Phylogenetic Relationships Among Extant Classes of Echinoderms, as Inferred from Sequences of 18S rDNA, Coincide with Relationships Deduced from the Fossil Record

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Abstract. In spite of the rich fossil record and multiple descriptions of morphological and embryological characteristics, the origin and subsequent evolution of echinoderms remain highly controversial issues. Using sequence data derived from 18S rDNA, we have investigated the phylogenetic relationships among five extant classes of echinoderms—namely, crinoids, asteroids, ophiuroids, echinoids, and holothurians. Almost complete sequences of 18S rDNA were determined for one species in each class, and phylogenetic trees were constructed both by the neighbor-joining method and by the maximum-likelihood method, with a hemichordate as an outgroup. The trees constructed by these methods support the hypothesis that the phylum Echinodermata can be subdivided into two subphyla, Pelmatozoa and Eleutherozoa. The class Holothuroidea, which has been the subject of debate with respect to whether the members are primitive or advanced echinoderms, did not occupy a primitive position but had an affinity for the class Echinoidea. Since both trees gave different branching topologies for the order of emergence of asteroids and ophiuroids, it seems likely that these two groups emerged within a very short period of time. A rough estimate of the timing of the divergence of the five classes from the present molecular analysis coincided with that deduced from the fossil record.

Introduction

Some of the most familiar seashore animals are echinoderms. There are about 7,000 living species, and they include the sea lilies and feather stars (the class Crinoidea), sea stars (the class Asteroidea), brittle stars (the class Ophiuroidea), sea urchins and sand dollars (the class Echinoidea), and sea cucumbers (the class Holothuroidea) (Barnes 1987; Brusca and Brusca 1990). In addition, another 13,000 species are known from fossil record derived from early Cambrian deposits. Echinoderms are characterized by a well-developed coelom, an endoskeleton composed of unique calcareous ossicles, a water vascular system, and pentamerous radial symmetry.

Echinoderms have been used frequently as materials for embryologic studies, and the patterns of development of many species have been described (Mortensen 1921; Davidson 1986; Raff 1987; Holland 1988; Strathmann 1988). In addition, there is a rich fossil record of the evolution of echinoderms because they have a rigid endoskeleton and are surrounded by many spicules that lie just under the surface of their bodies (Smith 1984). The fossil record is of great value for studies of echinoderm evo-
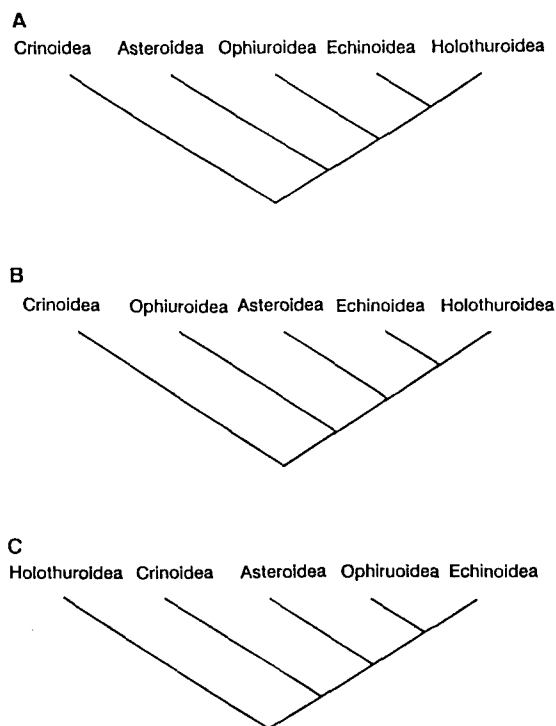
42

A
Crinoidea   Asteroidea Ophiuroidea Echinoidea Holothuroidea

B
Crinoidea   Ophiuroidea Asteroidea Echinoidea Holothuroidea

C
Holothuroidea Crinoidea   Asteroidea Ophiruoidea Echinoidea

Fig. 1. The three major phylogenetic trees for living echino-
derm classes that have been proposed to date. A is taken from
Paul and Smith (1984) and Smith (1988). B was derived from 18S
rRNA sequence data by Field et al. (1988), and C is taken from
Smiley (1986, 1988). One of the differences between the trees is
the position of the class Holothuroidea; this group is regarded as
the most advanced echinoderm in A and B, but as the most
primitive one in C.

lution (Paul and Smith 1984; Smith 1988). However,
the origin and subsequent evolution of echinoderms
remain highly controversial issues. The use of dif-
ferent morphological and embryological character-
istics in cladistic analyses has resulted in different
phylogenetic trees that attempt to relate the various
classes of echinoderms (e.g., Raff et al. 1988). As a
result, the evolution of echinoderms remains one of
the most important current problems in systematic
zoology.

