -GAGGCUUCC AG-----GAG UCGGGUUUGU UGAGAAUGCA GCCCAAGA-
Crinoid_sp.    GGGCGCG--U GGCGA------ --------------- CCACGCUUUU CAG-----GAG UCGGGUUUGU UGAGAAUGCA GCCCAAGA-
Sea_Star_sp.   GGCGUCUCG--- GGCGC------ --------------- C AAGGCCCGC GG-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
Asterina_sp.   GGGUCUCAGG GCCCU------ --------------- -GAGGCCACG AA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
S_purpuratus   GGGUCUCAGG GCCCU------ --------------- -GAGGCCACG AA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
Ardacia_sp.    GGGUCUCAGG GCCCU------ --------------- -GAGGCCACG AA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
O_esmarki      GGGUCUCAGG GCCCU------ --------------- -GAGGCCACG AA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
O_echinata     GGGUCUCAGG GCCCU------ --------------- -GAGGCCACG AA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-

Ptychodera_sp. -----CCGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
Crinoid_sp.    -----CCGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
Sea_Star_sp.   -----AGGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
Asterina_sp.   -----GGGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
S_purpuratus   -----AGGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
Ardacia_sp.    -----AGGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
O_esmarki      -----CCGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-
O_echinata     -----CCGGU GGAAACUC- CAUCAAGGC UAA-----GAG UCGGGUUGU UGAGAAUGCA GCCCAAGA-

Ptychodera_sp. A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
Crinoid_sp.    A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
Sea_Star_sp.   A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
Asterina_sp.   A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
S_purpuratus   A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
Ardacia_sp.    A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
O_esmarki      A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-
O_echinata     A-GGGAAA-G UU-GAAA-AG AACUUUGA-A G-AGAG-A-- GUU--CAAG- AGUACGUGAA A-CUG---CU GAGAAGCAA-

Ptychodera_sp. ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
Crinoid_sp.    ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
Sea_Star_sp.   ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
Asterina_sp.   ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
S_purpuratus   ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
Ardacia_sp.    ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
O_esmarki      ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
O_echinata     ---CCGGUGG GCCCGC---- --GGGCCGGU UGCCCACCCG ----ACCCGU CUUGAACAC GGCACAAGGA GUCUA-ACAC-
Appendix 3 (Continued).

