THE MITOCHONDRIAL GENOME MAP OF THE CRINOID, *FLOROMETRA SERRATISSIMA*

by

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ABSTRACT

A major portion (approximately 14.5 kb) of the mitochondrial genome of the crinoid, *Florometra serratissima*, has been sequenced and a complete gene map has been developed. The genome contains the genes for 13 proteins, small and large ribosomal RNAs (12S and 16S rRNAs), and 22 transfer RNAs (tRNAs) as well as a putative control region for initiation of replication (D-loop). The crinoid mitochondrial gene order is: [D-loop]-T-E-12S-F-L_{UUR}-G-16S-Y-ND2-I-ND1-COI-R-ND4L-COII-K-A8-A6-COIII-S_{UCN}-ND3-ND4-H-S_{AGN}-ND5-ND6-Cytb-P-Q-N-L_{CUN}-A-W-C-V-M-D, where the single letters indicate tRNA genes. Previous analyses have demonstrated that there has been a mitochondrial gene rearrangement that separates four echinoderm classes into two groups. The classes Asteroidea (sea stars) and Ophiuroidea (brittle stars) contain a 4.6 kb inversion in their mitochondrial genomes in comparison to the Echinoidea (sea urchins) and Holothuroidea (sea cucumbers). This inversion includes most of the tRNA genes, the 16S rRNA gene, and the genes for the NADH dehydrogenase subunits 1 and 2. In the case of brittle stars there has been a subsequent inversion event within this region. The mitochondrial gene order of *F. serratissima* (class Crinoidea) displays additional inversions within this 4.6 kb region not seen in the other classes. The remaining mitochondrial gene order of the five echinoderm classes is conserved. Both gene order and gene sequencing may be used to examine the relationship of the crinoids to the other echinoderm classes as

well as the potential phylogenetic pathways of echinoderm mitochondrial genome rearrangements.

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INTRODUCTION

Crinoidea Evolution and General Biology

Historically, there have been five extant classes of echinoderms considered: the Asteroidea (sea stars), the Echinoidea (sea urchins), the Holothuroidea (sea cucumbers), the Ophiuroidea (brittle stars) and the Crinoidea (feather stars). Recently, Baker et al. (1986) established a sixth class, the Concentricycloidea (sea daisies) following a discovery of new benthic specimens off the coast of New Zealand. Within the Phylum Echinodermata, the classes are subdivided into the mostly extinct, stalked Subphylum Pelmatozoa and into the free-living, stemless echinoderms of Subphylum Eleutherozoa. The class Crinoidea is the only living representative of the Pelmatozoa and is the oldest of the extant echinoderm classes. Fossil evidence indicates that the divergence between the crinoids and the other classes appears to have taken place before the end of the Lower Cambrian, about 550 million years ago (mya) (Smith, 1988). The oldest crinoid-like fossil known, *Echmatocrinus brachiatus* Sprinkle, was found in the Burgess Shale of British Columbia and is dated back to the Middle Cambrian (Sprinkle, 1973), yet the first distinct crinoid fossil does not appear until the beginning of the Ordovician period (Figure 1). At present, there are 21 species of Cambrian and Lower Ordovician crinoids known, plus a variety of fossil structures that suggest the presence of additional crinoid species (Donovan, 1986). The basic body plan for the Eleutherozoan echinoderms was established by the

Figure 1: Fossil record for echinoderms and vertebrates.

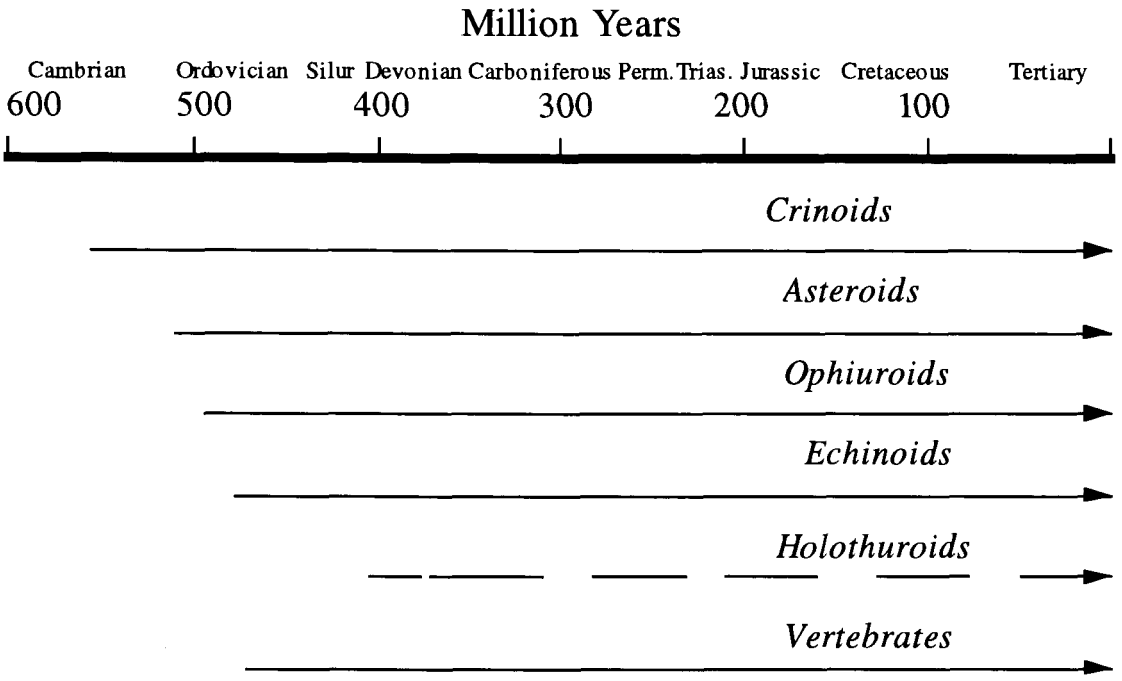


Figure 1. A summary of divergence times for the five major echinoderm classes based on fossil evidence (Smith et al., 1988). Crinoids appear first in the fossil record with the asteroids, ophiuroids, echinoids, and holothuroids appearing later.

end of the Lower Cambrian but, it was not until the Ordovician that the asteroids, ophiuroids and echinoids first appeared in the fossil record with the holothuroids appearing even later (Paul and Smith, 1984).

The stalked crinoids, like the other classes of the Pelmatozoa, flourished in Palaeozoic era during which over 6000 fossil crinoid species have been described (Moore and Jeffords, 1968). Crinoids increased in abundance and diversity up to the Mississipian (Early Carboniferous) about 350 mya, until the Mid-Late Triassic when a massive extinction event resulted in only a few representative groups remaining (Simms, 1988). An indication of the abundance of crinoids in this era is seen in the Mississipian Burlington Limestone layer, a middle Palaeozoic deposit that occurs in southeastern Iowa, western Illinois and Missouri, 99% of which is comprised of fossil crinoid remains, including approximately 300 different species (Macurda and Meyer, 1983). The deposit averages 40 to 50 meters in thickness and covers tens of thousands of square kilometers.

A typical Palaeozoic crinoid is divided into three morphological regions: the calcareous plated calyx or theca, which has been used for most fossil classification purposes (Hyman, 1955 and Sprinkle, 1987), the food-gathering arms that branch (with or without pinnules) off the side or top of the calyx and a stalk, with associated cirri that attaches the base of the calyx to the substrate (Sprinkle, 1987). The stalk is flexible, being mostly composed of a series of calcareous discs (columnals) stacked one on top of the other. The feeding and reproducing part of the animal is situated at the top of the stalk. The digestive

system is tubular, consisting of a mouth, intestine and anus. The calyx, a cup-like structure, encloses the complete digestive system and is covered orally by a membrane, the tegmen, that bears the mouth (Hyman, 1955). Extant crinoid species maintain the general structures of fossil crinoids yet contain modifications of details, to different extents (Hickman, 1967).

The class Crinoidea is subdivided into four subclasses based on several features, particularly the arrangement of plates in the calyx, structure of the arms and nature of the tegmen (Sprinkle, 1987). Three of the subclasses, Camerata, Flexibilia and Inadunata are extinct and for the most part, restricted to the Palaeozoic era. The fourth subclass Articulata, first appeared in the Early Triassic (about 250 mya), and is generally thought to have evolved from an inadunate ancestor, or ancestors, at some point in the Late Permian or Early Triassic (Sprinkle, 1987; Paul and Smith, 1984; Macurda and Meyer, 1983; Meyer and Macurda, 1977; Nichols, 1966; Van Sant and Lane, 1964). However, others believe as it is difficult to identify a single character that is both specific to the articulates as well as being recognizable in fossil material, the ancestor to the articulates may be any of the extinct subclasses (Simms, 1988). The articulates flourished in the Jurassic and Cretaceous and have existed to present day. Originally, all articulate crinoids were stalked, but during the Mesozoic (Jurassic), some of them gave rise to stalkless articulates (Simms, 1988). Modern crinoids are represented by either the stalked sea lilies, belonging to the orders Isocrinida, Millericrinida, Bourgueticrinida and Cyrtocrinida (Breimer, 1978) or the free-living comatulids or feather stars in the

order Comatulida, which are stalked as juveniles, but break free later. The emergence of the feather star in the Jurassic, represents a major step in crinoid evolution. The absence of a stalk, combined with the development of muscular articulations, allowed this more mobile form to invade new habitats and eventually to become the more widely distributed modern crinoid (Macurda and Meyer, 1983). At present, there are about 650 species of living crinoids of which, only about 80 have the typical stalked pelmatozoan form and are restricted to deep waters, probably in a state of approaching extinction (Moore and Teichert, 1978; Hyman, 1955). Feather stars range from intertidal waters to great depths (Meyer et al., 1978), have undergone extensive speciation and appear on the upgrade at present (Hyman, 1955).

Present understanding of development in crinoids is derived from studies of comatulid crinoids or feather stars. Little or nothing is known about the embryology of the approximately 80 species of stalked sea lilies as they live too deep to study by conventional SCUBA diving and most dredged individuals are collected damaged and dying (Holland, 1991). Sexes are separate in crinoids, although a few hermaphroditic individuals have been observed in *Oxycomanthus japonica* (Dan and Dan, 1941), *Himerometra bartschi* (Vail, 1987), *Colobometra perspinosa* (Vail, 1987), *Oligometra serripinna* (Vail, 1987) and *Florometra serratissima* (Mladenov, 1986). Gonads are usually located in the genital pinnules on the lower half to two-thirds of each arm but in some species, the gonads are located within the arms (Hyman, 1955). Only one species of crinoid, the feather star *Florometra serratissima*, is found in

northeastern Pacific coastal waters (Mladenov, 1987), from California to Alaska at depths of 11 to over 1200 meters (Clark and Clark, 1967) with gravid females available at any time of the year (Mladenov, 1986). *F. serratissima* doliolaria larvae (nonfeeding vitellaria larvae surrounded by bands of cilia) begin to hatch 35 hours after fertilization and swim just below the surface of the water. After 4.5 days they begin to explore the substratum and then settle into aggregations where there is a rapid metamorphosis into the attached stalked cystidean form of developing feather stars. The non-feeding cystidean, increases in height (to approximately 1.8 mm) and transforms into a pentacrinoid: oral plates open, tube feet are extended and feeding begins (Mladenov and Chia, 1983). Over the course of weeks to months, the pentaradial arms, water vascular system, anus, and calyx develop and just prior to separation of the free-living feather star juvenile from its stalk, the pinnules and cirri develop from the arms and base of the calyx, respectively (Mladenov, 1987).

Mitochondrial Genomes

The mitochondrial genomes of metazoans are mostly closed circular DNA molecules that range in size from 14-17 kb. The complete sequences of mitochondrial DNA (mtDNA) are now available from a variety of different animals. There is a notable conservation of mitochondrial gene content in the metazoans, however, the arrangements of the genes within the mtDNA genomes vary greatly (Desjardins and Morais, 1990; Okimoto et al., 1992; Pääbo et al., 1991; Cantatore et al., 1989; Wolstenholme, 1992). In general,

the mitochondrial genome contains genes for: 13 proteins, the oxidative phosphorylation system cytochrome oxidase subunits I, II, and III (COI, COII, and COIII) and cytochrome b (Cyt b), the ATPase complex subunits 6 and 8 (ATPase6 and ATPase 8), and the respiratory chain NADH dehydrogenase subunits 1-6 and 4L (ND1-ND6 and ND4L); 22 transfer RNA genes (tRNAs); and small and large ribosomal RNA genes (12S and 16S rRNAs). In addition, there are usually one or more noncoding regions which have been demonstrated to contain signals for initiation of replication and transcription of the mtDNA in mammals and amphibians (Montoya et al., 1982, 1983; Clayton, 1982, 1984, 1991; Walberg and Clayton, 1981; Brown et al., 1986). In vertebrates, the main noncoding region that contains the replication origin of the heavy strand (O_H) and the promoters for heavy (HSP) and light (LSP)-strand transcription is often referred to as the displacement loop or D-loop region. This control region or D-loop is thus named because initiation of the heavy (H) strand replication results in the formation of a triple-strand structure, a displacement loop, with the newly sythesized H-strand paired to the L-strand. A second, but smaller noncoding region believed to contain the origin of the light strand replication (O_L) has also been located in mammals (Bibb et al., 1981; Anderson et al., 1981), including marsupials (Janke et al., 1994), and possibly in *Xenopus laevis* (Wong et al., 1983) and *Drosophila* (Clary and Wolstenholme, 1985), but has not been detected in birds (Desjardins and Morais, 1990). In other organisms, such as the invertebrates, there are similar noncoding regions present in the mtDNA that are often designated putative

control regions. Although some of the mtDNA genome size variations result from differences in gene lengths, most are attributed to differences in the length of the control region, some of which contain repeated sequences (Bermington et al., 1986; Moritz et al., 1987; Rand and Harrison, 1989; Buroker et al., 1990; Wolstenholme, 1992). In some instances, tandem duplications of coding regions have also occurred (Moritz and Brown, 1986,1987; Wallis, 1987; Moritz, 1991; Zevering et al., 1991).

In contrast to other mtDNAs, the animal mitochondrial genome is highly compact, with very few intervening sequences. Apart from the origin of replication regions, the genes coding for the transfer, ribosomal, and messenger RNAs do not contain any introns or untranslated regions. In most cases, genes are contiguous or separated by only a few nucleotides, while in others the genes overlap (Wolstenholme, 1992; Saccone and Sbisà, 1994). Some of the overlaps occur between the 3' ends of genes that are transcribed on opposite strands yet there are cases in which the overlap involves genes encoded on the same strand. One striking example is the overlap seen in the ATPase6 and ATPase8 genes. The initiation of the ATPase6 gene overlaps with the termination of the ATPase8 gene to varying degrees among metazoans (Anderson et al., 1981, 1982; Bibb et al., 1981; Roe et al., 1985; Jacobs et al., 1988a; Cantatore et al., 1989; Desjardins and Morais, 1990; Smith et al., 1990; Asakawa et al., 1995). In addition, the ND4 and ND4L genes have been seen to overlap by seven nucleotides in vertebrates, where they occur next to one another (Anderson et al., 1981). Complete, bicistronic

transcripts containing the ATPase6 and ATPase8 reading frames, and the ND4 and ND4L reading frames have been isolated from the HeLa cell mitochondria (Ojala et al., 1981) as well as two bovine proteins that correspond in size and sequence predicted by overlapping ATPase6 and ATPase8 genes (Fearnley and Walker, 1986), which demonstrate that translation of the ATPase6 protein must involve initiation within the ATPase8 reading frame. The condition of overlapping genes contained within the same transcript would require specific mechanisms by which independent complete translation products could be formed (Anderson et al., 1982). The mitochondrial genomes of sea urchins produce multicistronic, and probably overlapping transcriptional units which extend bi-directionally over the whole genome. It has been suggested that extensive RNA processing would be required to produce mature RNA molecules from the primary transcripts, at the post-transcriptional level (Cantatore et al., 1990; Jacobs et al., 1988a).

In contrast to the universal code, the genetic code in mitochondria is diverse and varies from phylum to phylum (Barrell et at., 1979; Jukes and Osawa, 1990; Osawa et al., 1992). Rather than being a stop codon, as seen in the universal genetic code, UGA specifies tryptophan in all animal mitochondrial genomes. In addition, the codons AGA and AGG are thought to specify stop codons in vertebrates (Anderson et al., 1981, 1982; Bibb et al., 1981; Roe et al., 1985; Gadaleta et al., 1989; Osawa et al., 1989; Desjardins and Morais, 1990) yet, specify serine in most of the invertebrates (Jacobs et al., 1988a; Cantatore et al., 1989; Himeno et al., 1987; Okimoto et al., 1992;

Asakawa et al., 1995) except cnidaria in which arginine is specified as in the standard universal code (Wolstenholme, 1992). In most metazoans, the codons AUA and AAA specify methionine and lysine respectively. In the echinoderms however, AUA specifies isoleucine and AAA, asparagine (Himeno et al., 1987; Jacobs et al., 1988a; Cantatore et al., 1989).

A second feature of the metazoan mtDNA genetic code is the variation in translation initiation and termination codons. It has been determined that all four AUN triplets can act as initiation codons (Bibb et al., 1981; Attardi et al., 1985; Jacobs et al., 1988a; Cantatore et al., 1989) as opposed to just AUG. Furthermore, in some cases the codon GUG is thought to be act as the initiation codon (Clary and Wolstenholme, 1985; Gadaleta et al., 1989; Jacobs et al., 1988a; Cantatore et al., 1989; Desjardins and Morais, 1990). Translation termination in metazoan mtDNA genomes is also mediated in some unique ways. In addition to the AGG and AGA stop codons utilized by vertebrates, metazoans use TAA or TAG as their termination codons. Some gene transcripts end with incomplete termination codons such UA or a single U. A complete termination codon results from post-transcriptional polyadenylation of individual transcripts (Anderson et al., 1981; Ojala et al., 1981; Clayton, 1991).

The diversity of the genetic code in animal mitochondria is considered to be directly related to the sequence and/or structural components of mitochondrial tRNA genes (Osawa et al., 1992). The metazoan mtDNA genomes contain the genes for usually only 22 tRNAs yet these are sufficient to decode the 13 protein genes found in most metazoan mitochondria due to a

more extended U:N wobble than that seen in the decoding of the universal genetic code. As a result, a single tRNA can read all codons of a four-codon family (Barrell et al., 1980; Anderson et al., 1981). However, it is uncertain if this is a result of the U, present in the wobble position of the anticodon, being able to bind with all four third-codon positions, or if two nucleotide pairs are sufficient to stabilize the anticodon-codon interaction (Lagerkvist, 1981). The presence of a reduced number of tRNAs is thought to have evolved from pressure for economization of genome size (Anderson et al., 1981, 1982; Attardi et al., 1985; Bibb et al., 1981; Clary and Wolstenholme, 1985; Roe et al., 1985; Wolstenholme et al., 1987; Gadelata et al., 1989; Desjardins and Morais 1990; Okimoto et al., 1992).