The phylogeny of the extant five classes of echin-
oderms has been debated for nearly a century, and
the debate has now converged on three main issues
(Fig. 1). One problem involves the order of emer-
gence of asteroids and ophiuroids (Fig. 1A and B).
Another problem, which is the most difficult, in-
volves the phylogenetic status of holothurians. The
orthodox view is that holothurians are an advanced
group of echinoderms (Fig. 1A and B; Paul and
Smith 1984; Smith 1988; Raff et al. 1988), but the
holothurians have also been considered to be a
primitive group (Fig. 1C; Smiley 1988). In holothu-
rians, no torsion of the body axis is apparent during
metamorphosis, while torsion is commonly ob-
served in asteroids, ophiuroids, and echinoids. In

addition, the structures of the radial water vessels
and hydrocoels seem primitive in holothurians. By
contrast, holothurians share some characteristics
with echinoids—for example, reduction in the area
of the aboral surface.

Recent advances in molecular biology have made
it possible to answer some of the problems posed by
evolutionary biologists. In particular, molecular
data, including the amino acid sequences of certain
proteins and nucleotide sequences of RNAs and
DNAs, provide very powerful tools with which to
deduce the phylogenetic relationships among ani-
mal groups because it is relatively easy to identify
homologous characteristics, and these molecular
characteristics can be interpreted more objectively
than others. In particular, sequence data derived
from the 18S rRNA (or rDNA) of the small riboso-
mal subunit can serve as a powerful basis for phy-
logenetic reconstructions and for independent vali-
dation of classic morphological and embryological
criteria (Pace et al. 1985; Raff et al. 1988). The pi-
oneer study by Field et al. (1988) of the molecular
phylogeny of animal groups suggested that (1)
echinoderms are monophylic in origin, (2) crinoids
are separate from the other classes of eleutherozo-
ans, (3) the 18S rRNA-derived phylogenetic tree is
hierarchical, and (4) the sequences from holothu-
rian species exhibit a higher rate of nucleotide sub-
stitution than sequences from other classes but ho-
lothurian sequences exhibit similarity to those of
echinoids. However, definitive conclusions must be
postponed until the entire sequences of 18S rRNAs
are obtained (Raff et al. 1988).

We have now extended the study of Field et al.
(1988) by determining and comparing the almost
complete nucleotide sequences of 18S rDNAs from
members of five classes of echinoderms. During our
molecular-phylogenetic studies of the evolutional
pathway from advanced invertebrates to primitive
chordates, we noticed that a hemichordate had an
18S rDNA sequence that closely resembled those of
echinoderms (Wada and Satoh, unpublished). Use
of the hemichordate as an outgroup for inferences
of echinoderm phylogeny resulted in an apparently
more appropriate interpretation of the relationships
among echinoderms. Furthermore, when we calcu-
lated from the sequence data the timing of the di-
vergence of each of the five echinoderm groups, our
estimate coincided with those deduced from the fos-
sil record (Smith 1988).

Materials and Methods

Biological Materials. The echinoderms examined in the present
study were the comatulid *Antedon serrata* (a member of the

Crinoidea), the starfish *Asterias amurensis* (Asteroidea), the brittle star *Ophioplocus japonicus* (Ophiuroidea), the sea urchin *Strongylocentrotus intermedius* (Echinoidea), and the sea cucumber *Stichopus japonicus* (Holothuroidea). The acornworm *Balanoglossus carnosus* (in the phylum Hemichordata) was also examined as an outgroup animal. All of the specimens were collected off the coast of Japan.

Isolation of DNA, Amplification of 18S rDNA and Sequencing of the Amplified DNA. High-molecular-weight genomic DNA was extracted from gonads or tissues by the method described previously (Wada et al. 1992). Almost the entire length of the 18S rDNA was amplified by the polymerase chain reaction (PCR) in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT, USA) using primer 0 (5'CTGGTTGATCCTGCCAG3') and primer 10 [5'CACCTACGGA(AT)ACCTTG3']. Amplifications were performed in 50 μl of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, with 0.2 mM each dNTP, 50 pmol primers, template DNA (5–50 μg), and 1 U *Taq* DNA polymerase (Toyobo Co. Ltd., Osaka, Japan). The temperature regimen for 30 cycles was 1 min at 94°C, 2 min at 42°C, and 3 min at 72°C.

Amplified DNA was purified by electrophoresis in a 0.8% agarose gel and treated with lambda exonuclease (BRL, Bethesda, MD, USA) to obtain single-stranded DNA (Higuchi and Ochman 1989). With the single-stranded DNA as template, the nucleotide sequence was determined by the dideoxy chain-termination method (Sanger et al. 1977) with Sequenase ver 2.0 (United States Biochemical Corp., Cleveland, OH, USA) and [35S]-dATP (Amersham Japan, Tokyo, Japan). In addition to primers 0 and 10, primer 8 (5'CCGGAGAGGGAGCCTGA3'), primer 7 (antisense analog of primer 8), primer 1 (5'CAGCAGC-CGCGGTAATT3'), primer 9 (antisense analog of primer 1), primer 3 (5'GCGAAAGCATTTGCCAA3'), primer 4 (antisense analog of primer 3), primer 5 [5'GAAACT(TC)AAAGGAAT3'], primer 6 (antisense analog of primer 5), and primer 2 [5'ACGGGCGGTGTGT(AG)C3'] were used for sequencing (Fig. 2).