**Ptychodera_sp.**
GUGCGCGA-- GUCACAGGCC CC--AGUCCC CUGGCCUGUG GGGCU--AGC -GAAG-GUGA -------AGGG CGGCC--CG

**Crinoid_sp.**
GCCCGCGA-- GUCGUAGGGA -------CUA GAAGCCCGAA GGGC--AAU -GAAG-GUGA -------AGGU CAGU--CC

**Sea_Star_sp.**
GCCCGCAA-- GUCGGAGGGC CG--CAAGUC GAGGCCCCGA GGCGC--AAC -GAGA-GUGA -------AGGC CGGCU--GC

**Asterina_sp.**
GCCCGCAA-- GUCAARGGGG CG---UUAC GAAACCCCAC GGCGC--AAU -GAAG-GUGA -------AGGU CGAU----UC

**S_purpuratus**
GCCCGCAA-- GUCGGAGGGC CG---UUAC GAAACCCCAC GGCGC--AAU -GAAG-GUGA -------AGGU CGAU----UC

**O_esmarki**
ACACGCAA-- GUCGAAGGGG CC---UAUC GAAGCCCCUA GGCGC--AAU -GAAA-GUAG ----CAAGGG CGGUG---UA

**O_echinata**
ACUGCAAA-- GUCGAAGGGG CC---UAUC GAAGCCCCUA GGCGC--AAU -GAAA-GUAG ----CAAGGG CGGUG---UA

**Ptychodera_sp.**
GUGCGCGGCA GUGGGAGGCC CGGCCGCGGCC GGGCCGGGAC GCACACCG-- ----GCAGA UCUC--UCU GGCGCCGCGG

**Crinoid_sp.**
GUCUGGCCGA GGUGGGAUCC GGGCCCCGGC GGGCCGGGAC GCACACCG-- ----GCAGA UCUC--UCU GGCGCCGCGG

**Sea_Star_sp.**
GUCCGGCCGA GGCGGGAUCC GGGCCCCGGC GGGCCGGGAC GCACACCG-- ----GCAGA UCUC--UCU GGCGCCGCGG

**Asterina_sp.**
GUGGCAGAAG GCAGGAAAGG ACGCGUUUGG ACCCGAAAGA UGGUG----AA CU-AU-G CCCC GGGUAGAGG AAGCAGGAGG

**S_purpuratus**
GUGAGGCGGA GGAAGAGCGG GCACGCUAGG ACCCGAAAGA UGGUG----AA CU-AU-G CCCC GGGUAGAGG AAGCAGGAGG

**Arbacia_sp.**
GAGCGCGGA GUAAGAGCGG GCACGCUAGG ACCCGAAAGA UGGUG----AA CU-AU-G CCCC GGGUAGAGG AAGCAGGAGG

**O_esmarki**
GGCCGCCCGA GGUGGGAUCC CUGCCCUCUG G--GUGGGGC GCACCACCGC CC---CGUCU CGUGC----- GCUUUGCUCG

**O_echinata**
GGCCGCCCGA GGUGGGAUCC CUGCCCUCUG G--GUGGGGC GCACCACCGC CC---CGUCU CGUGC----- GCUUUGCUCG

**Ptychodera_sp.**
AAGCCAGAGG

**Crinoid_sp.**
AAGCCAGAGG

**Sea_Star_sp.**
AAGCCAGAGG

**Asterina_sp.**
AAGCCAGAGG

**S_purpuratus**
AAGCCAGAGG

**Arbacia_sp.**
AAGCCAGAGG

**O_esmarki**
AAGCCAGAGG

**O_echinata**
AAGCCAGAGG

**Ptychodera_sp.**
AAGCCAGAGG

**Crinoid_sp.**
AAGCCAGAGG

**Sea_Star_sp.**
AAGCCAGAGG

**Asterina_sp.**
AAGCCAGAGG

**S_purpuratus**
AAGCCAGAGG

**Arbacia_sp.**
AAGCCAGAGG

**O_esmarki**
AAGCCAGAGG

**O_echinata**
AAGCCAGAGG

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Appendix 3 (Continued).

Ptychodera_sp.  GC-G--AGGA CCGCC----- A-CCGGG-CG CUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
Crinoid_sp.     GC-G--AGGU CCGAA----- A-CCAUGGCA UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
Sea_Star_sp.    GC-G--AGCU CCGCA----- G-CCGGGGCG UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
Asterina_sp.    GC-G--AGUG CCGCA----- A-CCGGGGCA UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
S_purpuratus    GC-G--AGUG CCGCA----- A-CCGGGGCA UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
Arbacia_sp.     GC-G--AGUG CCGCA----- A-CCGGGGCA UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
O_esmarki       GC-G--AGUG CCGCA----- A-CCGGGGCA UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--
O_echinata      GC-G--AGUG CCGCA----- A-CCGGGGCA UUGACGCAAU GU--GAU-UU C-UGCCC-AG UGCUCUGAAU GU-CAAAG--

Ptychodera_sp.  --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
Crinoid_sp.     --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
Sea_Star_sp.    --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
Asterina_sp.    --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
S_purpuratus    --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
Arbacia_sp.     --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
O_esmarki       --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--
O_echinata      --UGA--AGA AAU-------- ---UCAA--- -UCAGAAGCGG GGUAAACGGC --GGGAGUAA -CUUUGACUC UCUUAGGU--

Ptychodera_sp.  AGCCAAUGGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
Crinoid_sp.     AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
Sea_Star_sp.    AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
Asterina_sp.    AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
S_purpuratus    AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
Arbacia_sp.     AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
O_esmarki       AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU
O_echinata      AGCCAAAUGC CUC--GCAUU CUAUU-AGU GACG-CCGCA UAAUGGAUUA ACGAGAUACC C-ACUGGCCU UAUCUAUCAU

Ptychodera_sp.  CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
Crinoid_sp.     CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
Sea_Star_sp.    CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
Asterina_sp.    CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
S_purpuratus    CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
Arbacia_sp.     CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
O_esmarki       CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
O_echinata      CUAGCGAAAC CACA-GCCAA -GGG--AAGCG -GC-UUGG-- --CAGAAUUA GCGGGGAAG AAGACCCUGU UGACCUUGAC
Appendix 3 (Continued).

Ptychodera_sp. UCUAGUCU---GACUCUGUG AAGGGACAUG GGAGGUGUAG GAUAGGUGGG AGGCCUCCG---------G GCCGC-CGG-
Crinoid_sp. UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG AAUAGGUGGG AGGCCUCA---------C GCCGC-CGG-
Sea_Star_sp. UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG GAUAGGUGGG AGGCCGCGGU---------CG GCCGC-CGG-
Asterina_sp. UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG GAUAGGUGGG AGGCCGCGGU---------C GCCGC-CGG-
S_purpuratus UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG GAUAGGUGGG AGGCCGCGGU---------C GCCGC-CGG-
Arbacia_sp. UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG GAUAGGUGGG AGGCCGCGGU---------C GCCGC-CGG-
O_esmarki UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG GAUAGGUGGG AGGCCGCGGU---------C GCCGC-CGG-
O_echinata UCUAGUCU---GACCUUGUG AAGGACAAGUG AGGGGUGUAG GAUAGGUGGG AGGCCGCGGU---------C GCCGC-CGG-

Ptychodera_sp. UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
Crinoid_sp. UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
Sea_Star_sp. UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
Asterina_sp. UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
S_purpuratus UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
Arbacia_sp. UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
O_esmarki UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC
O_echinata UGAAA-UACC-AUCUCUCU-GAUCGUGU-U UCUCUCU GGAGAC--GGACGUGGG ACAGGUG------GGCAGGGCGC

Ptychodera_sp. UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
Crinoid_sp. UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
Sea_Star_sp. UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
Asterina_sp. UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
S_purpuratus UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
Arbacia_sp. UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
O_esmarki UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---
O_echinata UGAAATUCUCUT-GACUCUCUG UACUCUCUG GAACUUCGUG AGACGUG---GAGACGUG---GAACGUG---GAACGUG---

Ptychodera_sp. UGAAUACCUCAGUCAA-GGCGC UCAAGGACGUG AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
Appendix 3 (Continued).