In addition to the reduced number of tRNA genes, the metazoan mtDNA encode variations in the standard, cloverleaf tRNA, secondary structure seen in nuclear tRNA genes. Most of the vertebrate and invertebrate mtDNA tRNAs can fold into the four-armed secondary structure but, there is usually variation in the size and sequence of the loops of the dihydrouridine (DHU or D) and TΨC (or T) arms. In general, the cloverleaf structure is maintained except there is usually a reduction in size of the tRNA, several universal bases are missing, some mismatches and unconventional pairings are present, and there is a high proportion of AT nucleotides (Attardi et al., 1985; Cantatore et al., 1989; Wolstenholme, 1992). It has been hypothesized that the weakened tRNA secondary structures resulting from unorthodox base pairings may be stabilized more by tertiary interactions or base stacking rather than hydrogen

bonds (Ueda et al., 1985; Roe et al., 1985; Kumazawa et al., 1989; Yokogawa et al., 1989; Wakita et al., 1994). Although the mtDNA tRNA genes vary in comparison to the universal tRNA cloverleaf sequence and structure, some generalities can be made. A comparison of *Paracentrotus lividus* mtDNA tRNAs with those of *Drosophila yakuba*, *Xenopus laevis,* and *Homo sapiens* reveal that the anticodon loop and stems, and the DHU stems are the most conserved regions of mtDNA tRNAs, where the TΨC stems and amino acid stems are more variable (Cantatore et al., 1989). In addition, the most striking example of tRNA structure variation is seen in the metazoan tRNA $serine_{AGN}$, in which the tRNA is smaller than any of the others and the DHU arm is missing .

The metazoan mitochondrial gene order and organization seems to vary from phylum to phylum. Gene rearrangement consists of a different distribution of genes between the two strands (polarity inversion), gene transpositions and even gene loss, in some cases (Saccone and Sbisà, 1994). The arrangement of genes in most vertebrates appears to be relatively stable, however, some variations have been noted in birds (Desjardins and Morais, 1990, 1991), and marsupials (Pääbo et al., 1991). In the phylum Echinodermata, complete mitochondrial genetic maps have been published for the sea urchins *Strongylocentrotus purpuratus* (Jacobs et al., 1988a), *Paracentrotus lividus* (Cantatore et al., 1989), and *Arbacia lixula* (De Giorgi et al., 1996) as well as the sea star, *Asterina pectinifera* (Asakawa et al., 1995). In addition, partial sequence data from another sea star, *Pisaster ochraceus,* has been published (Jacobs et al., 1989; Smith et al., 1990). Although previously studies of sea

urchin mtDNA (Roberts et al., 1983; Jacobs and Grimes, 1986; Jacobs et al., 1988b; Cantatore et al., 1987a) had suggested that its gene order was different, the sea urchin, *S. purpuratus,* published mtDNA map (Jacobs et al., 1988a) clearly demonstrated the presence of a novel gene order in comparison to the previous ones seen in vertebrates and *Drosophila*. In addition, utilizing protein and rRNA genes, it was proposed that the sea urchin pattern could be related to the vertebrate pattern with only a few rearrangements (Jacobs et al., 1988a; Cantatore et al., 1987a, 1989). The mitochondrial gene orders of the other two published sea urchin species *A. lixula* and *P. lividus* are the same as that of *S. purpuratus* which indicates stability of mtDNA gene order within the sea urchins. Previously, Smith et al. (1989) demonstrated that a major inversion event had occurred in two of the echinoderm classes. The sea stars (class Asteroidea) contained a 4.6 kb inversion encompassing portions of the tRNA cluster, the large ribosomal RNA gene, and the ND1 and ND2 genes in comparison to the sea urchins (class Echinoidea). This inversion was seen in two sea star orders (Forcipulatida and Valvatida). In addition, PCR and sequence data indicated that the sea cucumbers (class Holothuroidea) maintained the sea urchin mitochondrial gene order, but the brittle stars (class Ophiuroidea) demonstrated the basic sea star pattern (Smith et al., 1993). Therefore, although there is variation within the phylum Echinodermata, it seems that within classes the mitochondrial gene order is relatively stable. However, preliminary data indicates the presence of a novel tRNA duplication event within the sea cucumber genus *Cucumaria* with respect to other

holothuroids (Allan Arndt, pers. comm.). This 4.6 kb inversion creates two groupings of echinoderms: the echinoid/holothuroid lineage and the asteroid/ophiuroid one. This study was undertaken to determine the mtDNA genome order of the fifth echinoderm class, Crinoidea and its possible grouping to either lineage. Once the mtDNA gene order of the five major echinoderm classes is known, then potential phylogenetic pathways of echinoderm mitochondrial genome rearrangements may be postulated. In order to determine the crinoid mtDNA gene order, a strategy of DNA sequencing of products from PCR amplification across potential gene junctions and partial genomic clones was undertaken. The DNA sequence accumulated encodes approximately 90% of the crinoid mtDNA genome including the complete sequences of 22 tRNA genes, 11 protein coding genes and both ribosomal RNA genes, as well as partial sequences of the remaining two protein coding genes. This sequence data allows us to extensively examine echinoderm phylogenies and compare tRNA structures across echinoderm classes.

MATERIALS AND METHODS

Specimens

Florometra serratissima individuals were obtained by SCUBA in Howe Sound, around Bowen Island, B.C. and were also supplied by the Bamfield Marine Station, Bamfield, B.C.

DNA Isolations

1) Total DNA preparation from complete animals

Individual *Florometra* were frozen in liquid N_2 for five minutes and then ground to a fine powder in a frozen cold motor and pestle, adding liquid N_2 occasionally. The ground powder was added to 10 ml of Protease K buffer (100mM Tris-Cl pH 8; 200mM NaCl; 50mM EDTA pH 8; 1% SDS (sodium dodecyl sulfate); and 200 µg/ml Protease K (Sigma)) heated to 65° C and digested at 65° C for one hour with occasional mixing. After digestion was complete, one-half of the digestion volume of 1xTE (10mM Tris-Cl; 1mM EDTA pH 8) equilibrated phenol was added and mixed gently for five minutes. The tubes were centrifuged in a Beckman JS-13.1 rotor, 4° C for 10 minutes at 8000 rpm and the aqueous phase was removed to a fresh tube. The organic layer was re-extracted with the addition of 10 ml Protease K buffer (no Protease K added), mixed for five minutes and centrifuged as before. The re-extracted

aqueous phase was combined with the first aqueous phase and extracted with one-half volume of a 1:1 phenol:sevag{chloroform/isoamyl alcohol (24:1)} mixture. The tube was mixed gently for five minutes and centrifuged as before. The phenol:sevag extractions were continued until the interface between the aqueous and organic layers was clean. The aqueous layer was extracted with CTAB/NaCl (10% (w/v) Cetyl Trimethyl Ammonium Bromide in 0.7M NaCl): a one-sixth volume of 5M NaCl was added first and mixed well, then a one-eighth (original) volume of CTAB/NaCl was added. The tube was incubated at 65° C for 30 minutes and extracted with an equal volume of sevag twice (or until the interface was clean). The aqueous phase was transferred to a fresh tube and precipitated overnight at -20° C with the addition of 2.5 volumes of 95% ethanol. The DNA was pelleted in a Beckman JS-13.1 rotor, 13,000 rpm, 4° C for 20 minutes and the pelleted DNA was rinsed twice in 70% ethanol, re-centrifuged and let to air dry. The dry pellet was taken up in 1xTE containing 20 μg/μl RNAase and stored at 4° C.

2) Total DNA preparation of eggs from gravid females

During dissection of live crinoids, large amounts of eggs were released. The isolated *Florometra* eggs were digested in a final volume of 10 ml Protease K buffer (100mM Tris-Cl pH 8; 100mM NaCl; 50mM EDTA; 1% SDS; 200 μg/ml Protease K) for two hours at 65°C. The digested eggs were extracted with an equal volume of 1xTE equilibrated phenol until the aqueous

and organic layer interface was clean and extracted with an equal volume of 1:1 phenol:sevag. The organic and aqueous phases were separated by centrifugation in a Beckman JS-13.1 rotor, 20 °C, for 10 minutes at 8000 rpm. The aqueous layer was extracted with CTAB/NaCl (10% (w/v) Cetyl Trimethyl Ammonium Bromide in 0.7M NaCl): a one-sixth volume of 5M NaCl is added in first and then a one-eighth (original) volume of CTAB/NaCl is added. The tube was incubated at 65° C for 35 minutes, mixed occasionally and extracted with 0.5 volumes of sevag and centrifuged as before. The supernatant was precipitated with the addition of 2.5 volumes 95% ethanol at -20 °C overnight. The DNA was pelleted using a Beckman JA-13.1 rotor 13000 rpm, 4 °C, for 20 minutes, rinsed in 70% ethanol and then re-centrifuged for five minutes. The dry pellet was taken up in 1 ml of 1xTE and left at 4 °C.

Southern Blots

Total DNA preparations were single (and double) restriction digested in 100 µl volumes containing 10 µg DNA, 3 µl enzyme #1, (3 µl enzyme #2), and 10 µl of the appropriate 10x React Buffer for two hours at 37 °C, at which time an additional 3 µl of enzyme #1 (and #2) were added. The following enzymes were used: Kpn I, Pst I, Sst I, EcoR I, and Xho I and all restriction digests were carried out in specific React Buffers according to the manufacturer's

specification (Gibco BRL). After the second amount of enzyme(s) had been added, the digestion continued at 37 °C for another two hours, heated to 65° C for 15 minutes and stored on ice. The digested DNA was electrophoresed on 0.7% agarose gels in 1xTAE buffer (40mM Tris-Cl-acetate; 2mM Na_2EDTA) containing ethidium bromide and photographed under short U.V. wavelength light (302 nm). Prior to transfer of the gel, it was treated as follows: acid depurinated in 500 ml of 0.25M HCL for 20 minutes, rinsed with ddH_2O once, denatured in 300 ml denaturing buffer (1.5M NaCl; 0.5M NaOH) for 20 minutes, rinsed with ddH_2O twice and finally neutralized in two times 300 ml neutralizing buffer (1M NH_4OAc; 0.2M NaOH) for 30 minutes each time. The DNA was transferred to HybondN+ membrane (Amersham) overnight by capillary action in 2 liters of transfer buffer (1M NH_4OAc; 0.1M NaOH). The transferred DNA was U.V.-crosslinked to the membrane using an UV Stratalinker 2400, according to the manufacturer's specifications (Stratagene) and the membranes were vacuum dried at 80 °C for two hours and stored between pieces of 3mm Whatman. Hybridization, probing and detection were carried out as outlined in the chemiluminesence, ECL random prime labeling and detection systems kit protocol (Amersham). Probes used were both isolated PCR products and isolated inserts from identified Hind III clones (previously made by Sharon Gorski).

Polymerase Chain Reaction (PCR®)

1) Oligonucleotide Primers

All PCR primers were prepared in one of the following ways: on an Applied Biosystems oligonucleotide synthesizer at Simon Fraser University by the IMBB oligo service; by the Gibco BRL Custom-Made Primer service; or by the DNAgency oligo service (Philadelphia). *Florometra* specific primers that span gene junction regions were either designed from sequence obtained from Hind III clones, made previously by Sharon Gorski, or from sequence using internal gene primers and PCR. Internal gene primers had been designed earlier, from either consensus echinoderm regions, the sea cucumber *Cucumaria miniata* (Allan Arndt, pers. comm.) or were obtained from published sequences (see Table 1). Primers designed from *Florometra* sequence are distinguished from other primers with the prefix "fs". All PCR primer sequences and map positions, based on *Strongylocentrotus purpuratus* mitochondrial genome map data (Jacobs et al., 1988a), are shown in Table 1.

2) Thermal Cycle Amplification

Florometra DNA was amplified using the Polymerase chain reaction (PCR) in 50 µl reaction volumes containing 20mM $(NH_4)_2SO_4$; 75mM Tris-HCl pH 8.8; 0.01% Tween-20; 2.5mM $MgCl_2$; 0.2mM each of the four dNTP's; 2.0mM

Table 1. PCR primers

Name	Primer Sequence and Position[a]
fsCytb 3'	$_{15325}$TGGTATTTTCTTTTTGCTTATG$_{15346}$
12Sai[b]	$_{490}$CAAACCAGGATTAGATACCCTGTTAT$_{515}$
12Sbi[b]	$_{874}$GAGAGTGACGGGCGATGTGT$_{855}$
16Sb[c]	$_{5297}$GACGAGAAGACCCTGTGGAGC$_{5317}$
16Sr[c]	$_{5710}$ACGTAGATAGAAACTGACCTG$_{5690}$
fs16Sc	$_{5149}$GTTTAACTTTTGTCACTGGG$_{5130}$
fsND2f	$_{3258}$GCCATAACAAGAAAACACTG$_{3277}$
fsIle	$_{3203}$CATAGAGTTTGTGAGGATTTGA$_{3182}$
fsCOI 5'bk	gagaa**ggtacc**$_{6073}$CACGAGGAAAAGCCAAATCAGGAG$_{6050}$
COIef[c]	$_{6001}$ATAATGATAGGAGG(A/G)TTTGG$_{6020}$
COIer[c]	$_{6692}$GCTCGTGT(A/G)TCTAC(A/G)TCCAT$_{6673}$
fsCOI 3'	$_{6598}$GGAATGGTTTATGCTATGGTTG$_{6619}$
fsCOII 5'	$_{7929}$AAGAATAAAAGCAGGCACTACAG$_{7907}$
fsCOII 3'	$_{8083}$ACTGATGATTTGACTTTTGG$_{8102}$
fsCOIII 5'bk	atacaggtacc$_{9519}$CACGAATAACATCACGCCAC$_{9500}$
COIIIf[d]	$_{9354}$CATTTAGTTGATCCTAGGCCTTGACC$_{9379}$
COIIIr[d]	$_{10075}$CAAACCACATCTACAAAATGCCAATATC$_{10048}$
fsCOIII 3'	$_{9878}$TGCTCCTTTTACCATTTCTGATAG$_{9901}$
fsND5 5'	$_{12116}$AATTTAGAAGATTTAAGAGAATGTG$_{12092}$

[a] DNA primer locations are designated using nucleotide positions from the sea urchin, *Strongylocentrotus purpuratus* mitochondrial genome (Jacobs et al., 1988a). All primers are listed 5' to 3'. Lower case letters indicate random 5' sequence and a Kpn I restriction site (bold) added to some primers.

[b] Palumbi et al., 1991.

[c] Primers designed from consensus echinoderm sequence or from *Cucumaria miniata* (Allan Arndt, pers. comm.).

[d] ÒFoighil and Smith, 1995.

each of the two primers; approximately 10 ng DNA; and 0.2 units of Ultra Therm Thermophilic DNA Polymerase (Bio/Can Scientific). Thirty rounds of amplification were carried out in a GTC-2 Genetic Thermal Cycler (GL Applied Research Inc.) under the following conditions: an initial cycle of denaturation at 94° C for 90 seconds, annealing between 50-58° C (depending on the primer utilized) for 30 seconds, and extension at 72° C for 1-3 minutes (approximately 45 seconds per kilobase (kb) PCR product); followed by 29 cycles of 94° C for 30 seconds, 50-58° C for 30 seconds and 72° C for 1-3 minutes; with the final cycle extension time lengthened to 10 minutes. The PCR products were electrophoresed in 0.7% agarose gels containing 1XTAE buffer, stained with ethidium bromide, and were either photographed under short U.V. wavelength light (302 nm), or agarose gel slices containing the product band were excised under long U.V. wavelength light (360 nm) and the DNA isolated. The DNA was purified from the agarose plug by centrifugation through silanized glass wool plugs for 20 minutes at 6000 rpm in a desktop microcentrifuge. The eluted DNA was precipitated overnight at -20 °C by increasing the salt content to 1M NH_4OAc and adding 2.5 volumes of 95% ethanol. The precipitated DNA was centrifuged in a desk top microcentrifuge for 20 minutes at top speed and the pellet was rinsed in 70% ethanol and re-centrifuged for five minutes. The dry pellet was taken up in 1xTE, or ddH_2O and stored at -20° C.

Cloning

1) mtDNA clones

Previously, isolated *F. serratissima* mtDNA was used to prepare four Hind III clones in the plasmid vector pUC19 by Sharon Gorski. Of the four, only one had been identified as containing cytochrome b sequence homology and tRNA gene similarity. Table 2 contains a list of these clones and their sizes.

Table 2: Florometra mtDNA Hind III clones

Name	**Size**	**Insert sequence**
FSHD2	1800bp	cytochrome b/tRNA genes
FSHD4	373bp	unknown
FSHD8	1379bp	unknown
FSHD16	2100bp	unknown

2) PCR cloning

The isolated amplified bands from the PCR products were cloned using one of two methods: the TA cloning method of Marchuk et al. (1990) utilizing the plasmid vectors, pUC19 or pBluescript II KS(+); or by using the pCR-Script Amp SK(+) Cloning Kit according to the manufacturer's protocol (Stratagene).

3) Subcloning mtDNA and PCR clones

The Hind III plasmid clones (Sharon Gorski) and the PCR clones were digested with several different restriction enzymes (Hind III, EcoR I, Sst I, Kpn I, Alu I) according to the manufacturer's specifications (Gibco BRL). Once internal restriction sites had been identified, the digested fragments were isolated through electrophoresis on 0.7% agarose gels; the agarose gel slices containing the fragment were excised under long U.V. wavelength (360 nm), and the DNA was purified through centrifugation in silanized glass wool columns and precipitation in 1M NH_4OAc and 2.5 volumes of 95% ethanol. In addition, one of the plasmid vectors pUC19, PVZ1 or pBluescript KS(+) was restriction digested with the appropriate enzyme and heat inactivated according to manufacturer's specifications. The digested vector and isolated fragment were ligated in 20 µl volumes overnight, at 14° C with the addition of 1 unit of T4 DNA ligase and 4 µl 5X T4 DNA Ligase Buffer (Gibco BRL). The ligations were used to transform DH5α competent cells. Transformations were done as follows: 5 µl of the ligation reaction was added to 100 µl competent cells (10^6-10^7 transformation efficiency) and left on ice for 30 minutes; the cells were heat-shocked at 42° C for 90 seconds and stored on ice for 2 minutes; 900 µl of sterile LB was added; and the cells were placed in a shaking 37° C incubator for one hour at which time 50 µl, 100 µl, and 200 µl was spread plated on LB-Agar plates containing 100 µg/ml Ampicillin, 40 µg/ml X-Gal and 160 µg/ml

IPTG and incubated overnight at 37° C. Single white colonies were streaked onto LB-Agar plates containing 100 µg/ml Ampicillin and grown overnight at 37° C. Single colonies were selected to inoculate 5 ml of LB and grown overnight at 37° C in a shaking incubator. The plasmid DNA was isolated using the alkaline lysis procedure outlined in Maniatis, et al. (1982) and stored at -20° C. Positive subclones were screened by restriction digest with appropriate restriction enzyme(s) and electrophoresis on agarose gels.