Comparison of Sequences and Inferences About Phylogeny. Sequences were aligned on the basis of the maximum nucleotide similarity. Using the aligned sequences, we calculated evolutionary distances in a pair-wise manner, as described by Jukes and Cantor (1969). The phylogenetic tree was constructed from an analysis of results by the neighbor-joining method of Saitou and Nei (1987). The degree of support for internal branches of the tree was further assessed by a bootstrapping technique (Felsenstein 1985). The phylogenetic tree was also constructed by the maximum-likelihood method (Felsenstein 1981) using the DNAML program from the PHYLIP package, version 3.4 (Felsenstein 1989).

Results

Phylogenetic Relationships Among the Five Extant Classes of Echinoderms

Genomic DNA was extracted from members of each of the five classes of echinoderms and from a hemichordate. In each case, almost the entire length of the 18S rDNA was amplified by PCR and the complete nucleotide sequences of the amplified products of PCR, except for the 5' and 3' termini,

were determined directly without recourse to cloning. Figure 2 shows the sequences of 1,720 nucleotides which have been aligned on the basis of maximum nucleotide similarity. This alignment reveals that the sequences are highly conserved in some regions, while those in other regions are highly variable. In addition, it is obvious that the holothurian exhibits a higher degree of nucleotide substitution than sequences from other classes, as pointed out in the previous study of 18S rRNA (Field et al. 1988).

Table 1 shows the structural similarities and evolutionary distances that were calculated in a pairwise fashion for the sequences that are aligned in Fig. 2 by use of the formula of Jukes and Cantor (1969). From this table it is evident that the rate of evolution of holothurians is considerably higher than that of the other groups. This difference may be one of the reasons for the difficulties encountered in attempts to determine the phylogenetic position of this animal group. This rapid rate of evolution of holothurians was confirmed by an analysis of at least two other species (*Cucumaria chronhjelmi* and *Paracandina chilensis*) of different orders (data not shown). Therefore, for further analyses, we used sequences of clearly aligned regions only.

A phylogenetic tree (Fig. 3A) was constructed by the neighbor-joining method (Saitou and Nei 1987) by referring to the distances given in Table 1. Another tree (Fig. 3B) was also constructed by the maximum-likelihood method (Felsenstein 1981), which is thought to be able to provide true topology regardless of inconstancies in rates of evolution (Hasegawa et al. 1991). The phylogenetic relationships between members of five classes of extant echinoderms were deduced by comparing these two trees.

First, among the five groups, the early divergence of the crinoid lineage (the Pelmatozoa) from other groups (the Eleutherozoa) was evident, and it was supported by a relatively higher value after "bootstrapping" (56.0). Therefore, echinoderms can be subdivided into two subphyla, the Pelmatozoa and the Eleutherozoa.

Second, in eleutherozoans, it was evident that echinoids and holothurians form a group that is discrete from asteroids and ophiuroids. The high value obtained by bootstrapping (69.4) supports this grouping. With respect to the phylogenetic position of asteroids and ophiuroids, the neighbor-joining tree and the maximum-likelihood tree had different topologies (compare Fig. 3A and B). The neighbor-joining tree suggested the early divergence of ophiuroids from three other classes of eleutherozoa, in accordance with the results of analysis of the sequence data for 18S rRNA (cf., Fig. 1B; Field et al.

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                                       40                                      80
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius GAAACTGCGGGATGGCTCATTAAATCAGTTATGGTTCATTGGATC*GAGTCCCCCCGA*CATGGATAACTGTGGTAATTC
As.amurensis        ATC                          C     G   CA    **TT
An.serrata          A*                           C     A T     TA ** T
Op.japonicus        A*              C            C     A       TG * T
Sti.japonicus       A ATC        G               T     GA A    TT**AT C
Ba.carnosus         AA*                          C   T G  GT A    TT   T

                                       120                                     160
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius TAGAGCTAATACATGCGTCCAAGCGCCGACTTTCC*AGAAGGCGTGCTTTTATTAGGAACAAGACCAGCCGG*******
As.amurensis                  AAT       C A G G         C            A C GG
An.serrata                   AA         CG* G                     G  AT  G
Op.japonicus                 CA         C A G                        A TC G
Sti.japonicus                CAA        CC T GG G A       C       G    C GTC CTTT
Ba.carnosus                  AAGG       GCG GG              *  C     AT   GTCC

                                       200                                     240
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius *TCTCGGCCGGCCACACTGGTGAACTCTGGATAACACAGCCGA**TCGCACGGTCTTTGCACCGGCGACGGATCCTTC*G
As.amurensis    **  C    TC GT              CTG             C
An.serrata      *  T C   CT C CT            TTT  T          A*                       A
Op.japonicus    *  T T    TT GT             TT   A          A C          A           A
Sti.japonicus   CGGGGTC G T TC G        A   TCAT    GA        *          CTC        AA
Ba.carnosus     TCG GCC    T C    C          TTG  G      C  CG  G        A  *