Ptychodera_sp. GCUCGGC- GG-------AC GGAACC C--G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
Crinoid_sp. GCUAGC-GA GG-------AC GGAACC G----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
Sea_Star_sp. GCUCAGC-GA G-------AC GGAACC G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
Asterina_sp. GCUCAGC-GA G-------AC GGAACC G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
S_purpuratus GCUCAGC-GA G-------AC GGAACC G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
Arbacia_sp. GCUCAGC-GA G-------AC GGAACC G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
O_esmarki GCUCAGC-GA G-------AC GGAACC G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU
O_echinata GCUCAGC-GA G-------AC GGAACC G-----GUAG AGCA--AAAG GGC------- AAAAG-CUC- GCUU-GAUUU

Ptychodera_sp. U--GAUUUC -AGUG-----C GAUA-CAGA CGGU-GAAA-- GC--G-GGAC C-UACGAUC UUUUGA----- UG---UU---
Crinoid_sp. U--GAUUUC -AGUA-----U GAUA-CAGA CGGU-GAAA-- GC--G-UGGC C-UACGAUC UUUUGA----- CC---UU---
Sea_Star_sp. U--GAUUUC -AGUA-----U GAUA-CAGA CGGU-GAAA-- GC--G-GGAC C-UACGAUC UUUUGA----- CC---UU---
Asterina_sp. U--GAUUUC -AGUA-----U GAUA-CAGA CGGU-GAAA-- GC--G-GGAC C-UACGAUC UUUUGA----- CC---UU---
S_purpuratus U--GAUUUC -AGUA-----C GAUA-CAGA CGGU-GAAA-- GC--G-UGGC C-UACGAUC UUUUGA----- CC---UU---
Arbacia_sp. U--GAUUUC -AGUA-----U GAUA-CAGA CGGU-GAAA-- GC--G-UGGC C-UACGAUC UUUUGA----- CC---UU---
O_esmarki U--GAUUUC -AGUA-----C GAUA-CAGA CGGU-GAAA-- GC--G-UGGC C-UACGAUC UUUUGA----- CC---UU---
O_echinata U--GAUUUC -AGUA-----Y GAUA-CAGA CGGU-GAAA-- GC--G-UGGC C-UACGAUC UUUUGA----- CC---UU---

Ptychodera_sp. AGGGGUUCCA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
Crinoid_sp. AGGAGUUUA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
Sea_Star_sp. CGGAGUUGA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
Asterina_sp. CGGAGUUGA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
S_purpuratus CGGAGUUGA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
Arbacia_sp. CGGAGUUGA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
O_esmarki CGGAGUUGA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA
O_echinata CGGARUUGA AGCAAGAGGU GUCAGA- AA AGUACCACA GGGAU--AAC UGGCUUG--U GGC-GGCCA ACG-GCUAA

Ptychodera_sp. AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C GCAAG-GCAG
Crinoid_sp. AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-G GCAAG-GCAG
Sea_Star_sp. AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C GCAAG-GCAG
Asterina_sp. AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C RCAAAG-GCAG
S_purpuratus AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C ACCAAG-GCAG
Arbacia_sp. AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C ACCAAG-GCAG
O_esmarki AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C ACCAAG-GCAG
O_echinata AGCGACGU-- CGCUUUU--G AUC-CUUCGA U--GUGGCC- UCUCUCAAUC AUUGCC-AAG CAGAA-UU-C ACCAAG-GCAG
Appendix 3 (Continued).

Ptychodera_sp. UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Crinoid_sp. UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Sea_Star_sp. UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Asterina_sp. UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
S_purpuratus UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Arbacia_sp. UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
O_esmarki UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
O_echinata UGGAUGUUC ACCCCACU-AAG -AAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA

Ptychodera_sp. CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Crinoid_sp. CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Sea_Star_sp. CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Asterina_sp. CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
S_purpuratus CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
Arbacia_sp. CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
O_esmarki CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA
O_echinata CUGAUGAAG-----CCCCUC GUCUCAGACG -GAAGGGAACG -UGAGCUUGG GUUACGAC-C -GUCCUGAGAC AGGU-UAGU- UUACCCCUA

Ptychodera_sp. UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Crinoid_sp. UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Sea_Star_sp. UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Asterina_sp. UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
S_purpuratus UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Arbacia_sp. UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
O_esmarki UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
O_echinata UAAAGCCGCUUG GCCGAUAGGC CAAUGGCUAAGG CAGCUACCUC CCUGGGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA

Ptychodera_sp. UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Crinoid_sp. UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Sea_Star_sp. UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Asterina_sp. UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
S_purpuratus UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Arbacia_sp. UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
O_esmarki UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
O_echinata UUAAGACUG CAUUG-UCAGG AUGCAGACG CUCGUAGACG AGGAAAGUCG CUGUGGCUU ACGACUGUAG UCAUAAGCUU GCUCAAGAGA
Appendix 3 (Continued).