Sequencing PCR products, PCR clones, Hind III clones, and subclones

All DNA sequences were obtained using the chain termination method of Sanger et al. (1977) applying: the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemicals/Amersham); the Sequenase PCR product Sequencing Kit (U.S. Biochemicals/Amersham); and through Automated sequencing, ABI (Applied Biosystems) protocols. In addition, sequencing of plasmid clones using the Sequenase Version 2.0 system included 10% DMSO (Terry Snutch, pers. comm.) in the reaction mixture. Aside from the sequences obtained using the standard plasmid primers M13 Forward, M13 Reverse, T3, and T7 and specific PCR primers utilized in the amplification (Table 1), internal sequencing primers were designed to acquire the rest of the PCR product sequence (primer walking) for larger fragments. After one segment had been sequenced, a primer was designed from the end of that sequence and then

used to prime another sequencing reaction further in the clone. In most cases, primer walking occurred from both ends of the clones until the two strands overlapped. Internal sequence was also obtained through sequencing subclones when internal restriction sites were found within the clones. In most cases, two PCR clones were sequenced and any nucleotide discrepancies were resolved by sequencing a third PCR clone. Table 3 contains a list of the sequencing primers used. The prefix "fs" denotes those primers designed from *F. serratissima* sequence. The other primers were either designed from consensus echinoderm sequence or from the sea cucumber, *Cucumaria miniata* (Allan Arndt, pers. comm.).

Sequence Analysis

Florometra mtDNA sequences were analyzed using Delaney (Delaney Software Ltd.), ESEE sequence alignment programs (Cabot and Beckenbach, 1989), network searches Blastx, Blastn, Blastp, and FASTA @ ncbi.nlm.nih.gov. Sequences containing tRNA genes were identified and folded into their secondary structures and compared to the tRNA sequences and structures found in other metazoans. *Florometra* DNA sequence encoding mtDNA proteins was translated using the accepted echinoderm mtDNA genetic code demonstrated in *Paracentrotus lividus* (Cantatore et al, 1989), *Strongylocentrotus purpuratus* (Jacobs et al., 1988a) and *Asterina pectinifera* (Himeno et al., 1987; Asakawa et al., 1995).

Table 3. Sequencing Primers

Name	Sequence and Position[a]
Cytb 5f[b]	$_{15518}$CATGAATTGGAGGCCAACC$_{15536}$
Trp[b]	$_{1580}$GTTAACTAAACTGAAAGCCTTCAAAG$_{1605}$
Val[b]	$_{1756}$AAGCGACTCTTTTACACAG$_{1738}$
Metb[b]	$_{1842}$CATTCTTGGGATATGAGCC$_{1824}$
fsAsp	$_{1889}$TAGTGATTATGGTTTGAC$_{1906}$
fsDloop[c]	TTGGGTATTTTTCTGTTAGT
fsGlu	$_{993}$AAACCTCAATAAGAATGCTC$_{1012}$
fs12S 3'	$_{733}$AAATAAGTGGGCTACAATAC$_{752}$
fs12S 5'	$_{340}$CGCGGTGGCTGGCACG$_{325}$
16Scuc[b]	$_{5569}$TGACAAIIAGGATTGCGACC$_{5588}$
fs16Sd	$_{4885}$GTTAAGTTATTGTCTTGT$_{4868}$
fsTyr	$_{1945}$ACAAAATGGCTGAAAGACTA$_{1964}$
fsND2fb	$_{3595}$CATGACAAAAGATAGCACCAAC$_{3616}$
fsND2fc	$_{3944}$TCTATCACTTGGCGGTTTAC$_{3963}$
fsND1a	$_{2868}$ATTATTTTAGCGTATTCTG$_{2850}$
fsND1 5'	$_{2580}$ACTGCTCGTATTCCACCTA$_{2562}$
fsCOI 5'a	$_{5813}$GTAGAAAACAACCAACGACT$_{5794}$
fsCOI 3'b	$_{6954}$TTCTGGTTTTACTCATTG$_{6971}$
fsCOIend	$_{7171}$GTCGGAGTGTTTTATTAC$_{7188}$
COII(169)[b]	$_{7744}$GCATCCTCCCCTCTTATGGAG$_{7764}$
fsCOII 3'b	$_{8285}$TTTATGGTCAATGTTCTG$_{8302}$
fsA6b	$_{8853}$CCTCCCCAAGAAGTCTGTA$_{8836}$
fsA6a	$_{9190}$GACCAAATAGTAGTAGCAAG$_{9171}$
fsCOIIIend	$_{10102}$GATGAGGTAGATAAAAGAGAGG$_{10123}$
fsND4a	$_{10855}$GTGTATTACTTATTTTTGA$_{10873}$
fsND4new	$_{10968}$ATCTCGTTGGGGTTCTCA$_{10985}$
fsND4b	$_{11388}$TGGTATAATTTGTTTTTGTC$_{11407}$
fsND5+	$_{13109}$GTCAGTTAGGGTTAATGGTAGTATC$_{13133}$
fsND5-	$_{13133}$GATACTACCATTAACCCTAACTGAC$_{13109}$

[a]DNA primer locations are designated using nucleotide positions from the sea urchin, *Strongylocentrotus purpuratus* mitochondrial genome (Jacobs et al., 1988a). All primers are listed 5' to 3'.

[b]Primers designed from consensus echinoderm sequence or from *Cucumaria miniata* (Allan Arndt, pers. comm.).

[c]fsDLoop primer was designed from *F. serratissima* unassigned sequence I (UAS I) and therefore has no designation point in the *S. purpuratus* mtDNA genome. Its location is noted in Figure 5 within the UAS I region.

RESULTS

1) Southern Blots

Figure 2 depicts the derived restriction map of *F. serratissima* with approximate gene locations from Southern blots of both single and double restriction digests of total *F. serratissima* DNA (Southern Blots not shown). There is only one Kpn I restriction site in the mtDNA genome, located within the Cytochrome b gene (Cyt b). A Kpn I and Sst I double restriction digest revealed that the 12S and 16S ribosomal RNA genes as well as the ND1-Isoleucine tRNA-ND2 gene composite (ND1 I ND2) were all contained within a 5.8 kb segment of the genome, while the ND5 gene was located within a second 4.1 kb Kpn I and Sst I fragment. The Cyt b gene is located within both of these fragments. A Southern blot using the COI gene located it to a third 3.8 kb Kpn I and Sst I fragment, however Southern blots from a Sst I single digest and Sst I / EcoR I, and Sst I / Xho I double digests demonstrated that this 3.8 kb fragment containing the COI gene, consisted of Sst I ends. In addition, a Sst I / Pst I double digest indicated the presence of a Pst I site 0.5 kb in from one of the Sst I sites of this fragment. A Kpn I / Pst I double digest probed with COI placed this Pst I site towards the ND5 side of the mtDNA genome. A second Pst I site was found 0.6 kb outside the Sst I site of the 4.1 kb Kpn I / Sst I fragment containing the ND5 gene and one of Cyt b segments. Finally, an EcoR I site was discovered 0.8 kb from this second Pst I site towards 3.8 kb COI Sst I site (see Figure 2).

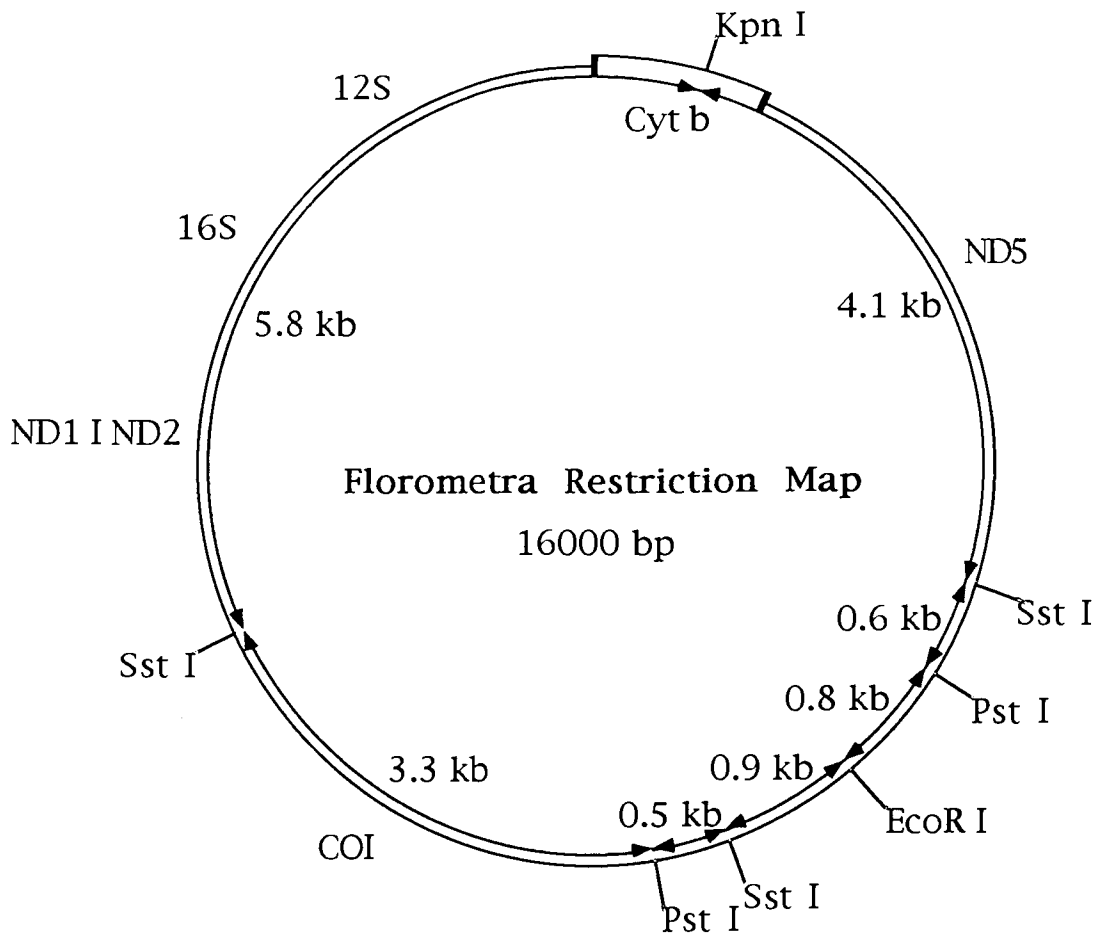


Figure 2. A restriction map of the *Florometra serratissima* mtDNA genome denoting general locations of genes. Numbers inside the circle indicate approximate kilobases (kb) separating the restriction sites.

2) Identification of the Hind III mtDNA clones

The four *F. serratissima* mtDNA Hind III clones (Table 2) made previously by Sharon Gorski were identified through DNA sequencing. The clones FSHD4 (373 bp) and FSHD8 (1379 bp) have been completely sequenced and the insert sequence identified. The clone FSHD4 includes the 3' end of the ND1 gene, the tRNA gene for isoleucine (I), and the 5' end of the ND2 gene all in the same transcriptional polarity. The clone FSHD8 contains the 3' end of the 16S rRNA gene, the tRNA genes for glycine (G), $leucine_{UUR}$ (L), and phenylalanine (F), and the 5' end of the 12S rRNA gene all in the same transcriptional polarity as well (see Figure 3). The clones FSHD2 (1800 bp) and FSHD16 (2100 bp) have been partially sequenced but the insert sequence has been identified. The FSHD16 clone contains the 3' ends of the ND6 and the ND5 genes at one end in opposite transcriptional polarity, and the 5' end of the ND5 gene with a portion of the tRNA gene for $serine_{AGN}$ (S) at the other end in the same transcriptional orientation. The FSHD2 clone contains the beginning of the ND6 and Cyt b genes at one end, in opposite transcriptional polarities and the tRNA genes for proline (P), glutamine (Q), asparagine (N), $leucine_{CUN}$ (L), alanine (A), and part of tryptophan (W) at the other end in alternating transcriptional polarities (see Figure 3).

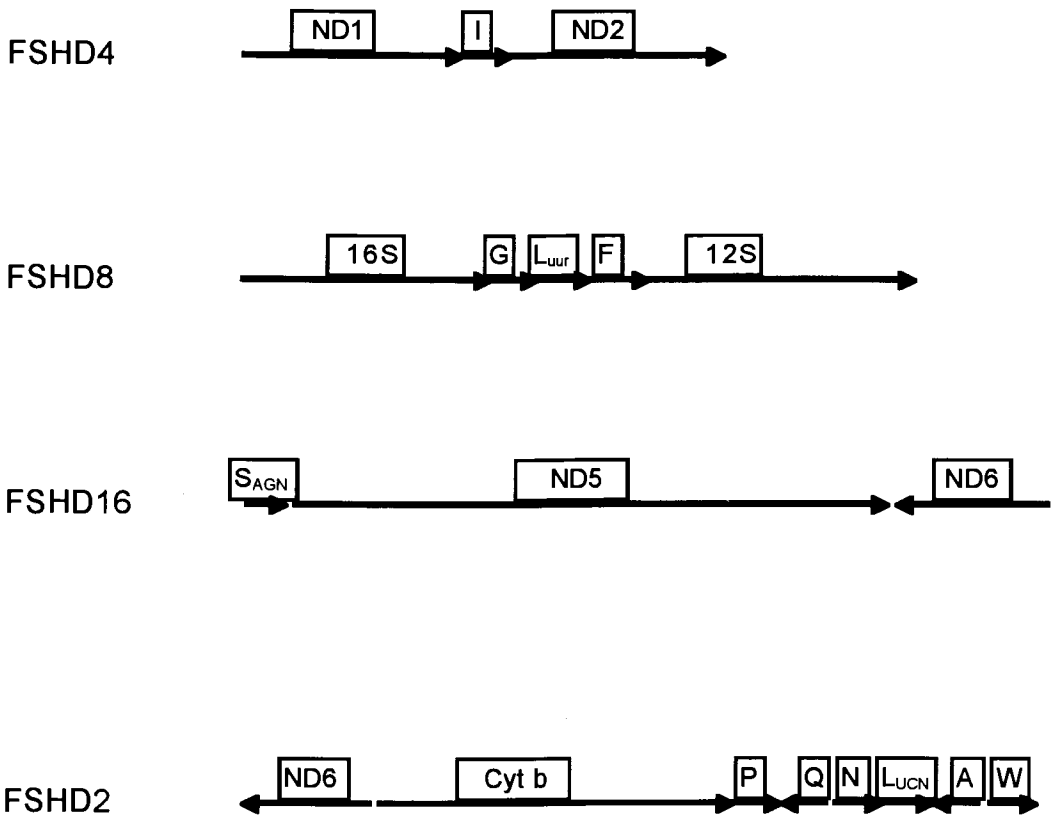


Figure 3. Gene order and transcriptional polarities from four *Florometra serratissima* mtDNA Hind III clones (made previously by Sharon Gorski). Single letters indicate tRNA genes and arrows indicate transcriptional polarity.

3) PCR products

PCR products were obtained from amplification either across gene junctions or within specific genes. Primers used in each amplification (Table 1), the region amplified and size of the PCR products are all shown in Table 4. Most of the PCR products shown in Table 4 have been cloned but all of the amplified regions have been sequenced completely. In all cases, single PCR product bands were obtained in the amplification reactions.

Table 4. PCR products and fragment sizes

Primers used	**Region Amplified**	**size**
fsCytb 3' / 12Sai	Cyt b / tRNAs / Dloop / 12S junctions	2088 bp
12Sai / 12Sbi	12S gene internal	398 bp
12Sbi / 16Sb[a]	12S / F / L_{UUR} / G / 16S junctions	1482bp
16Sb / 16Sr	16S gene internal	382 bp
fs16Sc / fsND2f	16S / Y / ND2 junctions	1932 bp
COIef / COIer	COI gene internal	672 bp
fsIle / fsCOI 5'bk	I / ND1 / COI junctions	1426 bp
fsCOI 3' / fsCOIIIr[a]	COI / R / ND4L / COII / K / A8 / A6 / COIII	3444 bp
fsCOI 3' / fsCOII 5'	COI / R / ND4L / COII junctions	1325 bp
fsCOII 3' / fsCOIII 5'bk	COII / K / A8/ A6 / COIII junctions	1436 bp
fsCOIII 3' / fsND5 5'	COIII / S_{UCN} / ND3 / ND4 / H / S_{AGN} / ND5	2270 bp
COIIIf / COIIIr[a]	COIII gene internal	695 bp

[a]PCR products not cloned

Figure 4 illustrates some of the gene junction PCR products outlined in Table 4. Lanes 1 and 8 contain 300 ng of the 1 kb ladder as marker lanes, where lanes 2 through 7 contain the amplification products from the primer combinations of: fsCytb 3' / 12Sai, fsND2f / fs16Sc, fsIle / fsCOI 5'bk, fsCOI 3' / fsCOII 5', fsCOII 3' / fsCOIII 5'bk, and fsCOIII 3' / fsND5 5', respectively.

4) Subcloning

The mtDNA clone, FSHD2, has been subcloned into the plasmid vector pUC19 using an internal Kpn I site (within the Cyt b gene) found in the clone. The mtDNA clones FSHD16 and FSHD2 have been subcloned into the Sma I site of the plasmid vector PVZ1 using internal Alu I restriction sites. In addition, the PCR clone obtained from the fsCOIII 3' / fsND5 5' amplification has been subcloned twice into the plasmid vector pUC19 utilizing an internal EcoR I and an internal Sst I site, located within the product. The fsCOI 3' / fsCOII 5' PCR clone has also been subcloned using an internal Hind III restriction site found within this product.