                                       280                                     320
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius AATGTCTGCCCTATCAACTT*TCGATGGTACGTTATGCGCCTACCAT*GGTCGTCACGGGTAACGGAGAATCAGGGTTCG
As.amurensis                                                   A
An.serrata                     T                              TG
Op.japonicus    C                                              A
Sti.japonicus   A    T                    A       *  T      T G
Ba.carnosus     G                                          G  T A          G

                                       360                                     400
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
                     Primer8(7)
Str.intermedius ATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCCCGACACGG
As.amurensis        T                                                              *
An.serrata          T                                                      T     A
Op.japonicus              T                                                T
Sti.japonicus                                                       A      T
Ba.carnosus                                                               T

                                       440                                     480
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius GGAGGTAGTGACGAAAAATAACAATACAGGACTCTTTCGAGGCCCTGTAATTGGAATGAGTACACTTTAAATCCTTTAAC
As.amurensis                 G                          G
An.serrata                          T    C
Op.japonicus
Sti.japonicus        A       G       C       CG        G C
Ba.carnosus                                                               A

                                       520                                     560
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
                                         Primer1'(9)
Str.intermedius GAGGATCCACTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGTAGCGTATATTAAAGCTGTTGCAG
As.amurensis        T T                                      A            AT  C
An.serrata          T T                                      A
Op.japonicus        T                                                 T   C
Sti.japonicus       T                                        C        TT  C
Ba.carnosus         T T                                      A