Ptychodera_sp.  C-UUUAU--- CGUACCAGA U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCAA-UGA GCAGCCGACCU

Crinoid_sp.    C-UUGGAGU CAGGUUAC-- U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCAA-CAA GCAGCCGACU

Sea_Star_sp.   C-CUUGAUGC GAUCUC--- U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCA-CAU GCAGCCGACU

Asterina_sp.   C-UUGGAGU CAGGUUAC-- U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCA-CAU GCAGCCGACU

S_purpuratus   C-UUAAUGU CAGCUUUCU U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCA-CAU GCAGCCGACU

Arbacia_sp.    C-UUAAUGU CAGCUUUCU U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCA-CAU GCAGCCGACU

O_esmarki      C-UUUAUGU CAGCUUUCU U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCA-CAU GCAGCCGACU

O_echinata     C-UUUAUGU CAGCUUUCU U-UUAUAGG GAUAACUGG GUAUUCUAG AGCUAAUACA UGGCA-CAU GCAGCCGACU

Ptychodera_sp. CGCGGGG-GG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

Crinoid_sp.    CGG-GGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

Sea_Star_sp.   UAGGGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

Asterina_sp.   UAGGGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

S_purpuratus   UAGGGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

Arbacia_sp.    UAGGGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

O_esmarki      UAGGGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

O_echinata     UAGGGAAGG CGUGCUUUA UAGGAA-A U-GACAAUC CGGCUUCG AGG--CUC C CGCA-CGUC C CUGCA-GG

Ptychodera_sp. UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

Crinoid_sp.    UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

Sea_Star_sp.   UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

Asterina_sp.   UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

S_purpuratus   UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

Arbacia_sp.    UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

O_esmarki      UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

O_echinata     UCUGGAAUAC UUGG------- CGGACGCAG GGCGCGGCG CCGGCGACG AUCUUCG-GA GGUGCUCGG U-AUCAACUU

Ptychodera_sp. U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

Crinoid_sp.    U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

Sea_Star_sp.   U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

Asterina_sp.   U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

S_purpuratus   U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

Arbacia_sp.    U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

O_esmarki      U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG

O_echinata     U-CGAUGGUA CUGUUCGAC CUGUACG AGAACGAG GGAUUCG GAGCAACG GAGGGAAGG
Appendix 3 (Continued).

Ptychodera_sp.  CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
Crinoid_sp.    CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
Sea_Star_sp.   CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
Asterina_sp.   CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
S_purpuratus   CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
Arbacia_sp.    CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
O_esmarki      CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG
O_echinata     CCUGAGAAAC GGCUACCACA U-CCA-AGGA AGGCAGCAGG CGCGCAAAUU ACCCAUUCCC GACGAGGGA GGUAGUGACG

Ptychodera_sp.  AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
Crinoid_sp.     AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
Sea_Star_sp.    AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
Asterina_sp.    AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
S_purpuratus    AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
Arbacia_sp.     AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
O_esmarki       AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG
O_echinata      AAAAUAACCA AUACAGGACU CUUUCAAGGC CC-UCCAAAUU GGAAUGAGUA CACUUAAAU AAAAAGCUCG

Ptychodera_sp.  GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
Crinoid_sp.     GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
Sea_Star_sp.    GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
Asterina_sp.    GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
S_purpuratus    GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
Arbacia_sp.     GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
O_esmarki       GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG
O_echinata      GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU AUAUUAAUGC UGUUGCAGGU AAAAAGCUCG

Ptychodera_sp.  UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
Crinoid_sp.     UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
Sea_Star_sp.    UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
Asterina_sp.    UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
S_purpuratus    UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
Arbacia_sp.     UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
O_esmarki       UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
O_echinata      UAGUUGGAUC CUUUGCC--- -AGGCUCGCG CC---GCGCGGU AAAAAGCUCG
Appendix 3 (Continued).

Ptychodera_sp. UCGGUCCUUGU--- -UCCCGGGU GCUCUUAAGCU GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
Crinoid_sp. UCGCUUUGU--- -UCACCCGGU GCUCUUAAGU AA-GUGGCAG G-GGGCCAG GAACGCUUAC UUUGAAAAAA
Sea_Star_sp. UCGGUCCGU --- -CCCGCCGU UCUCUUGACU GA-GUGGCGG G-GGUGACCG AAACGCUUAC UUUGAAAAAA
Asterina_sp. UCGGUCCGU --- -CCCGCCGU UCUCUUGACU GA-GUGGCGG G-GGUGACCG AAACGCUUAC UUUGAAAAAA
S_purpuratus UCGGUCCGU --- -CCCGCCGU UCUCUUGACU GA-GUGGCGG G-GGUGACCG AAACGCUUAC UUUGAAAAAA
Arbacia_sp. UCGGUCCGU --- -CCCGCCGU UCUCUUGACU GA-GUGGCGG G-GGUGACCG AAACGCUUAC UUUGAAAAAA
O_esmarki GCGGUUUGU--- -CGCCCGGGU UCUCUUAACU GA-GUGGCGG G-GGUCGACG AAACGCUUAC UUUGAAAAAA
O_echinata GCGGUUUGU--- -CGCCCGGGU UCUCUUAACU GA-GUGGCGG G-AACGACCG AAACGCUUAC UUUGAAAAAA