5) DNA sequencing

The PCR products and product clones shown in Table 4, as well as the mtDNA clones FSHD4 and FSHD8, have been sequenced completely. In addition, partial sequences of FSHD2 and FSHD16 have been obtained. The sequences have been organized into three separate mtDNA fragments of varying lengths. Figure 5 is the translated *F. serratissima* sequence starting

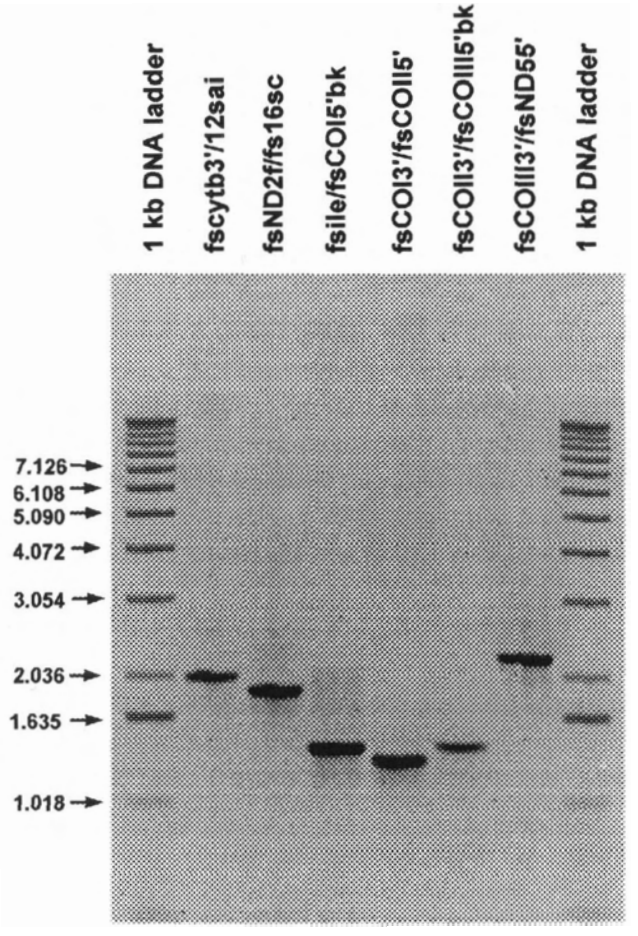


Figure 4. A representative gel of PCR products resulting from the use of primers that span gene junctions. Lanes 1 and 8 contain 300 ng of the the 1 kb ladder as marker lanes with sizes marked. Lanes 2 through 7 contain amplified products from the following primer sets: Lane 2, fsCytb 3' & 12Sai; Lane 3, fsND2f & fs16Sc; Lane 4, fsIle & fsCOI 5'bk; Lane 5, fsCOI 3' & fsCOII 5'; Lane 6, fsCOII 3' & fsCOIII 5'bk; and Lane 7, fsCOIII 3' & fsND5 5'.

from part way through the Cyt b gene and continues to the beginning of the ND5 gene. It includes the complete sequences for 10 protein coding genes, 22 tRNA genes and 2 ribosomal RNA genes, and is comprised of 13,371 nucleotides. Figure 6 consists of the translated regions of the beginning of the Cyt b gene, the complete ND6 gene, and the end of the ND5 gene. Figure 7 contains 647 nucleotides of translated ND5 gene. All three of the figures indicate PCR and sequencing primer positions (Tables 1 and 3), some restriction site locations, and transcriptional polarity of the genes. In addition, there are three regions of unassigned sequence (UAS) larger than 20 nucleotides (nt) that have not been identified and are designated UAS I (382 nt), UAS II (77 nt), and UAS III (73 nt). UAS I is located between the aspartic acid and the threonine tRNA genes (Figure 5), UAS II is situated between the 5' ends of the ND1 and COI protein genes (Figure 5), and UAS III is found between the 5' ends of the Cyt b and the ND6 genes (Figure 6).

Figure 8 shows the cloverleaf folded secondary structures of the 22 *F. serratissima* tRNA genes. Figure 9 is the derived *F. serratissima* mtDNA genome map generated from the sequence data and gene orders shown in Figures 5, 6, and 7. All transcriptional polarities are indicated by arrows and tRNA genes are represented by their single letter abbreviations. The boxes represent the approximate lengths of the individual genes and unassigned regions.

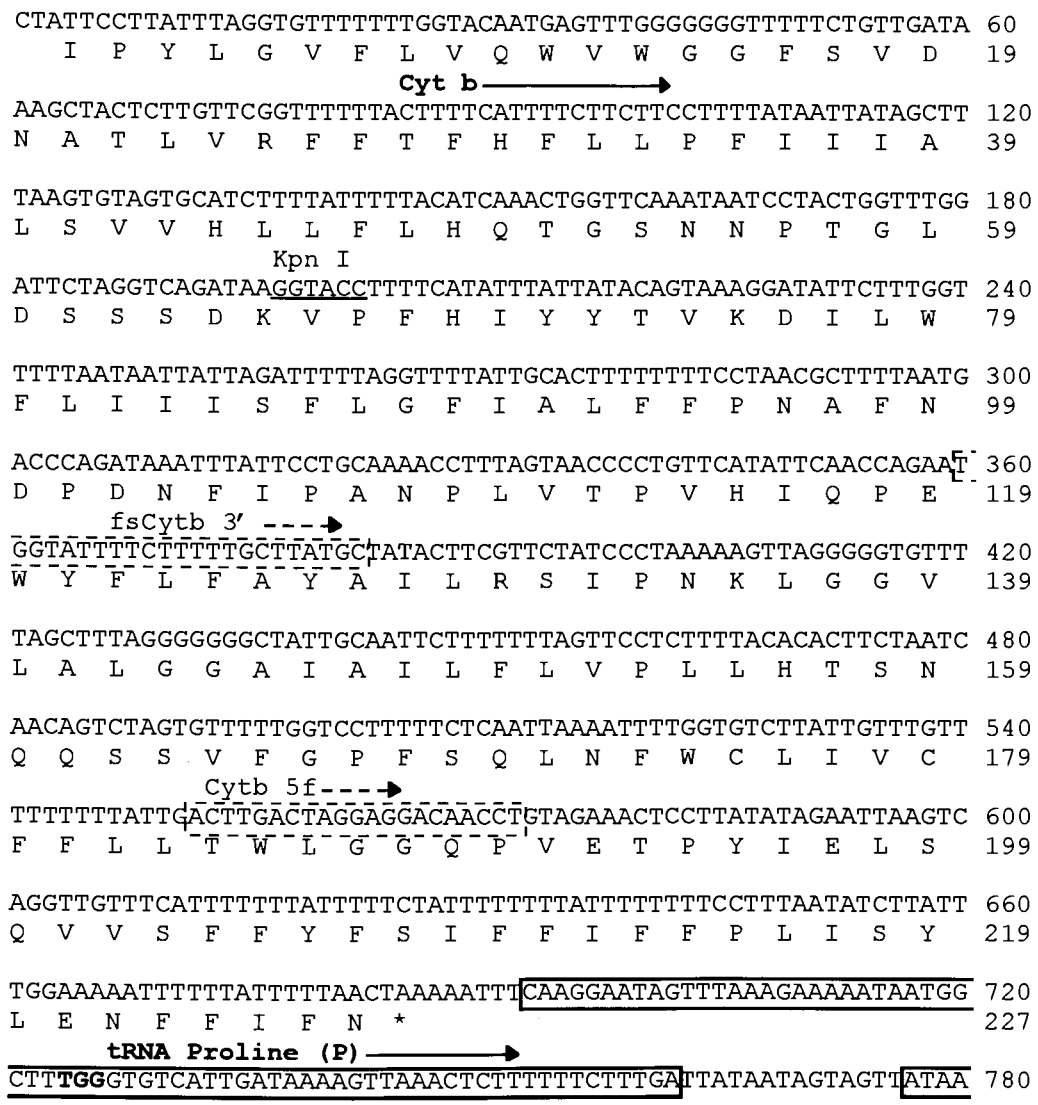
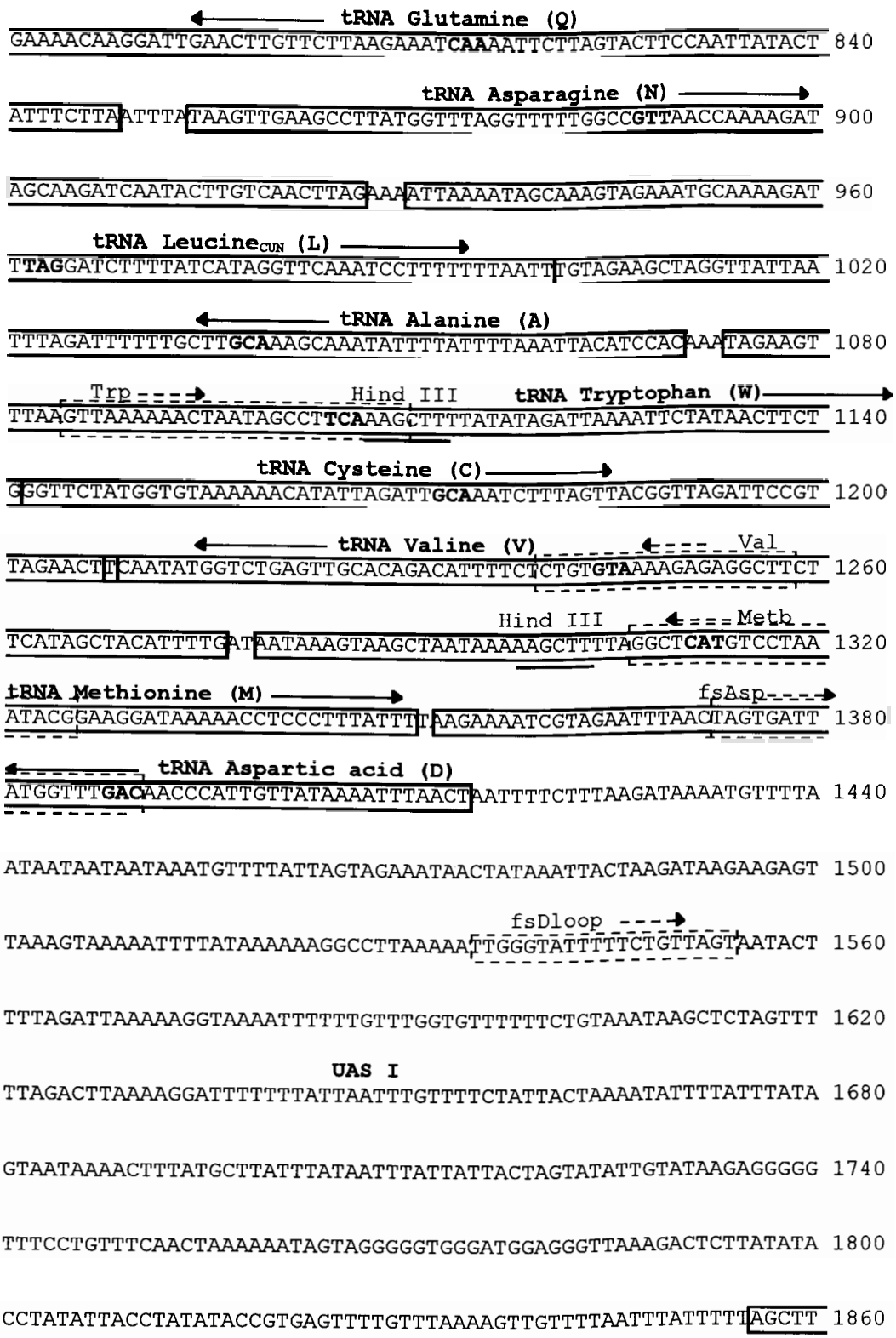


Figure 5. A compiled 13,371 nucleotide fragment of *Florometra serratissima* mtDNA sequence. Solid arrows indicate transcriptional polarity of individual genes. tRNA genes are boxed in solid lines and their anticodons are bold-faced. Protein coding genes are indicated by their putative start and stop codons. Stop codons are designated by asterisks. Their predicted amino acid sequences are shown one line below the DNA sequence. Amino acids are represented by their single letter abbreviations. Sequencing and PCR primer sequences (Tables 1 and 3) are boxed in dashed-lines and their direction is indicated by dashed arrows. The two unassigned regions of mtDNA sequence are denoted UAS I and UAS II. Restriction sites (Figure 2) are underlined.



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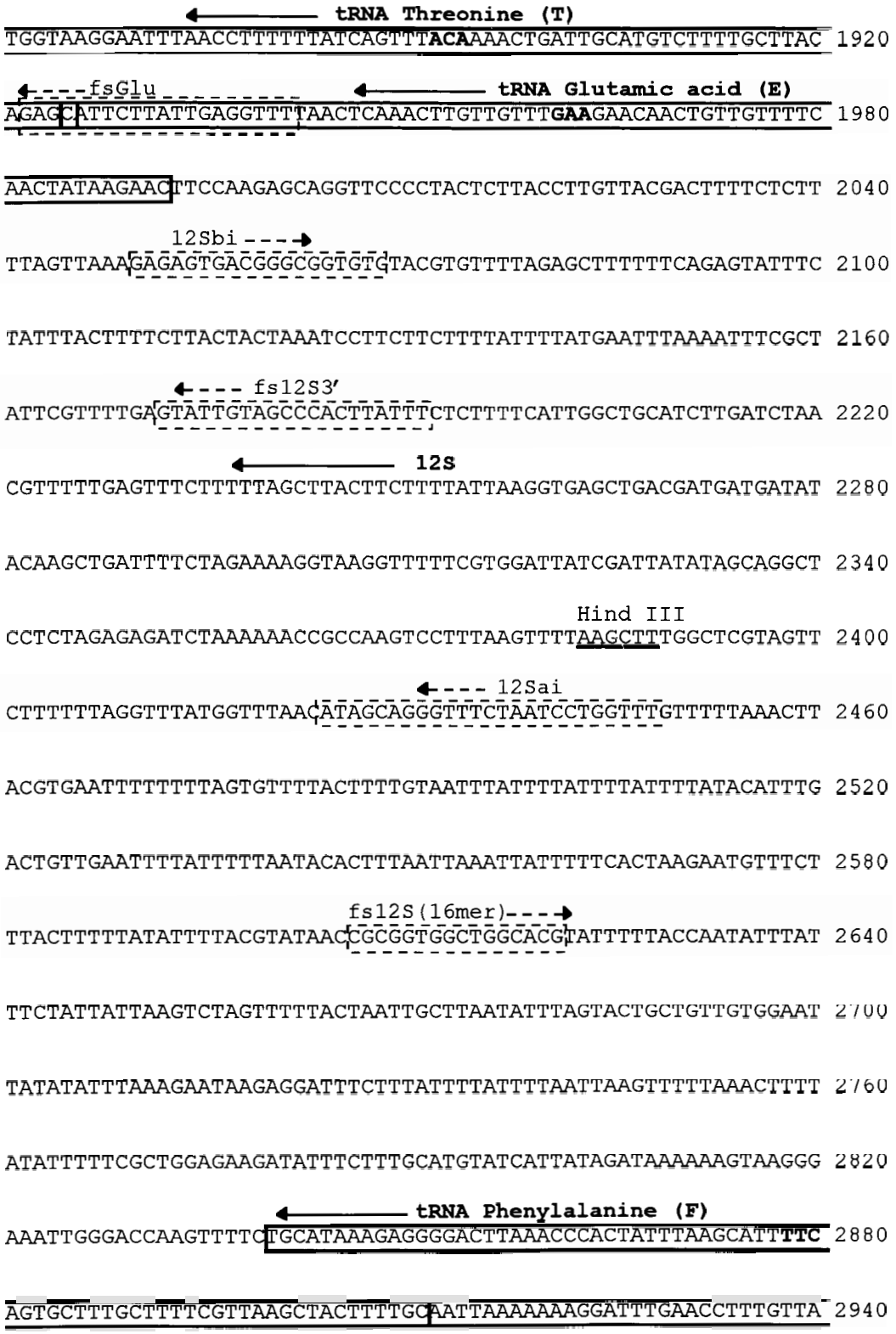

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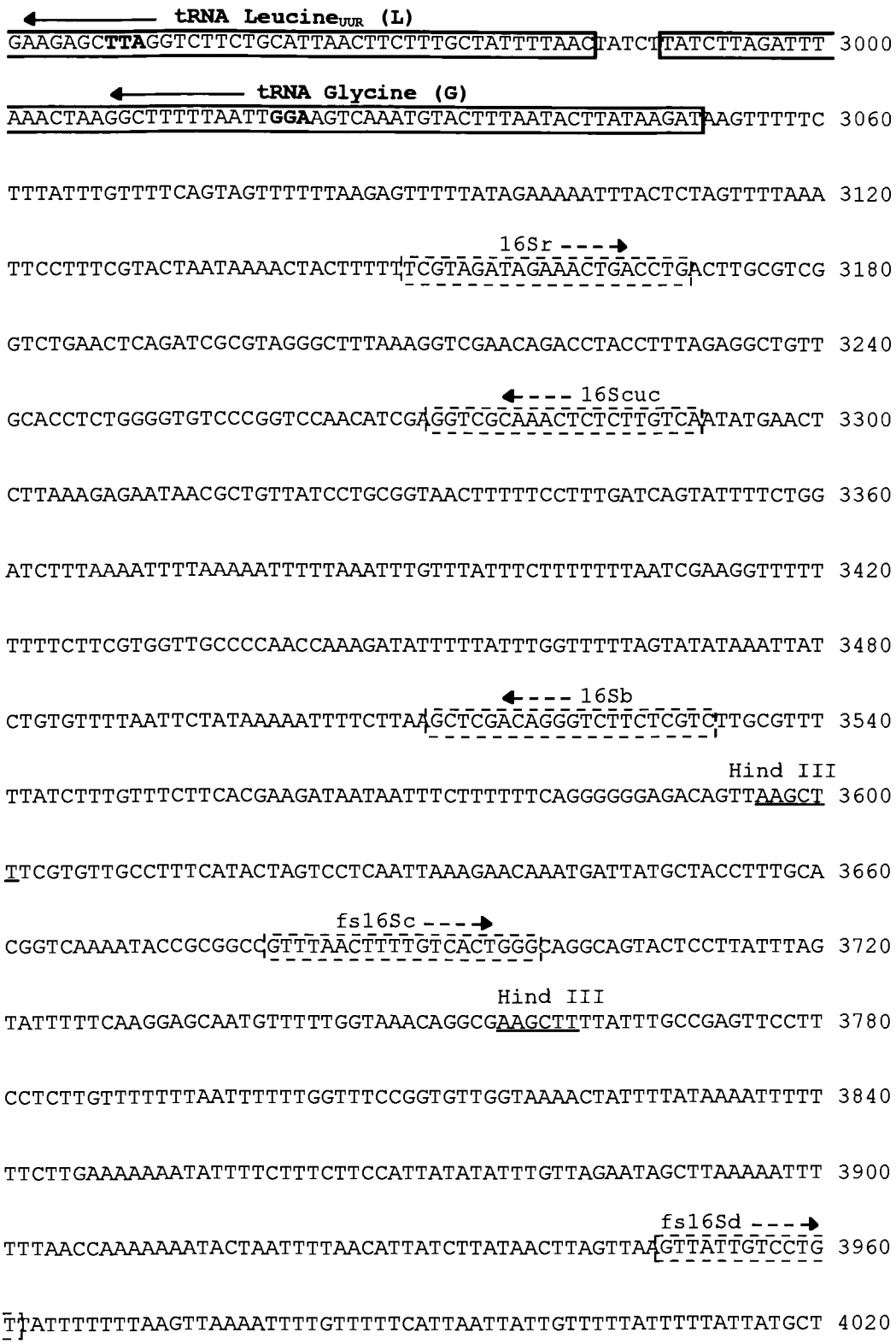