                                       600                                     640
           ...,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius TTAAAAAGCTCGTAGTTGGATCTTGGGCCCAGGCTG*CGGTCCGC**********CGTGTACGTGTACTGCA*GTCCTGG
As.amurensis                      G   CG  G                 ***AAGGCC C      CC *
An.serrata                       T  TC  T                   **GAGGT T*      CC
Op.japonicus             A     T     AA          **      CTCG AA GGGTGC*    TTT A   A
Sti.japonicus          C   T CT    GG  G G             TGAGCGGCACTGCCCGT TCCCCT CTCCCCGTCAA
Ba.carnosus     G             T       G                 CGAAA GCG GT C*      TT
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Fig. 2. Alignment of sequences of 1,720 nucleotides of 18S rDNA from the five echinoderms and the hemichordate analyzed in the present study. All the bases are shown for *Strongylocentrotus intermedius* and only bases different from these are shown for other species. *Asterisks* indicate deletions. The nine primers used for sequencing are shown by *lines*. Continued on pages 45–46.

```
                                    680                          720
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius ***************CCTTCCTCTCGGTTTTCGC*********CCGGTGCCCTTAATTGAGTGCCAGGAGAGGCCGGAA
As.amurensis                   C C                    T T   G C      G  C C ** A
An.serrata                       T        A           T             GG GTA
Op.japonicus                   C   G    G             TT   C        G TT C A  A
Sti.japonicus   ACGAGTCGTGGGAGATTT    CGG CAG GATT GGTTGGTCGT    T    C      GT C  A T
Ba.carnosus                    T  CT CT    GC  C          T   GC       G G T

                                    760                          800
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius CGTTTACTTTGAAAAAATTGGAGTGTTCAAAGCAGGCC*TCGCGCCTGAACAGCAGAGCATGGAATAATGGAATAGGACC
As.amurensis                                        GT A   G
An.serrata                           A                ATA       T
Op.japonicus                                         GCAACA
Sti.japonicus              G         A              C A AA T  C      TC       G
Ba.carnosus                         A                TCGAT    T  TCC

                                    840                          880
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius TCGGTTCTATTGCGTTGGTTTTCGGAACTCGAGGTAATGATTAAGAGGGACTGACGGGGGCATTCGTAT*TGCGGTGTGA
As.amurensis
An.serrata      T
Op.japonicus                          GTC           G
Sti.japonicus          T      C  GA  T CT         C       * T        C       C
Ba.carnosus            G         G G            A         C      C   C T

                                    920                          960
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
                                        Primer3(4)
Str.intermedius GAGGTGAAATTCTTGGATCGCCGCAAGACGAACGA*CTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGA
As.amurensis                                 C
An.serrata                                  C
Op.japonicus                                C     A              C
Sti.japonicus                       T      CC    A              C    G
Ba.carnosus              A       T       A T                              G

                                    1000                         1040
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius AAGTTAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACCATAAACGATGCCGACTGACGATCCGCCGGCGTTAC
As.amurensis                                               CG*    *
An.serrata                                               CG*   * C G       T
Op.japonicus                                        T   G ACG AT *
Sti.japonicus   CG  A        A           CG        A      CGTA T      C
Ba.carnosus              A         T               A   AG

                                    1080                         1120
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
                                                              Primer5(6)
Str.intermedius TCCCATGACGCGGCGGGCAGTC*TAAGGGAAACCAAAGTCTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTT
As.amurensis    A                  GC
An.serrata      A A                 C
Op.japonicus    A    A    TA  CT CC              T
Sti.japonicus                 ACTC CC  A
Ba.carnosus          CT      CT CC

                                    1160                         1200
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius AAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTCACCCGGCCC
As.amurensis                                                          G
An.serrata                          C
Op.japonicus
Sti.japonicus            .               T                   G  CT
Ba.carnosus

                                    1240                         1280
            ....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius GGACACAGTGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGA
As.amurensis
An.serrata      *                            T
Op.japonicus    A
Sti.japonicus                                T   A
Ba.carnosus     T
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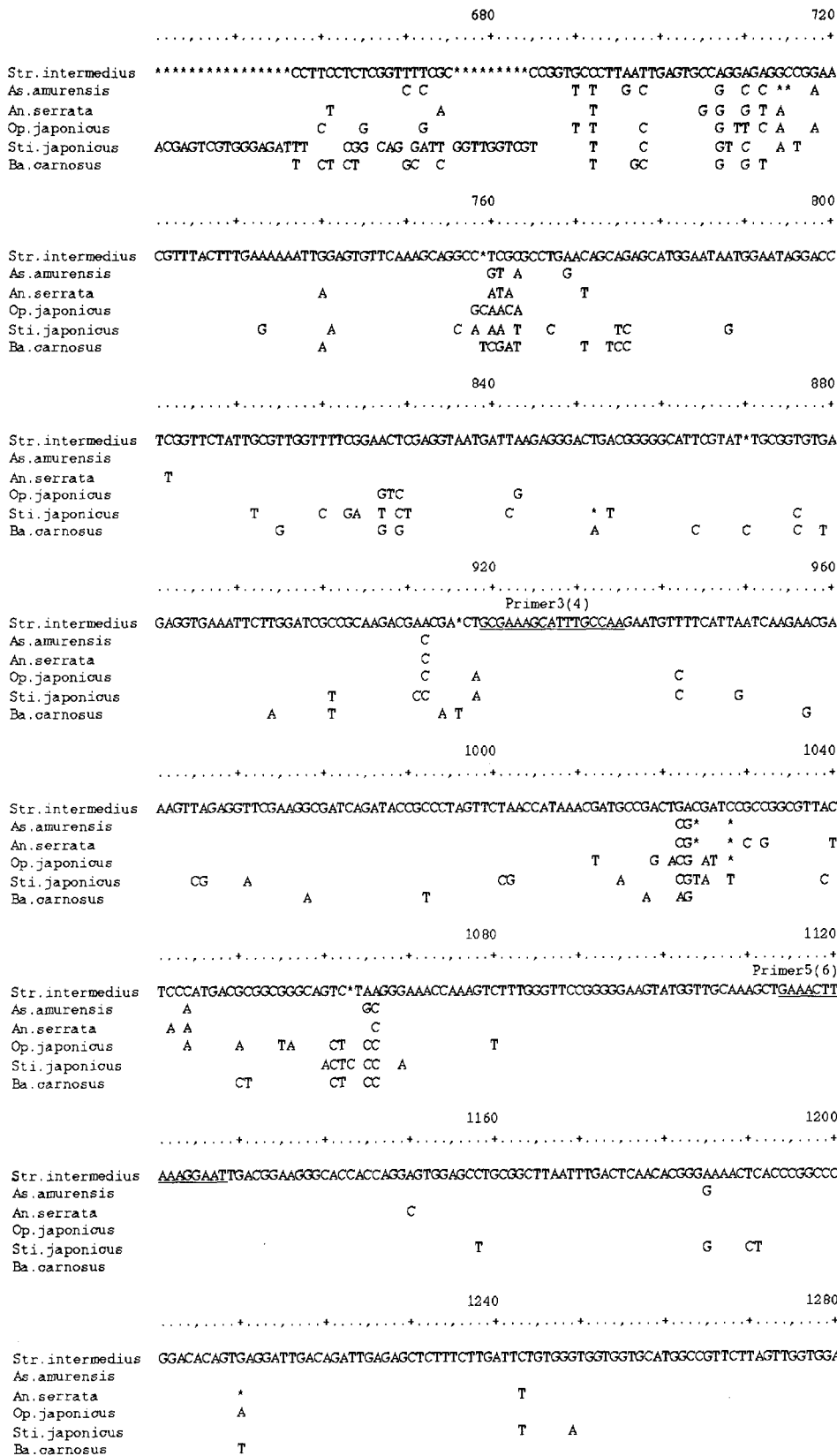
Fig. 2. Continued.

1988; Raff et al. 1988). The earlier divergence of ophiuroids was not, however, fully supported by bootstrapping (which gave a value of 34.0) in the neighbor-joining tree (Fig. 3A). By contrast, the maximum-likelihood tree suggested a common ancestry for asteroids and ophiuroids.

Third, with respect to the phylogenetic position of holothurians, both trees indicate that this group

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                                      1320                              1360
         ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius GCGATTTGTCTGGTTAATTCCGATAACGAACGAGACTCTGGCTTGCTAAATAGTTGCGCCACCCGCCG*CGTGCGGGTC*
As.amurensis                                                    C    GG    A CCGCC CT
An.serrata                                                          TT TG   G C CTT
Op.japonicus                                                          AG  *G C A
Sti.japonicus   T                                    T             CCG   T TG   A C CG
Ba.carnosus                                                        A  G    A   G CG C   C

                                      1400                              1440
         ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius AACTTCTTAGAGGGACAAGTGGCGTTTAGCCAGGCGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTTCGGGG
As.amurensis    G                 CG  C    C
An.serrata                        CG        C
Op.japonicus                   T           C
Sti.japonicus                A  T C   TA     G                                  C
Ba.carnosus                          CA                                         C

                                      1480                              1520
         ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius CGCACGCGCGCTACACTGGCGGAATCAGCGGGTACA*CTGCCCTTGGCCGGAAGGTCTGGGTAATCCGCTGAACCTCCTC
As.amurensis                   AA  G        GAT *C T   T T    C    C          C  T
An.serrata      GC            AAA        T TTG ***      AC         T         T T
Op.japonicus                  AA        T GTC * AT                T         T T
Sti.japonicus                C  G         T T   C    A    C        GA  T
Ba.carnosus                   AA          GTTG CT  G T  CT           A    T T T

                                      1560                              1600
         ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
Str.intermedius CGTGATGGGGATAGGGAGTTGCAATTATCTCCCTTGAACGAGGAATTCCCAGTAAGCGCGAGTCATCAGCTCGCGTTGAT
As.amurensis       C T      C      G     A           A
An.serrata        C A      C  T     T    C      G
Op.japonicus         T      A  T  G T                    AC        T
Sti.japonicus     C        C  T  G G               A              A
Ba.carnosus       C A      A  T    T                      A    T

                                      1640                              1680
         ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+
                               Primer2
Str.intermedius TACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGATCCTCGGAT****CGTCGG
As.amurensis                                                            C  GG C C
An.serrata                                                     A          ****C
Op.japonicus                                                             G C
Sti.japonicus                           C    AC      GC A    CGAC   T
Ba.carnosus     A                                          T        G C

                                      1720
         ....,....+....,....+....,....+....,....+....,....+
Str.intermedius CGTCGGACGGCTTTGCCGCCTCGC*TCGCATGTACGAGAA
As.amurensis    G CG  G  C   C G          T*GCGC CG
An.serrata      GCCGT T   T  CA   *****T     *GCGC   A
Op.japonicus    G    G C G  C    G    A   CT*GCGC
Sti.japonicus   GCA CCC      C G CGG T TCGA GCGTG
Ba.carnosus          GGTCG  C  G  AC        TTGCGC
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Fig. 2. Continued.

shares homology with echinoids. That is, holothurians do not occupy a primitive phylogenetic position among echinoderms. As mentioned above, the higher value after bootstrapping (69.4) in the neighbor-joining tree also supports a close relationship between holothurians and echinoids.

Timing of Divergence of the Five Classes, as Inferred from Sequences of 18S rDNA

Paul and Smith (1984) and Smith (1988) investigated the phylogenetic status of echinoderm fossil groups by incorporating data from the fossil record into the cladigram of the five extant classes. In addition, they estimated the timing of the divergence of each class by referring to the time between the latest emergence of the common ancestry of two groups and the emergence of the earliest fossil that has characteristics unique to one group (Smith 1988). The divergence times that they estimated are shown in Fig. 4A.