Ptychodera_sp. UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
Crinoid_sp. UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
Sea_Star_sp. UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
Asterina_sp. UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
S_purpuratus UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
Arbacia_sp. UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
O_esmarki UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
O_echinata UGGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA

Ptychodera_sp. UUGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
Crinoid_sp. UUGAAGGGU-- -UUCUGGCUU UCGGACUUCG GA-GUGGCGG G-GGUGGCGG GAACGCUUAC UUUGAAAAAA
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Arbacia_sp.     ACU-AACC-A  GGAU-UCCCU  ---AGU--AGC  -GGCGAGCGA  AGCGGGAAG-  --AGGC-CAG  CGGCCAA-UC  CCCGCCGCCC-
O_esmarki       ACU-AACC-A  GGAU-UCCCG  -CAGU--AGC  -GGCGAGCGA  AGCGGGAAG-  --AGGC-CAG  CGGCCAA-UC  CCCGCCGCCC-
O_echinata      UCU-AACU-A  GGAU-UCCCG  -CAGU--AGC  -GGCGAGCGA  AGCGGGAAG-  --AGGC-CAG  CGGCCAA-UC  CCCGCCGCCC-

S_hydriformis    ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
Cucumaria_sp.   ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
Ptychodera_sp.  ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
Crinoid_sp.     ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
Sea_Star_sp.    ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
Asterina_sp.    ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
S_purpuratus    ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
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O_esmarki       ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----
O_echinata      ---GAGUCGG GUUGUUUAG  AAUGCAGCCC  AAAGA-----  -CGGGUGGUA  AACUC-CAUC  CAAGCGUA-A  NUUAA-----

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Ptychodera_sp.  GCACGAGA-C  CGAUAGUAGA  C-----AAGU  ACCGUGA-GG  GAAC-GUU-G  AAA-AGAACU  CUGA-AG-A  AG-G--GUU-
Crinoid_sp.     GCACGAGA-C  CGAUAGUAGA  C-----AAGU  ACCGUGA-GG  GAAC-GUU-G  AAA-AGAACU  CUGA-AG-A  AG-G--GUU-
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O_echinata      GCACGAGA-C  CGAUAGUAGA  C-----AAGU  ACCGUGA-GG  GAAC-GUU-G  AAA-AGAACU  CUGA-AG-A  AG-G--GUU-
Appendix 4 (Continued).