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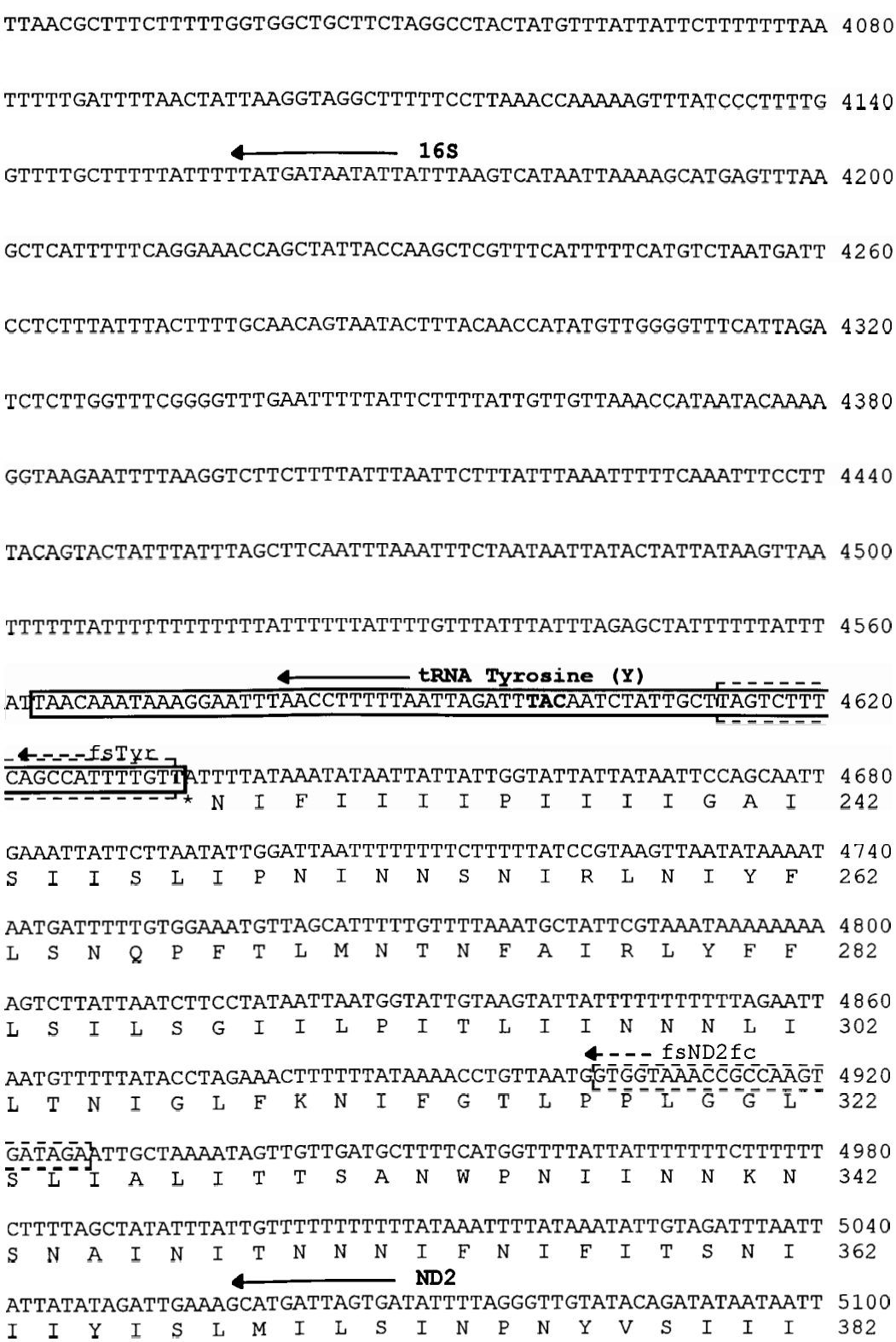

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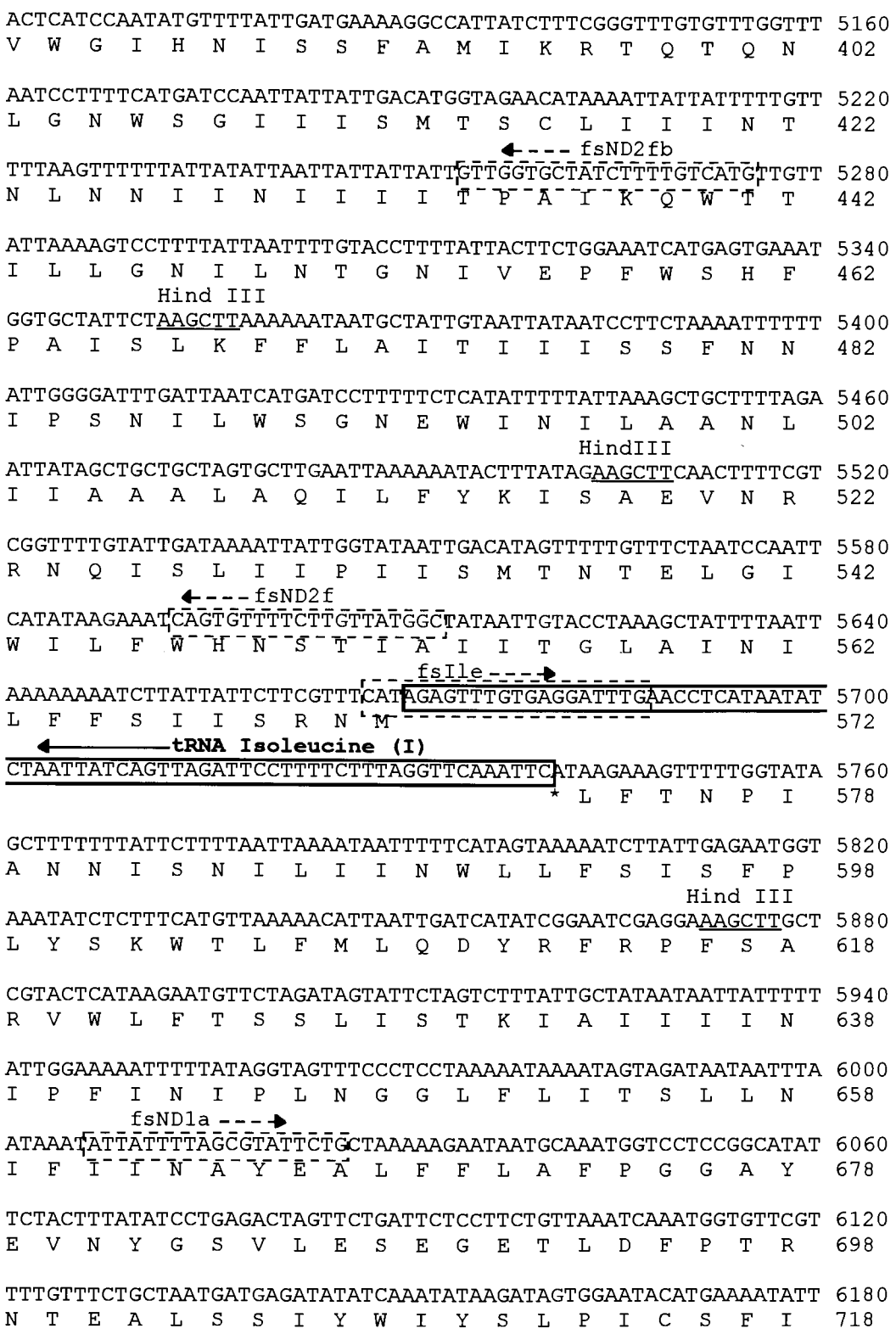

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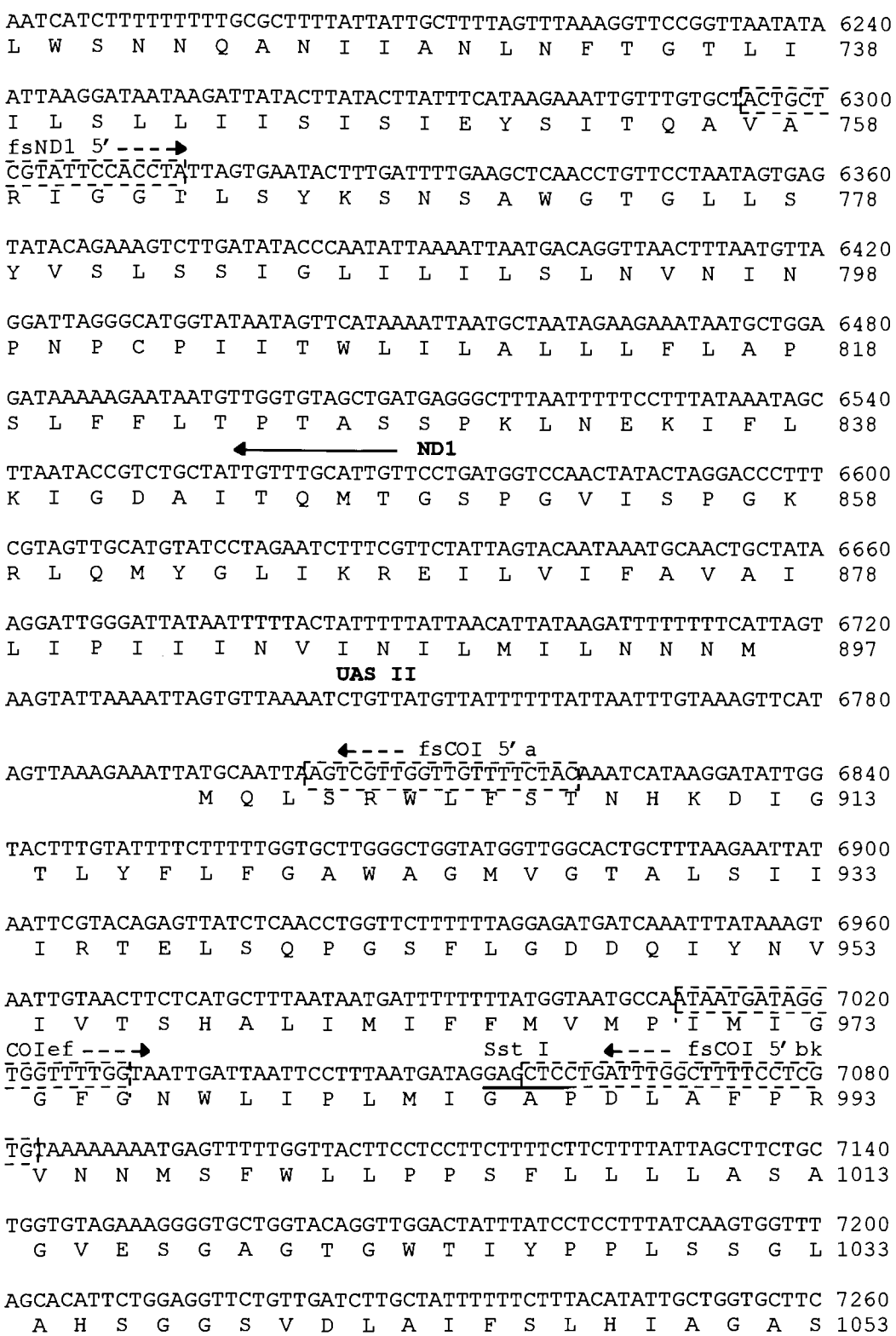

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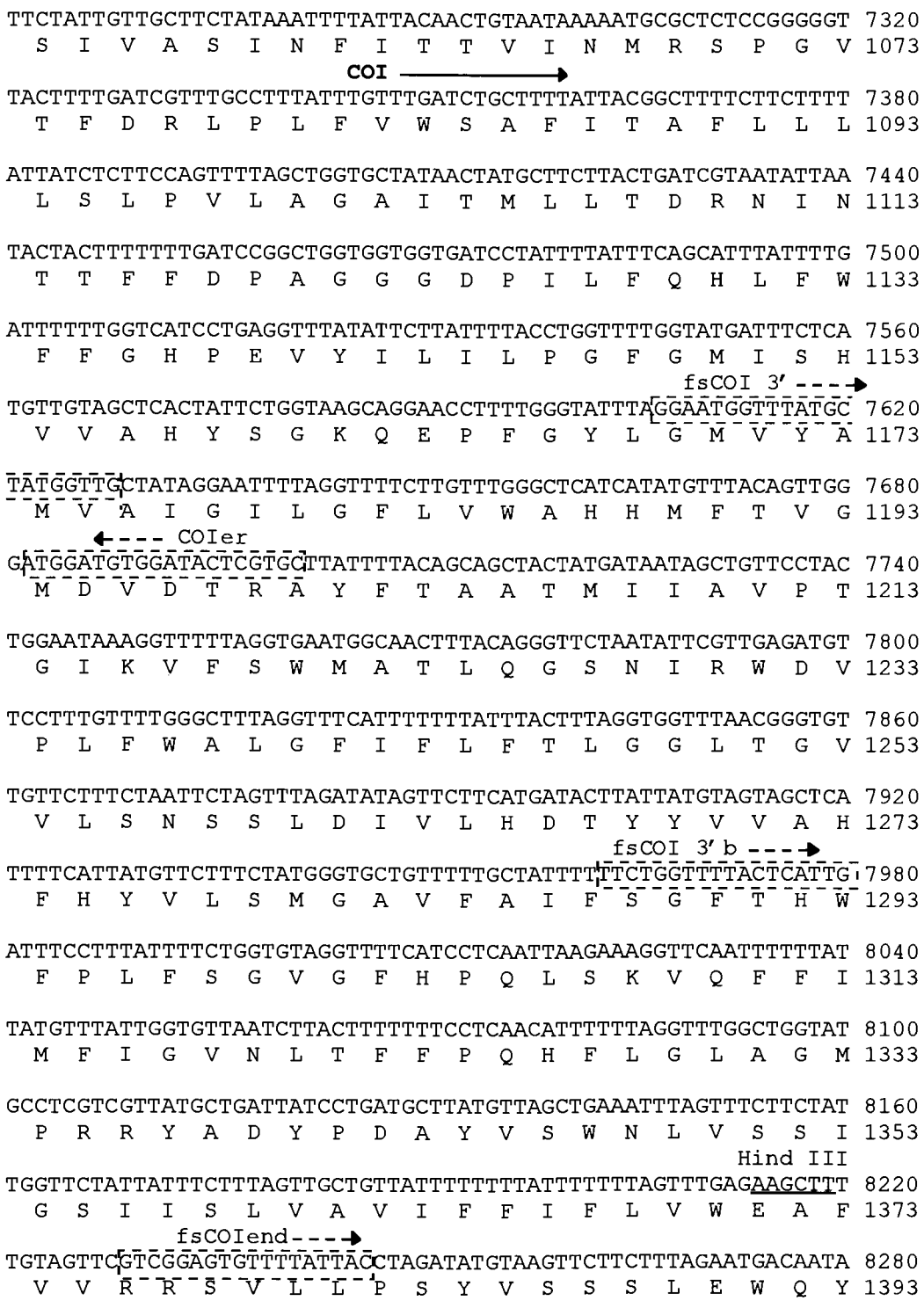

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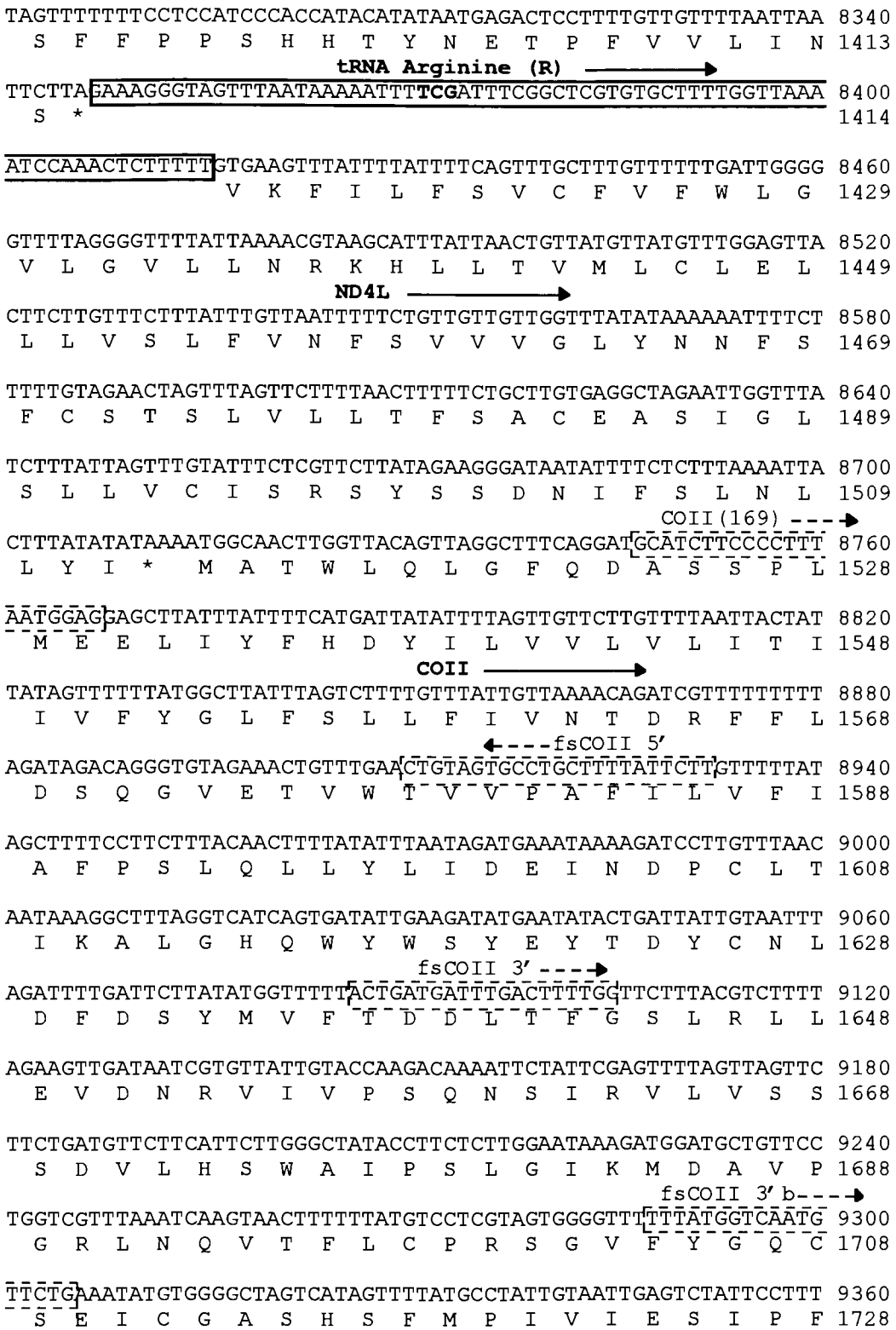