The present molecular-biological data indicated that the rate of evolution of 18S rDNA sequences in echinoderms other than holothurians is almost constant, which allowed us to estimate the timing of the divergence of echinoderm groups from the sequences of their 18S rDNAs. If we assume that the rate of evolution of the sequences is constant in crinoids, asteroids, ophiuroids, and echinoids, the averaged genetic distance between crinoids and the

Table 1. Structural similarities and evolutionary distance data for sequences of 18S rDNAs from echinoderms[a]

Species	Str. int.	As. amu.	An. ser.	O. jap.	Sti. jap.	B. car.
Strongylocentrotus intermedius		87	88	91	149	107
Asterias amurensis	0.0598		97	96	169	120
Antedon serrata	0.0605	0.0670		101	171	108
Ophioplocus japonicus	0.0626	0.0662	0.0699		167	110
Stichopus japonicus	0.1055	0.1209	0.1224	0.1193		184
Balanoglossus carnosus	0.0742	0.0838	0.0750	0.0764	0.1326	

[a] The lower left half of the table gives the numbers of substitutions, with gaps not included. The upper right of the table gives the evolutionary distances (average numbers of nucleotide substitutions per sequence position), as determined by the formula of Jukes and Cantor (1969)

A

B

0.02 unit
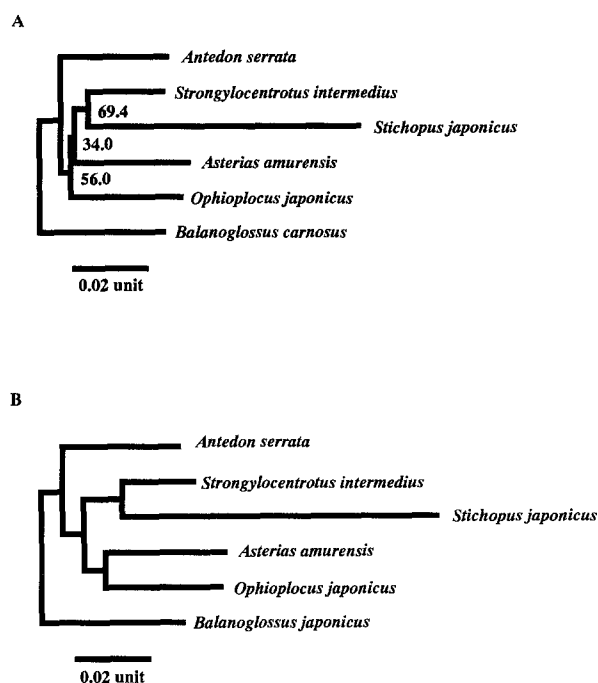
Fig. 3. Phylogenetic trees for extant classes of echinoderms, as deduced from comparisons of the aligned sequences of 18S rDNA (gaps are not included) shown in Fig. 2. Trees were constructed by (A) the neighbor-joining method and (B) the maximum-likelihood method. The *scale bars* indicate an evolutionary distance of 0.02 nucleotide substitutions per position in the sequence. Numbers at each branch in A indicate the percentage of times that a node was supported in 500 bootstrap pseudoreplications by the neighbor-joining method.

other three classes becomes 0.0658 substitution/site. This value corresponds to twice the length of time since the separation of the crinoid lineage from the eleutherozoan lineage. The genetic distance between echinoids and asteroids was calculated to be 0.0598 substitution/site and that between echinoids and ophiuroids to be 0.0626 substitution/site. With the further assumption that crinoids diverged from eleutherozoans 555 million years (MY) ago, the average rate of evolution was calculated to be 5.98 × 10^{-11} substitution/site/year. From the genetic distances, the timing of the separation of echinoids

from ophiuroids was estimated to be 523 ± 55 MY and that of echinoids from asteroids to be 500 ± 54 MY (Fig. 4B).

The timing of the divergence of echinoids from holothurians was calculated from the nucleotide substitutions that occurred in echinoids after the division from holothurians. The genetic distance can be calculated to be 0.0222 substitution/site. The divergence of these two groups was, therefore, estimated to have taken place 371 ± 62 MY ago (Fig. 4B).

Discussion

In addition to attempts at inferring phylogenetic relationships among the five extant classes of echinoderms from cladistic analysis of morphological and embryological characteristics and of the fossil record, several molecular approaches have also been exploited. For example, based on amino acid compositions of collagens, Matsumura and Shigei (1988) deduced that holothurians are related more closely to asteroids than to the other groups of echinoderms. In the present study, we analyzed the phylogenetic relationships among representatives of five extant classes of echinoderms by comparing the alignment of sequences of more than 1,700 nucleotides of 18S rDNAs. Unfortunately, the present study did not include the newly discovered class of living echinoderms, the Concentricycloidea (Baker et al. 1986). By taking a hemichordate as a reference group, we were able to deduce details of the evolution of living echinoderms. When either an amphibian or *Homo sapiens* was used as an outgroup animal, the results from the phylogenetic trees were much less conclusive.

The results of the present study of the almost complete sequences of 18S rDNAs basically support those of the previous study of partial sequences of 18S rRNA by Field et al. (1988). Both studies support the hypothesis that the phylum Echinodermata can be subdivided into two subphyla: The Pelmatozoa, which includes the class
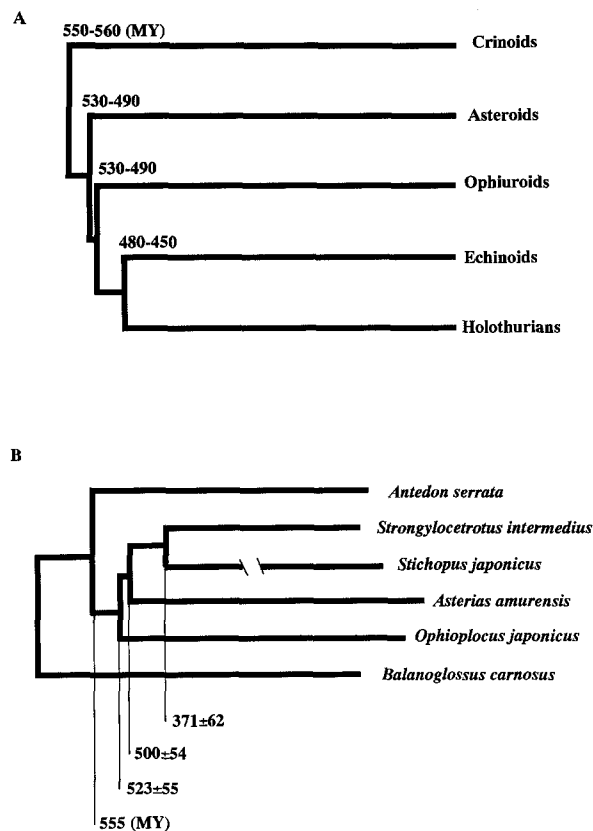
A

550-560 (MY) ———————————————— Crinoids

530-490 ———————————————— Asteroids

530-490 ———————————————— Ophiuroids

480-450 ———————————————— Echinoids

———————————————— Holothurians

B

Antedon serrata

Strongylocetrotus intermedius

Stichopus japonicus

Asterias amurensis

Ophioplocus japonicus

Balanoglossus carnosus

371±62

500±54

523±55