S_hydriformis  -CAAG-AGUG CGUGAAA-CC G---CCGAGA AGAAAA---- ---------- ---------- ---------- ----------
Cucumaria_sp.  -CAAA--UUA CGUGAAA-CU G---CCAAGA AGCAAA---- ---------- ---------- ---------- ----------
Ptychodera_sp. -CAAG-AGUA CGUGAAA-CU G---CUGAGA AGCAAA---- ---------- ---------- ---------- ----------
Crinoid_sp.   -CAAG-AGUA CGUGAAA-CC G---CUAAGA AGCAAA---- ---------- ---------- ---------- ----------
Sea_Star_sp.  -CAAG-AGUA CGUGAGG-CC G---AGUGAA AGCAAA---- ---------- ---------- ---------- ----------
Asterina_sp.  -CAAG-AGGA CGUGAAA-CC G---CCRAGA AGCAAA---- ---------- ---------- ---------- ----------
S_purpuratus  -CAAG-AGGA CGUGAAA-CC G---CCAAGA AGCAAA---- ---------- ---------- ---------- ----------
Arbacia_sp.   -CAAG-AGGA CGUGAAA-CC G---CCAAGA AGCAAA---- ---------- ---------- ---------- ----------
O_esmarki     -CAAU-AGGA CGUGAGG-CU G---CCGAGA AGUAAA---- ---------- ---------- ---------- ----------
O_echinata    -CAAU-AGGA CGUGAGG-CU G---CCGAGA AGUAAA---- ---------- ---------- ---------- ----------
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Cucumaria_sp. ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-GCAGGCCU GCGA--GUCG ACGGGA---- ---------- ----------
Ptychodera_sp. ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-ACAGUGGC GCAG--GUCG CAAGGC---- ---------- ----------
Crinoid_sp.   ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-ACACGGGC GCAG--GUCG CAGGGC---- ---------- ----------
Sea_Star_sp.  ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-CUCGGGCC GCAG--GUCG GAGGGC---- ---------- ----------
Asterina_sp.  ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-CUCGGGCC GCAG--GUCG GAGGGC---- ---------- ----------
S_purpuratus  ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-CACGGGCC GCAG--GUCG GAGGGC---- ---------- ----------
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O_esmarki     ACCCGUCUU GAAACCGGAC CAAGGAGGCU A-GGGCAAGC GCAA--GUCG AAGGGC---- ---------- ----------
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S_hydriformis ---AAC-GAA A-GUGA---A AGAGGGGCGG UGCUGGGACC CGAAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
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Ptychodera_sp. ---AGC-GAA G-GUGA---A AGAGGGGCA CGUUGGGAC CGAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
Crinoid_sp.   ---AAY-GAA A-GUGA---A AGAGGGGCA CCGUUGGGAC CGAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
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Asterina_sp.  ---AAY-GAG A-GUGA---A AGAGGGGCA CCGUUGGGAC CGAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
S_purpuratus  ---AUA-GAA A-GUGA---A AGAGGGGCA CCGUUGGGAC CGAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
Arbacia_sp.   ---AUA-GAA A-GUGA---C AGAGGGGCA CCGUUGGGAC CGAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
O_esmarki     ---AUA-GAA A-GUAG---A AGAGGGGCA CGCUAGGGAC CGAAGAUGG UG---AACUC AU-GCUUGAG CAGGAAGAG
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Crinoid_sp. CUGAAUGU-C AAAG----UG A--AGAAAU- ------------U CAA----CGA AGCGCGGGUA AACGGC--GG GAGUAA-CUA
Sea_Star_sp. CUGAAUGU-C AAAG----UG A--AGAAAU- ------------U CAG----CGA AGCGCGGGUA AACGGC--GG GAGUAA-CUA
Asterina_sp. CUGAAUGU-C AAAG----UG A--AGAAAU- ------------U CAA----CGA AGCGCGGGUA AACGGC--GG GAGUAA-CUA
S_purpuratus CUGAAUGU-C AAAG----UG A--AGAAAU- ------------U CAA----CGA AGCGCGGGUA AACGGC--GG GAGUAA-CUA
Arbacia_sp. CUGAAUGU-C AAAG----UG A--AGAAAU- ------------U CAA----UGA AGCGCGGGUA AACGGC--GG GAGUAA-CUA
O_esmarki CUGAAUGU-C AAAG----UG A--AGAAAU- ------------U CAA----CGA AGCGCGGGUA AACGGC--GG GAGUAA-CUA
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Cucumaria_sp. UGACUCUCUU AAGGU-AGCC AAAUGCCUC- -GUAUCUAU AA--AGUGCG -CGCAUGAAU GGAAUACGA GAUCCC-AC
Ptychodera_sp. UGACUCUCUU AAGGU-AGCC AAAUGCCUC- -GUAUCUAU AA--AGUGCG -CGCAUGAAU GGAAUACGA GAUCCC-AC
Crinoid_sp. UGACUCUCUU AAGGU-AGCC AAAUGCCUC- -GUAUCUAU AA--AGUGCG -CGCAUGAAU GGAAUACGA GAUCCC-AC
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112
Appendix 4 (Continued).

S_hydriformis  -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ CCAGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
Cucumaria_sp.  -GGUGU-CCC A----AGGA- GAGCUCAGC- GAGG------ ACAGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
Ptychodera_sp.  -GGUGU-CCU A----AGGC- GAGCUCGGC- GGGG------ ACGGAAACC CGC-----GU GGAGCA--AA AGGGC-----
Crinoid_sp.   -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ ACGGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
Sea_Star_sp.   -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ ACAGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
Asterina_sp.   -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ ACGGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
S_purpuratus   -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ ACGGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
Arbacia_sp.    -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ ACGGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
O_esmarki      -GGUGU-CCU A----AGGC- GAGCUCAGC- GAGG------ ACGGAAACCU CGC-----GU AGAGCA--AA AGGGC-----
O_echinata     -GGUGU-CCU A----AGGC- GACGCAAGGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----

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Ptychodera_sp. --AAAAG-CU C-GCUU-GAU UUU--GAUUU UC-AGUA--- -CGAAUA-CA GACCGC-GAA A-GC--G-GG GCC-UAACGA
Crinoid_sp.    --AAAAG-CU C-GCUU-GAU UUU--GAUUU UC-AGUA--- -UGAAUA-CA GACCGU-GAA A-GC--G-UG GCC-UAACGA
Sea_Star_sp.   --AAAAG-CU C-GCUU-GAU UUU--GAUUU UC-AGUA--- -UGAAUA-CA GACCGU-GAA A-GC--G-UG GCC-UAACGA
Asterina_sp.   --AAAAG-CU C-GCUU-GAU UUU--GAUUU UC-AGUA--- -UGAAUA-CA GACCGU-GAA A-GC--G-UG GCC-UAACGA
S_purpuratus   --AAAAG-CU C-GCUU-GAU UUU--GAUUU UC-AGUA--- -UGAAUA-CA GACCGU-GAA A-GC--G-UG GCC-UAACGA
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O_esmarki      --AAAAG-CU C-GCUU-GAU UUU--GAUUU UC-AGUA--- -UGAAUA-CA GACCGU-GAA A-GC--G-UG GCC-UAACGA
O_echinata     --AAAWG-CU S-GCUU-GAU UUU--GAUUU UC-AGUA--- -YGAAUA-CA GACCGU-GAA A-GC--G-UG GCC-UAACGA