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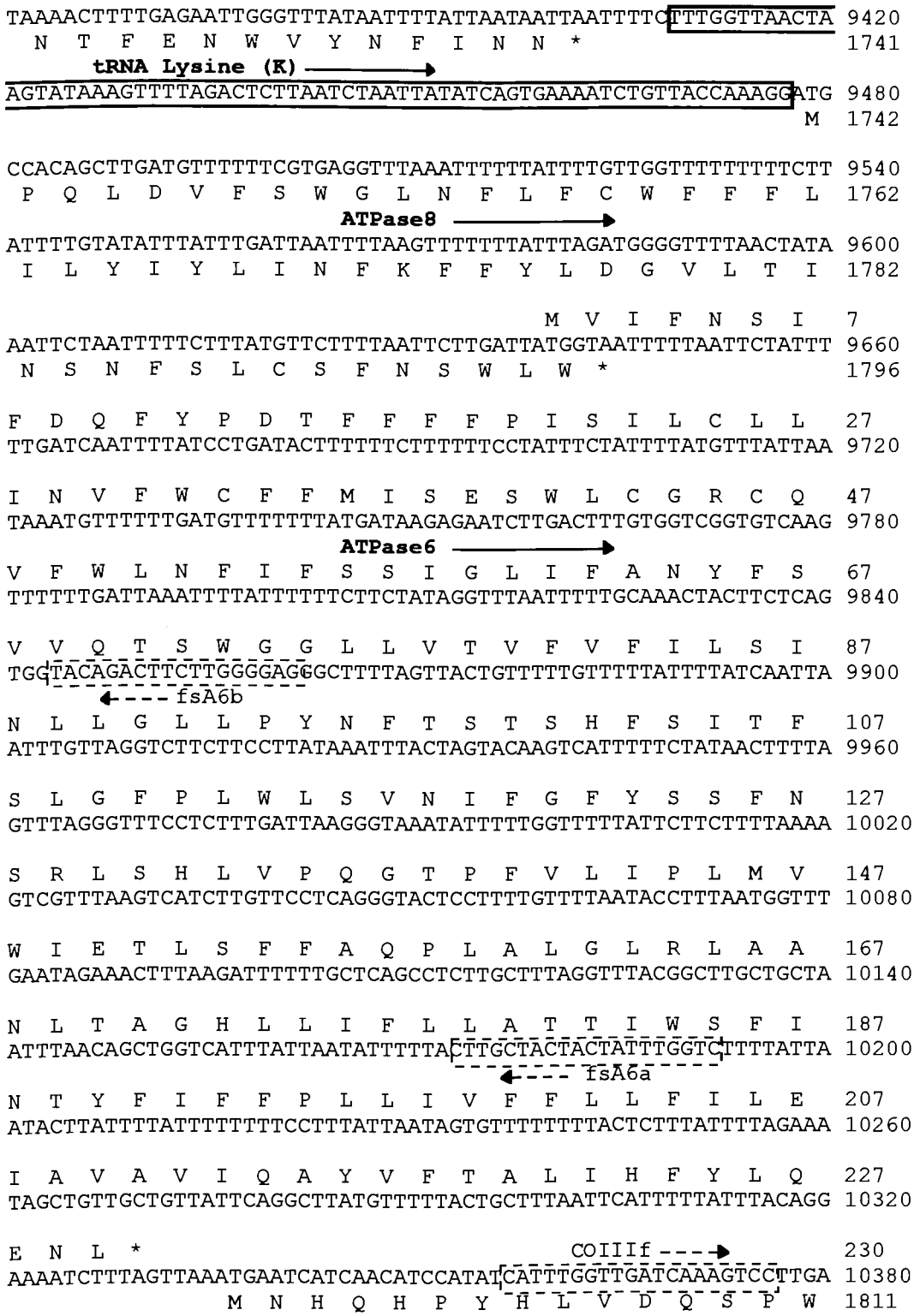

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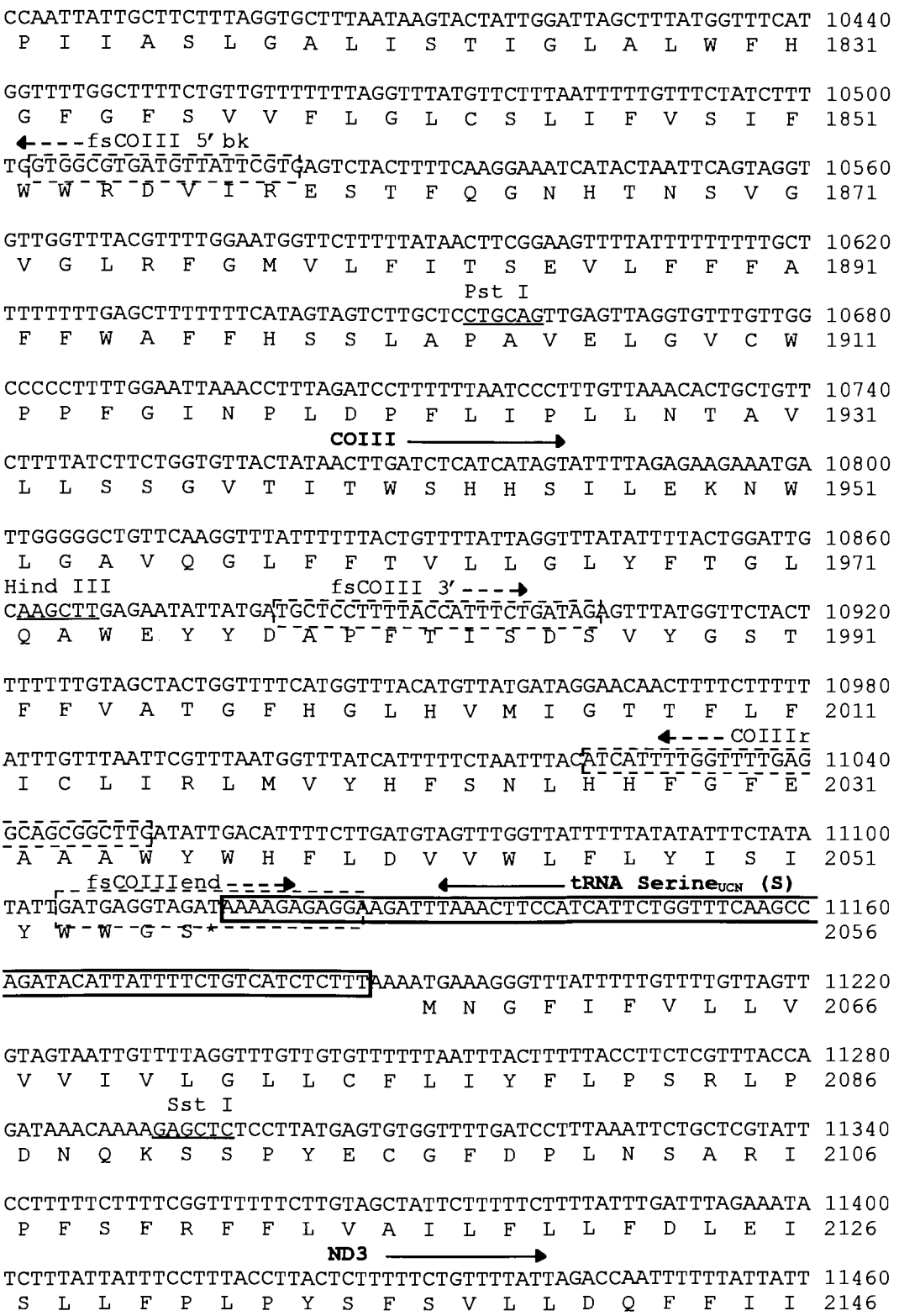

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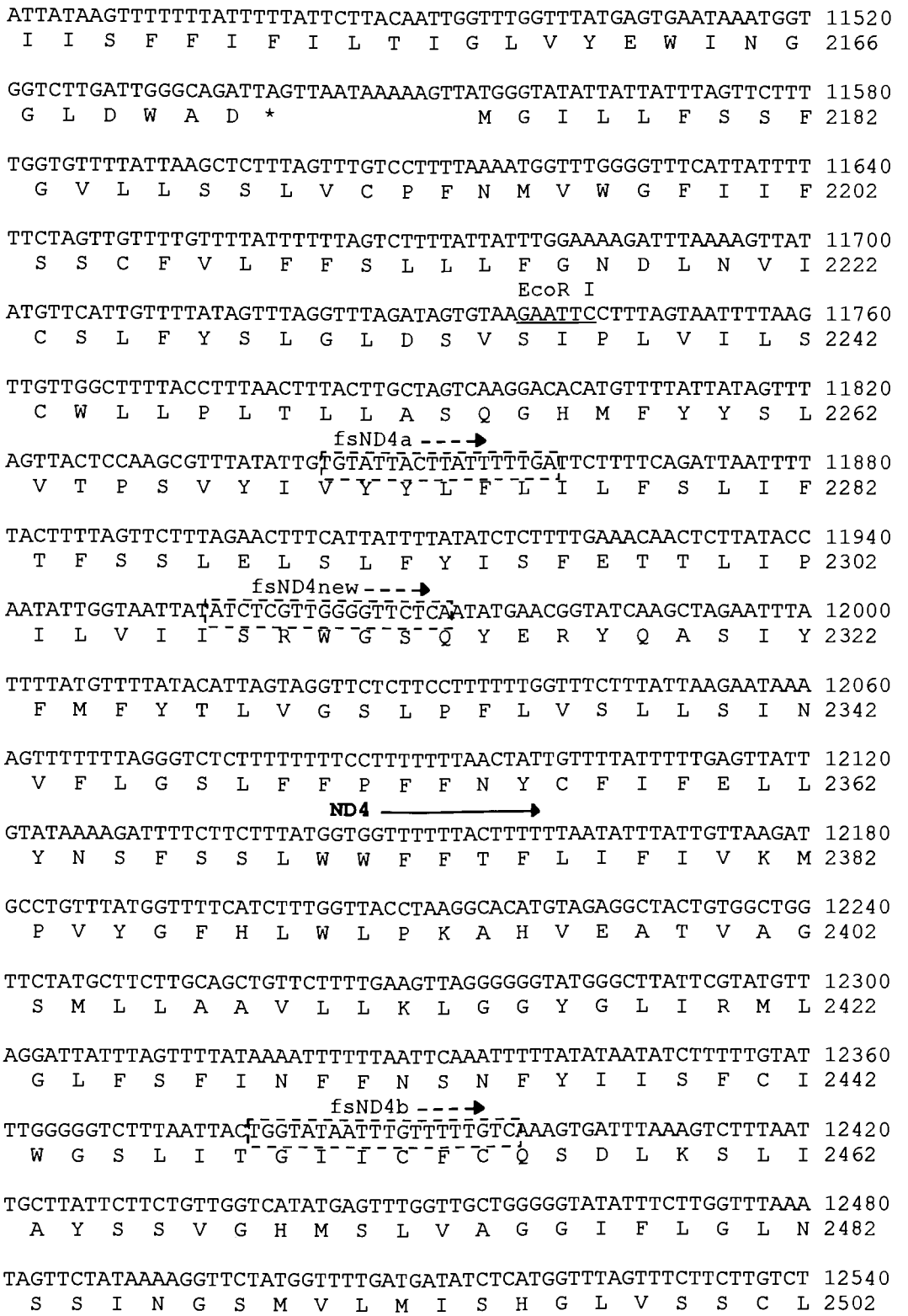

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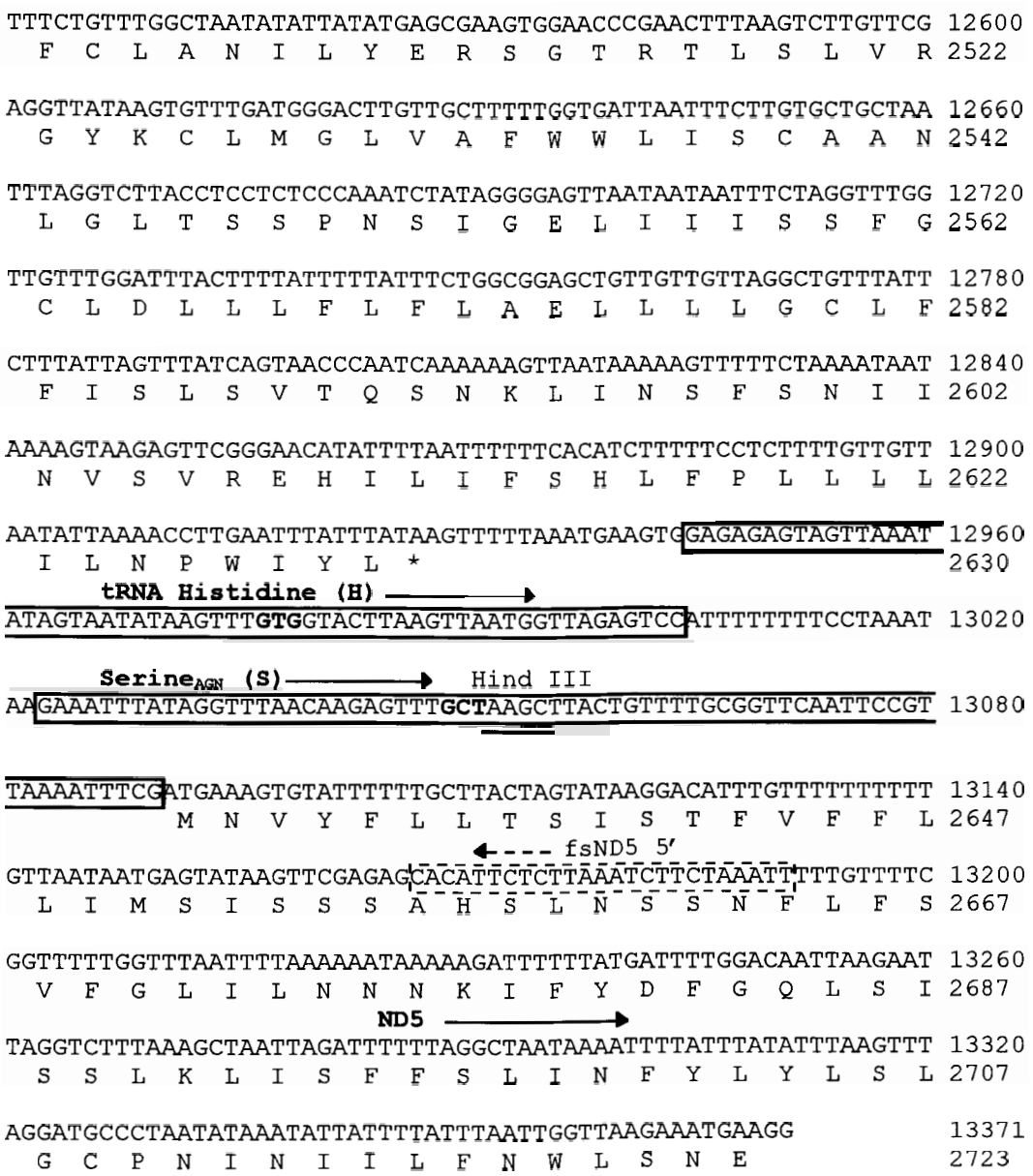

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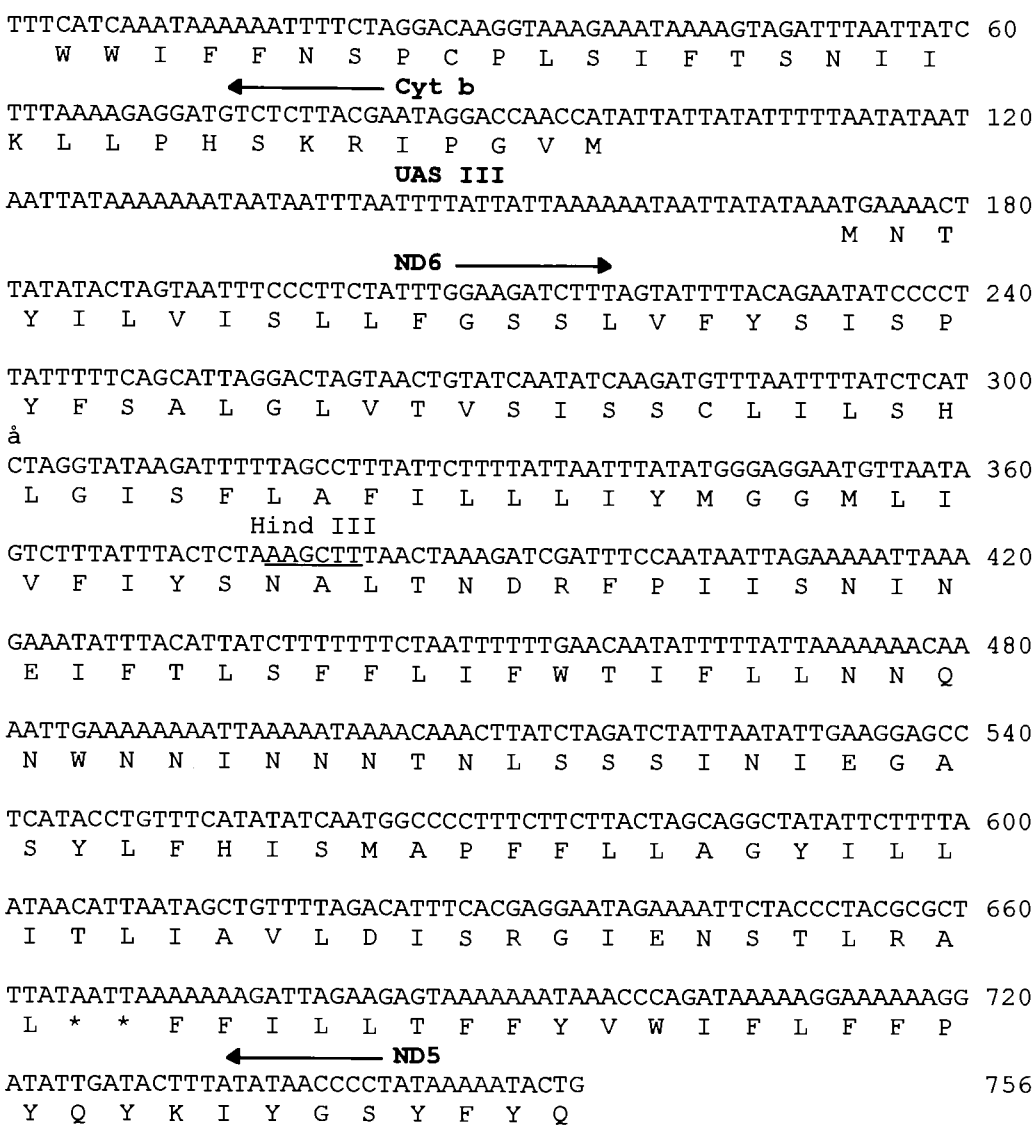

Figure 6. A 756 nucleotide fragment of *Florometra serratissima* mtDNA sequence compiled from the mtDNA Hind III clones FSHD2 and FSHD16 (Table 2 and Figure 3). Arrows indicate transcriptional polarity of the individual genes. The predicted amino acid sequence of the protein genes are shown one line below the DNA sequence. The stop codons of the ND6 and ND5 genes are denoted by asterisks. Amino acids are represented by their single letter abbreviations. The unassigned sequence between the 5' ends of the Cyt b and ND6 genes is labeled UAS III. The Hind III restriction within the ND6 gene is underlined.