555 (MY)

Fig. 4. Timing of the divergence of the five extant classes of echinoderms as deduced from (A) the fossil record (after Smith 1988) and (B) the 18S rDNA sequences obtained in the present study. The timing of divergences deduced from the fossil record is shown with definitive latest times of divergence and working estimates of the earliest times of divergence as the upper and lower limits, respectively. The timing of divergence, deduced from the present molecular analysis, was calculated only from the distance matrix presented in Table 1 and, therefore, does not depend upon the methods used for the construction of phylogenetic trees. The averaged genetic distance between crinoids and three other classes (holothurians not included) was 0.0658 substitution/site. This value corresponds to twice the time after crinoids separated from the other members of the eleutherozoan lineage—namely, 555 MY. Therefore, the averaged rate of evolution was estimated to be 5.98×10^{-11} substitution/site/year. Since the genetic distance between echinoids and ophiuroids was 0.0626 substitution/site and the distance between echinoids and asteroids was 0.0598 substitution/site, the divergences were estimated to have occurred 523 ± 55 MY and 500 ± 54 MY ago, respectively. The genetic distance in the echinoid lineage corresponding to the time after separation of echinoids from holothurians was calculated to be 0.0222 substitution/site and, therefore, the separation was calculated to have occurred 371 ± 62 MY ago.

Crinoidea, and the Eleutherozoa, which includes the classes Asteroidea, Ophiuroidea, Echinoidea, and Holothuroidea (e.g., Jefferies 1988). In the case of the eleutherozoans, the present molecular-phylogenetic study failed to determine the order of emergence of ophiuroids and asteroids, although it is clear that these two groups diverged earlier than another discrete group of echinoids and holothuri-

ans. The neighbor-joining tree and the maximum-likelihood tree had different topologies (Fig. 3). The bootstrap resampling in the neighbor-joining tree did not provide an answer to the question of the order of emergence of these two groups, although the present molecular data do not suggest the earlier emergence of asteroids than of ophiuroids. The earliest fossil records of asteroids and ophiuroids are found in the same beds (e.g., Smith 1988). Therefore, it is highly likely that these two groups emerged within a very short time period. This timing may explain why the order of emergence of these two groups cannot be inferred from 18S rDNA (or rRNA) sequence data. More molecular data with other probes are required if we are to clarify the speciation of the two groups that apparently occurred within a very short period of time.

The phylogenetic status of holothurians on the 18S rDNA trees (Fig. 3) shows that they are not primitive but have shared a common unique ancestry with echinoids until recently. One of the reasons for the inference that holothurians are primitive echinoderms involves the torsion of the body axis during metamorphosis, which is seen in asteroids, ophiuroids, and echinoids but not in holothurians (Smiley 1988). Since the present sequencing data do not support this inference, it is possible that torsion might have been lost during the evolution of holothurians. This hypothesis is consistent with the fact that torsion of the body axis is seen earlier than the acquisition of pentamery (Smith 1988). It is unlikely that acquisition of pentamery in holothurians and in the other echinoderms represents a convergence (Smith 1988). Therefore, the characteristics of holothurians that are regarded as primitive may be derived or ancestral characteristics that have been retained only by holothurians since their emergence.

As shown in Fig. 4, the timing of the divergence of echinoderm classes, as inferred from the fossil record and from substitutions in 18S rDNA, shows quite similar tendencies. Therefore, the present molecular study supports the earlier interpretation and characterization of the fossil record in cladistic analyses of echinoderm phylogeny (Paul and Smith 1984; Smith 1988). The divergence of ophiuroids and asteroids was almost simultaneous (Fig. 4). This conclusion also suggests that the emergence of these two groups occurred within a very short period of time. The timing of the divergence of echinoids from holothurians, as estimated from the present molecular data, is a little later than that deduced from the fossil record, perhaps because of the slower rate of evolution of echinoids. (The right end of the echinoid branch is a little shorter than those of the others.) From this reason, molecular sequence data may underestimate the time at which

divergence occurred, although it remains possible that the fossil record has been misinterpreted in this regard.

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