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Sea_Star_sp.   UCCUUUUGA- --CC---UU- --CGGGAUUC GAGCAGAGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----
Asterina_sp.   UCCUUUUGA- --CC---UU- --CGGGAUUC GAGCAGAGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----
S_purpuratus   UCCUUUUGA- --CC---UU- --CGGGAUUC GAGCAGAGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----
Arbacia_sp.    UCCUUUUGA- --CC---UU- --CGGGAUUC GAGCAGAGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----
O_esmarki      UCCUUUUGA- --CC---UU- --CGGGAUUC GAGCAGAGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----
O_echinata     UCCUUUUGA- --CC---UU- --CGGGAUUC GAGCAGAGG GUGUCAGA-- AAAGUUACCA CAGGGAU--A ACUGGCUG-----
Appendix 4 (Continued).

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Ptychodera_sp. -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
Crinoid_sp.  -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
Sea_Star.sp.  -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
Asterina_sp.  -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
S_purpuratus  -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
Arbacia_sp.  -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
O_esmarki     -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A
O_echinata    -UGGC-GGCC AAGCG--UUC AUAGCGACGU --CGCUUUUU -GAUC-CUUC GAU--GUCGG C-UCUUCCUA UCAUUGCG-A

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Ptychodera_sp. AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
Crinoid_sp.  AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
Sea_Star.sp.  AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
Asterina_sp.  AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
S_purpuratus  AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
Arbacia_sp.  AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
O_esmarki     AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG
O_echinata    AGCAGAA-UU -CGCCAAG-C GUUGGAUUGU UCACCCACU- AA-UAGGGAA CG-UGAGCUG GGUUUAGAC- C-GUCGUGAG

S_hydriformis  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Cucumaria_sp.  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Ptychodera_sp. ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Crinoid_sp.  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Sea_Star.sp.  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Asterina_sp.  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
S_purpuratus  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Arbacia_sp.  ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
O_esmarki     ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
O_echinata    ACAGGU-UAG U--UUUACCC UACUGAUGAG UAGUCAUAUG CUUGUCUCAA AGAUAAGGC AUGCAUGUC- UA--AGUACA
Appendix 4 (Continued).

S_{hydriformis}  AACUC-U-UA UGGGUGAA A CUGCAUAG-G GCUCAAUAAA UCAgcUGUUG UUUA-AUGAA G---CGAGU- -----UUAC
Cucumaria_{sp.}  UACGUGGAUA CACAGAGAAU CUGCAGAU-G GCUCAUUAAA UCAGUUAUGG UUUA-UUGGA G---AACAGU- -----UGCC
Ptychodera_{sp.}  AGCCUCGGUA CAAAGCGAAA CUGCGGAU-G GCUCAUUAAA UCAGUUAUGG UUCC-UUGGA G---CGUA- -----UUAC
Crinoid_{sp.}  AG-CU-UGUA CCAAGCGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA GC-GCAUC- ----CUAC
Sea_{Star}_{sp.}  AG-CU-U-U UCACAGCGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA GC-GCAUC- ----CUAC
Asterina_{sp.}  AG-CU-U-UC ACACAGGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA GC-GCAUC- ----CUAC
S_{purpuratus}  AG-CU-CGUC UCAG-CGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA GC-GCAUC- ----CUAC
Arbacia_{sp.}  AG-CU-C-GU CCAGCGGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA GC-GCAUC- ----CUAC
O_{esmarki}  AG-CU-UGUA UCAAGCGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA AC-GAGUU- ------UAC
O_{echinata}  AG-CU-UGUA UCAAGCGAAA CUGCGGAU-G GCUCAAUAAA UCAgcUGUUG UUCC-UUGGA AU-GAGUU- ------UAC

S_{hydriformis}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCUCACGGA- -AAGCGUGCU UUUAUUAGGA
Cucumaria_{sp.}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAU- UAAGCGCCGA CCCUUCGGGG GAAGCGUGCU UUUAUUAGGA
Ptychodera_{sp.}  UUGGAUAACU GUGGUAAUUC UAGAGCUAAUACAUGCGAC- CAAGCGCUGA CCUCACGGA- -AAGCGUGCU UUUAUUAGGA
Crinoid_{sp.}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAA- CAAGCGCUGA CCUCACGGA- -AAGCGUGCU UUUAUUAGGA
Sea_{Star}_{sp.}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCUCACGGA- -AAGCGUGCU UUUAUUAGGA
Asterina_{sp.}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCUCACGGA- -AAGCGUGCU UUUAUUAGGA
S_{purpuratus}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCUCACGGA- -AAGCGUGCU UUUAUUAGGA
Arbacia_{sp.}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCCUACGGA- -AAGCGUGCU UUUAUUAGGA
O_{esmarki}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCCUACGGA- -AAGCGUGCU UUUAUUAGGA
O_{echinata}  AUGGAUAACU GUGGUAAUUC UAGAGCUAAU ACAUGCGAC- CAAGCGCUGA CCCUACGGA- -AAGCGUGCU UUUAUUAGGA