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AAAACTGGTCTTTATTAGAAGTTTATGGTTTATTAAAAAAAGCTAATAGTTTAATAATAA 60
   N  W  S  L  L  E  V  Y  G  L  L  N  N  A  N  S  L  I  I   19
   EcoR I
AAGGAATTCAAAAAAGTGGGTTATTAGCTAGAATAGCAAAGTCTGCTCAGGTTGGTTTTC 120
N  G  I  Q  N  S  G  L  L  A  S  I  A  K  S  A  Q  V  G  F   39

ATCCTTGATTGCCAGCTGCTATGGAAGGTCCAACGCCTGTTTCTGCTTTATTACATAGGT 180
H  P  W  L  P  A  A  M  E  G  P  T  P  V  S  A  L  L  H  S   59
                        ND5 ──────►
CTACTATGGTTGTTGCTGGTGTCTTTTTTCTTATTCGATTAGGAAGTTTATTAAAATTTC 240
S  T  M  V  V  A  G  V  F  F  L  I  R  L  G  S  L  L  N  F   79

CTTCTTTTTTTTGTAGGTTTTGTTTATTTATTGGTGGAGTAACTTCTTTGTTTGCTGCAA 300
P  S  F  F  C  S  F  C  L  F  I  G  G  V  T  S  L  F  A  A   99

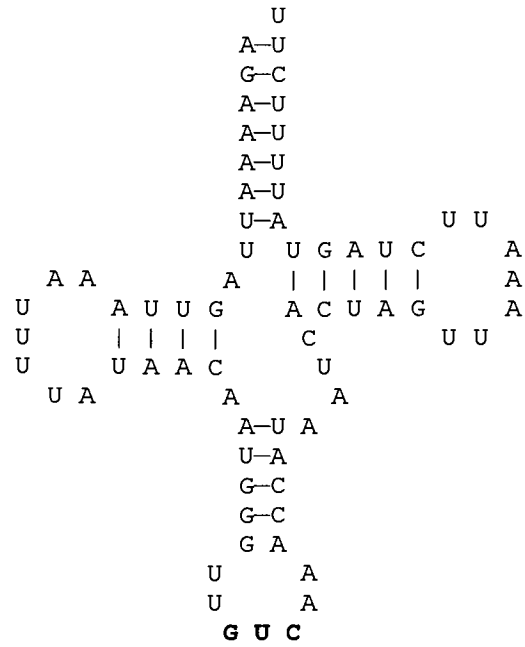
GTGTAGCTTTAGTTCAGCATGATATAAAGAAGATAATTGCTTATTCTACTACTAGTCAGT 360
S  V  A  L  V  Q  H  D  I  K  K  I  I  A  Y  S  T  T  S  Q  119
   fsND5+ - ►
TAGGGTTAATGGTAGTATCAATTGGTTTAGGAAGATATATAATTGCTTTTTTTCATATTT 420
L  G  L  M  V  V  S  I  G  L  G  S  Y  I  I  A  F  F  H  I  139
◄ - fsND5-
GTACTCATGCTTTTTTTAAGGCTATGCTTTTTCTTTGTTCTGGTAGAATAATTCATAGTT 480
C  T  H  A  F  F  K  A  M  L  F  L  C  S  G  S  I  I  H  S  159

TAAAAAAAGAGCAGGATATTCGAAAGATGGGAGGTTTATTTTTTCTTCTTCCAGTAACTA 540
L  N  N  E  Q  D  I  R  K  M  G  G  L  F  F  L  L  P  V  T  179

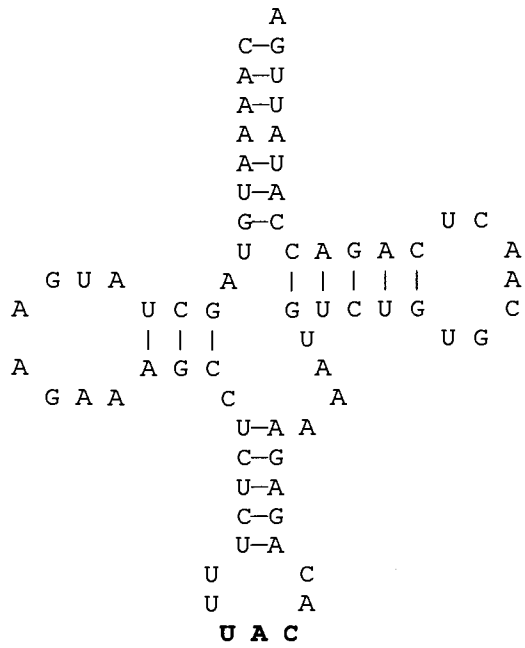
GCTCTTGTATTCTTTTAGGGAGTTTGGCTTTAGTTGGAACTCCTTTTTTAGCTGGTTTTT 600
S  S  C  I  L  L  G  S  L  A  L  V  G  T  P  F  L  A  G  F  199