S_{hydriformis}  ACAAGACCA GCCCGU----- ---GAACUCU AGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Cucumaria_{sp.}  ACAAGACCA GCCCGU----- ---GAACUCU AGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Ptychodera_{sp.}  CCAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Crinoid_{sp.}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Sea_{Star}_{sp.}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Asterina_{sp.}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
S_{purpuratus}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Arbacia_{sp.}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
O_{esmarki}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
O_{echinata}  ACAAGACCA AUCCG----- ---GAACUCU GGAUAACUUG U-------CGG AUGCAGCGGU UUUG-GACCG GCCAAGGAGU
Appendix 4 (Continued).

S_hydriformis  CUUC-AAAUG UCUGCCUU-A UCAACUGU-C GAUGUAGGU UAUGCCCUA CCA-U-GGUC GAACGGUGGC ACGGA--G-A
Cucumaria_sp.  CUUC-AAAAG UCUGCCUCU-A UCAACUUU-C GAUGGAGAAG UAGUGCCUA UCA-U-GGUC GUACGGUGUA ACGGA--G-A
Ptychodera_sp.  UUUC-AAGUG UCUGCCCU-A UCAACUUU-C GAUGGAGCU UAUGCCCUA CCA-U-GGUC GAACGGUGUA ACGGA--G-A
Crinoid_sp.   CUUC-AAAUG UCUGCCCUU-A UCAACUUU-C GAUGUAGCU UAUGCCCUA CCA-U-GGUC GAACGGUGUA ACGGA--G-A
Sea_Star_sp.   CUUC-GAAUG UCUGCCCU-A UCAACUUU-C GAUGGAGCU UAUGCCCUA CCA-U-GGUC GAACGGUGUA ACGGA--G-A
Asterina_sp.   CUUC-GAAUG UCUGCCCU-A UCAACUUU-C GAUGGAGCU UAUGCCCUA CCA-U-GGUC GAACGGUGUA ACGGA--G-A
S_purpuratus   CUUC-GAAUG UCUGCCCUU-A UCAACUUU-C GAUGGAGCU UAUGCCCUA CCA-U-GGUC GAACGGUGUA ACGGA--G-A
Arbacia_sp.    CUUC-GAAUG UCUGCCCU-A UCAACUUU-C GAUGGAGCU UAUGCCCUA CCA-U-GGUC GAACGGUGUA ACGGA--G-A

S_hydriformis  AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
Cucumaria_sp.  AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
Ptychodera_sp. AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
Crinoid_sp.    AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
Sea_Star_sp.   AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
Asterina_sp.   AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
S_purpuratus   AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A
Arbacia_sp.    AUCAGGGUUU GAAUCGGCU GAGGGAGCCU GAGAAACGGU UACCACAU-C CAAGAAGG CAGCAGCGC ACGGA--G-A

S_hydriformis  CAAUCCCAAU UCGGGGAGGU AGUGACGAAA AAUAACGAAU CAGCAGACCU UCAAAGGGUC U-GUAAUGCU AAGAGUCACA
Cucumaria_sp.  CACUCCCGCU CAGGGGAGCU AGUGACGAAA AAUAACGACU CAGCAGACCU UCAAAGGGUC U-GUAAUGCU AAGAGUCACA
Ptychodera_sp. CAUCCCCGGC AGGGGAGCU AGUGACGAAA AAUAACGACU CAGCAGACCU UCAAAGGGUC U-GUAAUGCU AAGAGUCACA
Crinoid_sp.    CAUCCCCGGC AGGGGAGCU AGUGAGGAAA AAUAACGACU CAGCAGACCU UCAAAGGGUC U-GUAAUGCU AAGAGUCACA
Sea_Star_sp.   CAUCCCCGGC AGGGGAGCU AGUGAGGAAA AAUAACGACU CAGCAGACCU UCAAAGGGUC U-GUAAUGCU AAGAGUCACA
Asterina_sp.   CAUCCCCGGC AGGGGAGCU AGUGAGGAAA AAUAACGACU CAGCAGACCU UC-GAGGCC U-GUAAUGCU AAGAGUCACA
S_purpuratus   CAUCCCCGGC AGGGGAGCU AGUGAGGAAA AAUAACGACU CAGCAGACCU UC-GAGGCC U-GUAAUGCU AAGAGUCACA
Arbacia_sp.    CAUCCCCGGC AGGGGAGCU AGUGAGGAAA AAUAACGACU CAGCAGACCU UC-GAGGCC U-GUAAUGCU AAGAGUCACA
O_esmarki      CAUCCCCGGC AGGGGAGCU AGUGAGGAAA AAUAACGACU CAGCAGACCU UC-GAGGCC U-GUAAUGCU AAGAGUCACA
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Appendix 4 (Continued).

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