ATTCTAAGGATTTAATTTTGGAATTAGGTCTTATTAGATTTTCTAAA               647
Y  S  K  D  L  I  L  E  L  G  L  I  S  F  S  N               215
```

Figure 7. A 647 nucleotide fragment of the *Florometra serratissima* ND5 gene compiled from the mtDNA Hind III clone, FSHD16 (Table 2 and Figure 3). Solid arrow indicates transcriptional polarity of the ND5 gene. The predicted protein amino acid sequence is shown on line below the DNA sequence. Amino acids are represented by their single letter abbreviations. Sequencing primers are boxed in solid lines and dashed arrows indicate their direction.

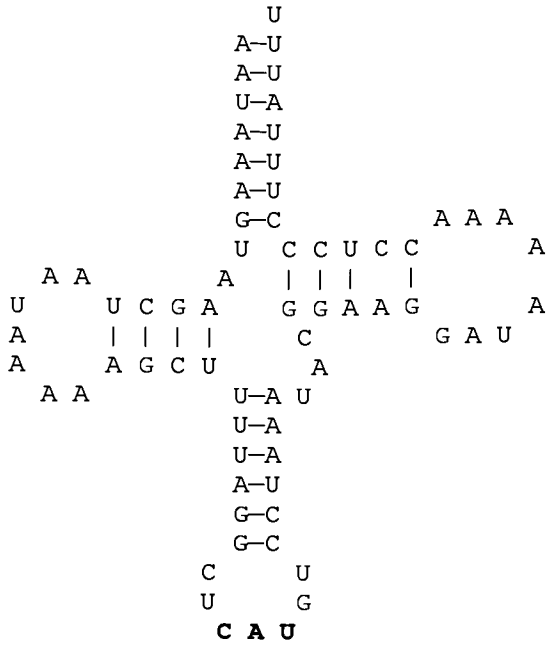


Aspartic Acid (D)

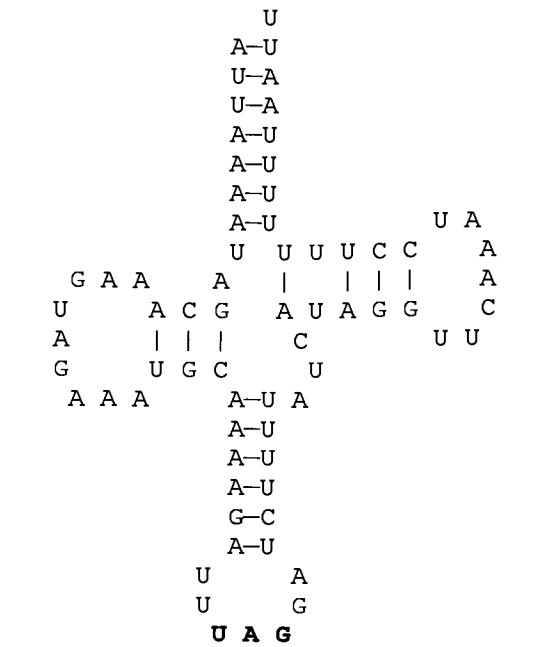


Valine (V)

Figure 8. Predicted secondary structures of the 22 *Florometra serratissima* mitochondrial tRNAs based on the sequence data of Figure 5. Standard (GC or AU) base-pairings are denoted by (–) and non-standard (GU) base-pairing by (+). Anticodons are in bold-face.

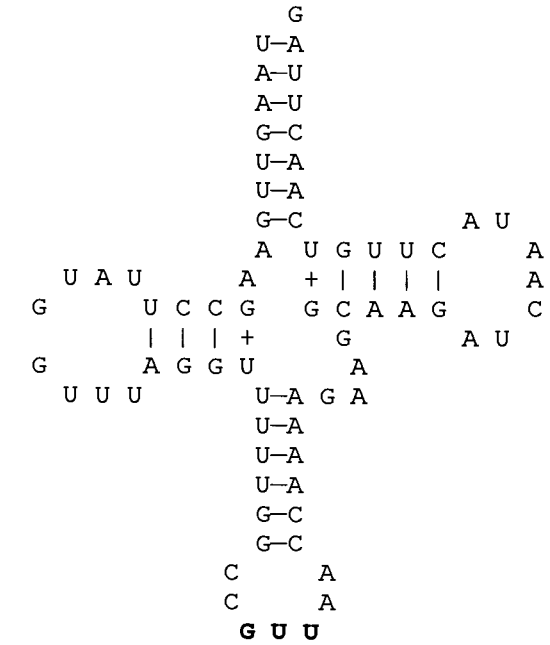


Methionine (M)

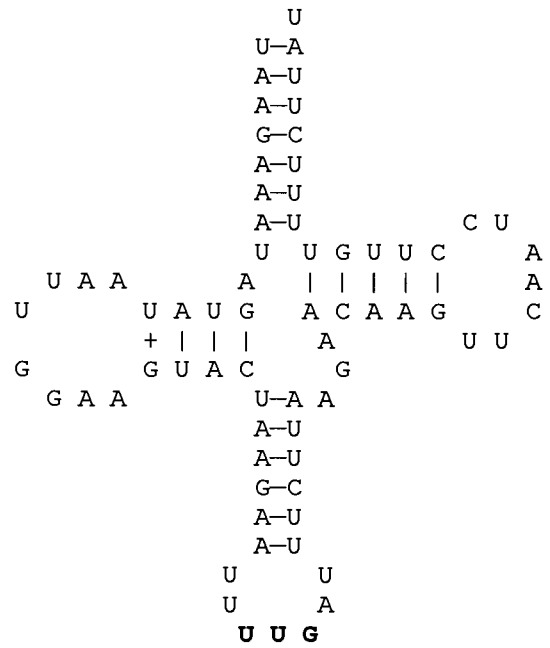


Leucine$_{CUN}$ (L)

Figure 8. continued.

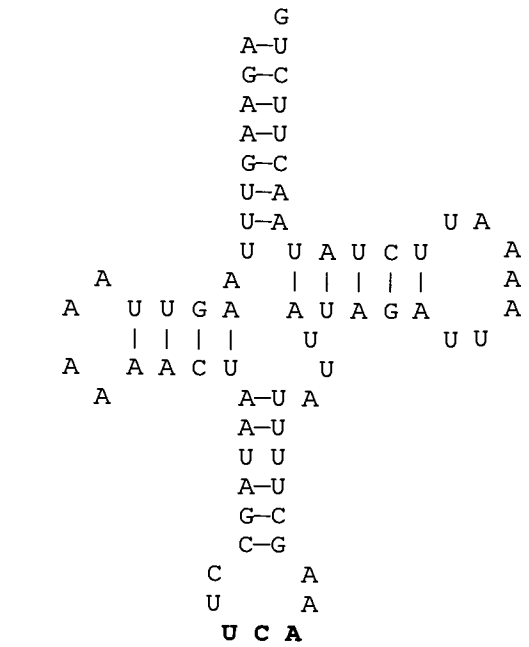


Asparagine (N)

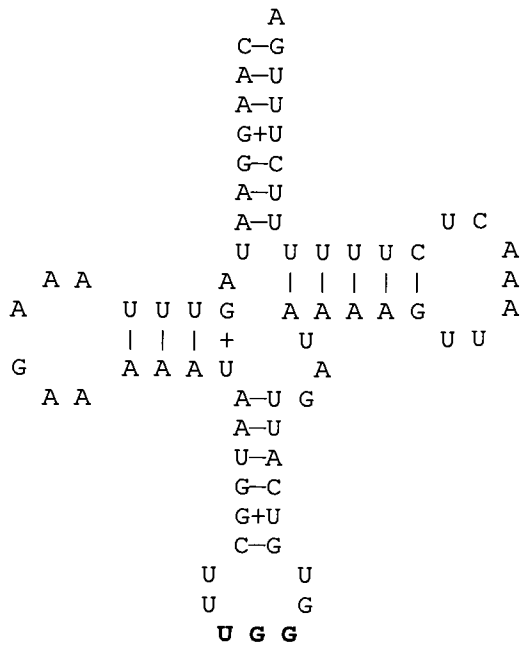


Glutamine (Q)

Figure 8. continued.

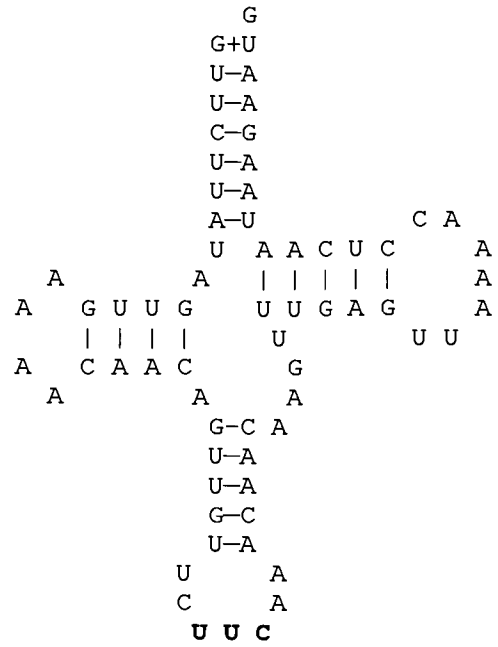


Tryptophan (W)

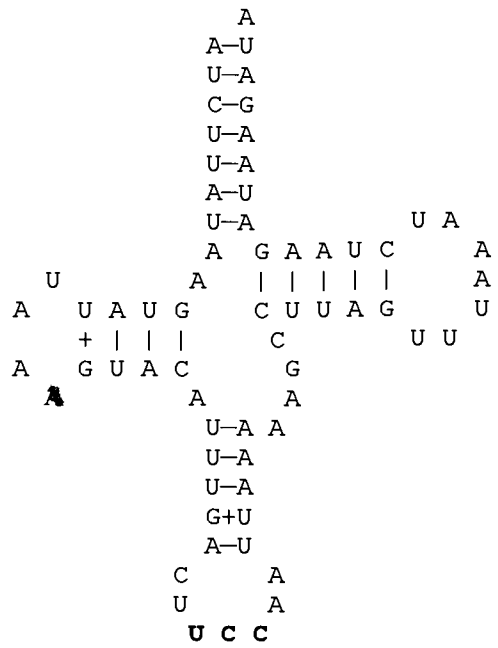


Proline (P)

Figure 8. continued.

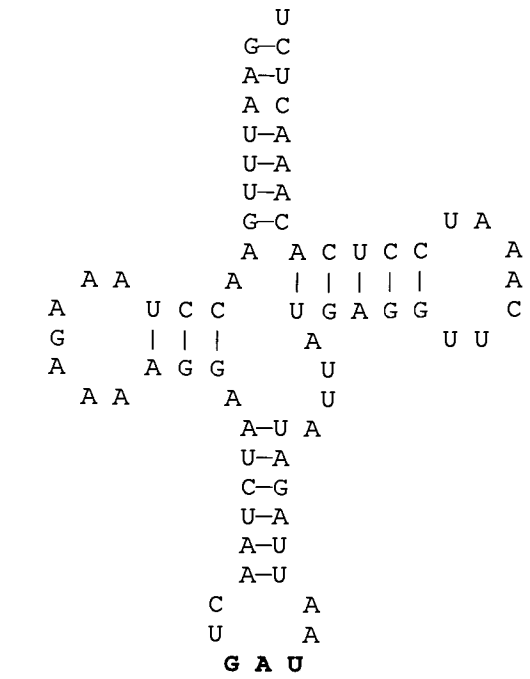


Glutamic Acid (E)

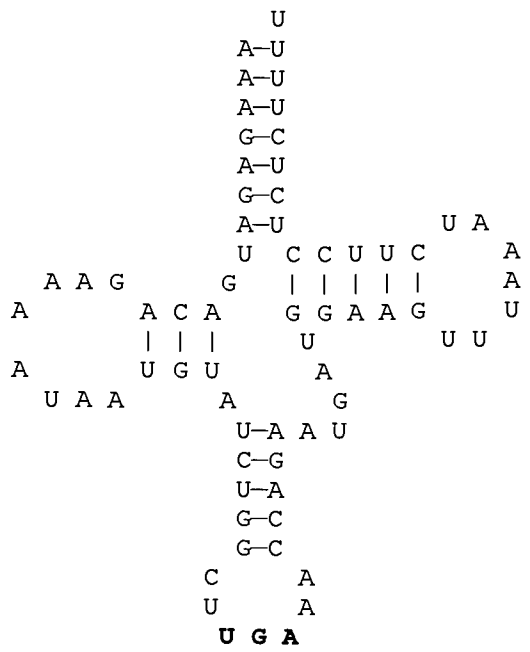


Glycine (G)

Figure 8. continued.

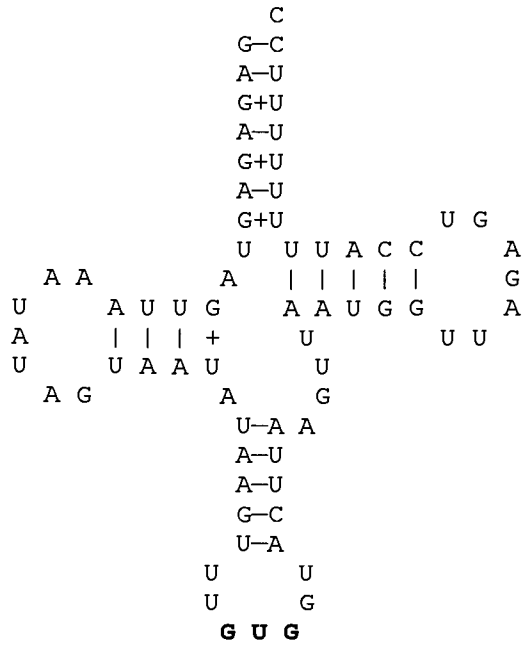


Isoleucine (I)

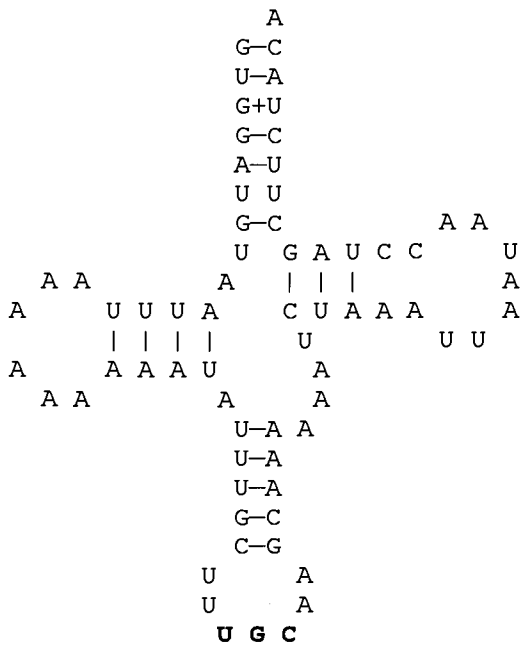


Serine$_{UCN}$ (S)

Figure 8. continued.

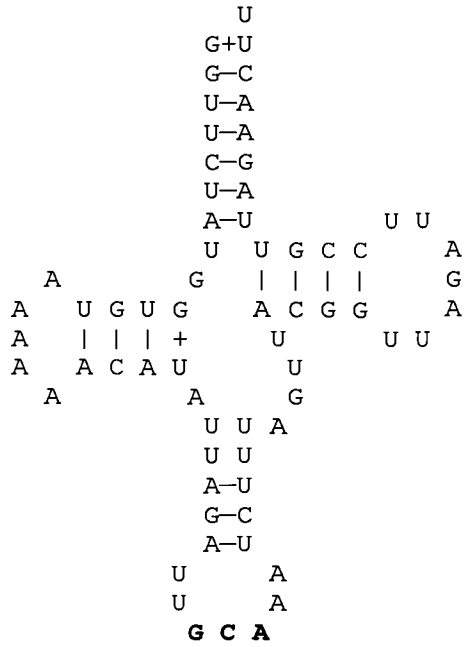


Histidine (H)

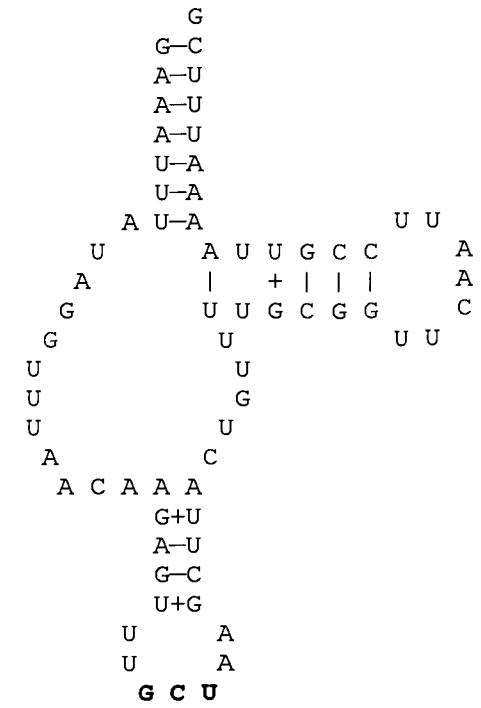


Alanine (A)

Figure 8. continued.

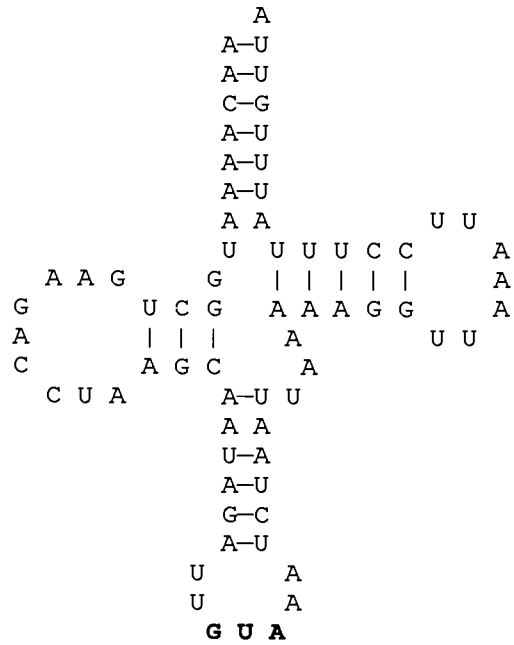


Cysteine (C)

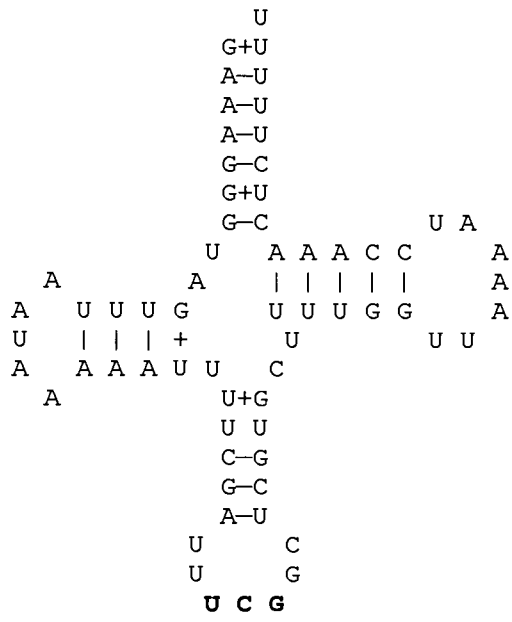


Serine$_{AGN}$ (S)

Figure 8. continued.

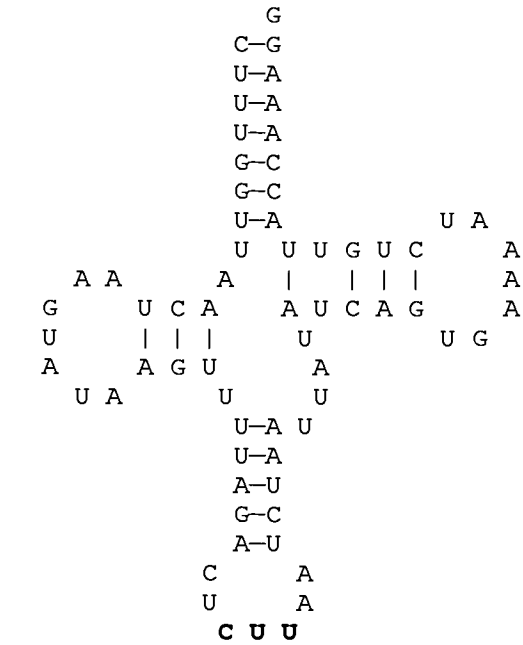


Tyrosine (Y)

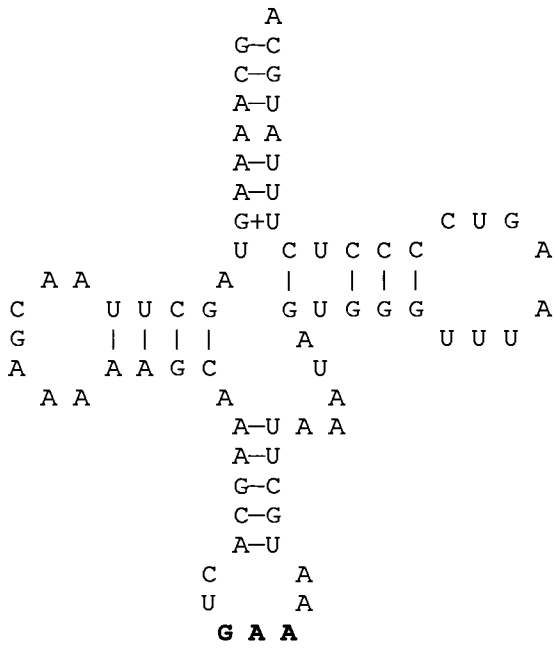


Arginine (R)

Figure 8. continued.

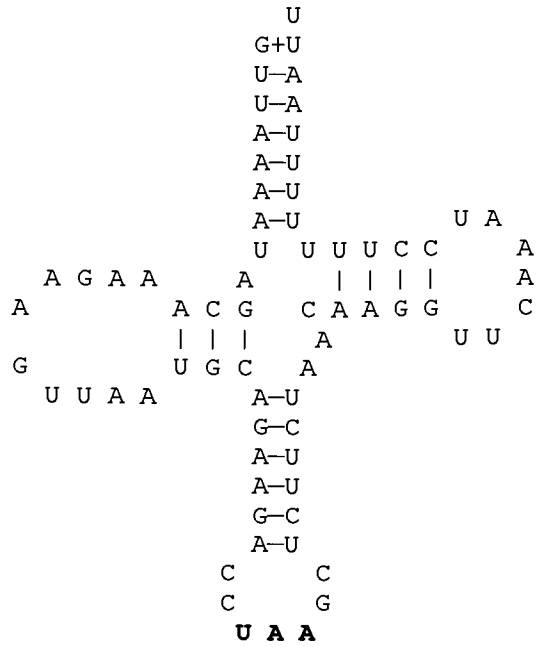


Lysine (K)

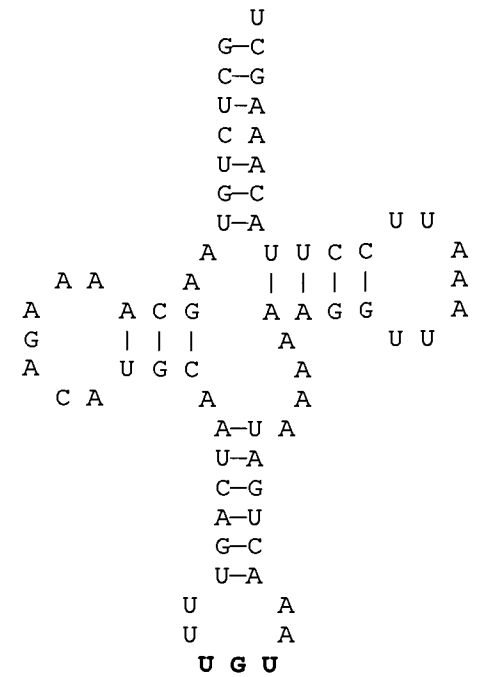


Phenylalanine (F)

Figure 8. continued.



Leucine$_{UUR}$ (L)



Threonine (T)

Figure 8. continued.

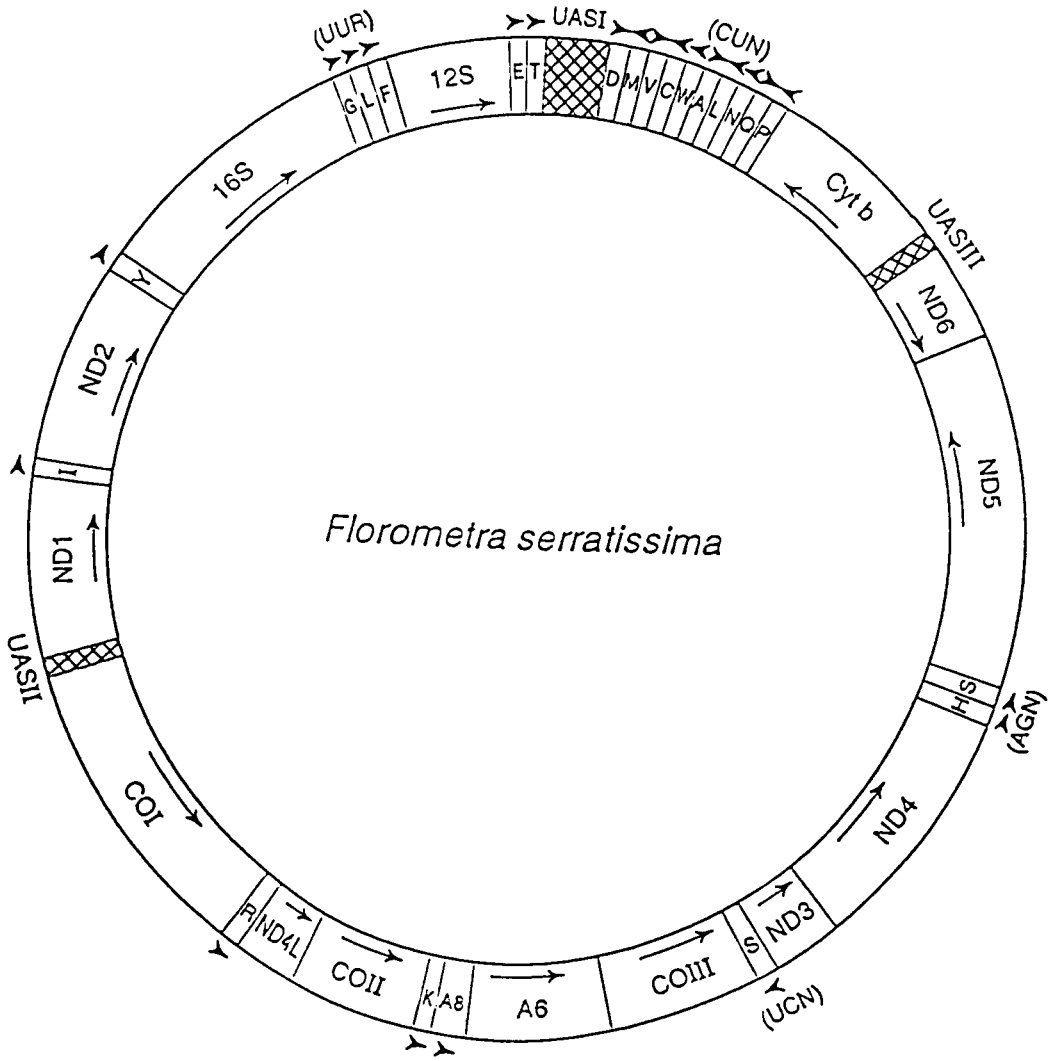


Figure 9. The mitochondrial genome map of the crinoid, *Florometra serratissima*. Arrows indicate the direction of transcription of each gene. Names of protein coding and ribosomal genes are abbreviated as given in the text, except A6 and A8 are used to denote ATPase subunits. tRNA genes are represented by their single letter codes. Serine and leucine tRNA genes are also identified by the codon family they recognize (in parentheses). Unassigned regions, UAS I, UAS II, and UAS III, are designated in hatched areas.

DISCUSSION

Florometra serratissima general mitochondrial DNA features

The purpose of this study was to determine the mtDNA gene order of the crinoid *Florometra serratissima* and its relationship to the other four major echinoderm classes: the asteroids, the echinoids, the holothuroids, and the ophiuroids. The complete mitochondrial DNA gene map has been determined for *F. serratissima* and is shown in Figure 9. The map illustrates the total 14,774 nucleotides of accumulated sequence data (Figures 5, 6, and 7) encoding the complete sequences of 11 protein coding genes, 22 tRNA genes and 2 ribosomal genes, as well as partial sequences of the final two protein coding genes. The *F. serratissima* mtDNA genome contains the same genes as seen in other complete echinoderm mtDNA maps (Jacobs et al., 1988a; Cantatore et al., 1989; Asakawa et al., 1995; De Giorgi et al., 1996; K. Beckenbach, pers. comm.) as well as vertebrates (Anderson et al., 1981,1982; Bibb et al., 1981; Roe et al., 1985; Gadaleta et al., 1989; and Desjardins and Morais, 1990) and *Drosophila* (Clary and Wolstenholme, 1985) which provides further evidence as to the consistency of gene content within animal mtDNA genomes. The conservation of mtDNA compactness seen in other metazoan genomes, has been maintained in *Florometra*. Three regions of unassigned sequence greater then 73 nucleotides have been found. The rest of the *Florometra* mtDNA genome contains intergenic spacers of 18 nucleotides or less, and in some instances gene overlaps occur (Figures 5 and 6). Overlaps

from genes transcribed on opposite strands are found in the tRNA genes cysteine and valine in which a one nucleotide overlap is seen and in the COIII and tRNA serine$_{UCN}$ genes where there is an overlap of two nucleotides (see Figure 5). Overlaps are also seen with genes transcribed on the same strand. The 3' end of the glutamic acid tRNA gene and the 5' end of the threonine tRNA gene may overlap by one nucleotide, depending on the tRNA structure of glutamic acid (discussed below). However, the 3' end of the ATPase8 and the 5' end of the ATPase6 genes, which are transcribed on the same strand, overlap by 7 nucleotides. Similar ATPase8 and ATPase6 gene overlaps are seen in most metazoans but, the degree of the overlap varies (Anderson et al., 1981, 1982; Bibb et al., 1981; Roe et al., 1985; Jacobs et al., 1988a; Cantatore et al., 1989; Desjardins and Morais, 1990; Smith et al., 1990; Asakawa et al., 1995; and De Giorgi et al., 1996).

Protein Coding Genes-Initiation Codons

Although it had been demonstrated earlier that the mitochondrial genetic code varies between phyla (Barrell et al., 1979; Jukes and Osawa, 1990; and Osawa et al., 1992), an accepted echinoderm code has been proposed (Himeno et al., 1987; Jacobs et al., 1988a; Cantatore et al., 1989; Asakawa et al., 1995; and De Giorgi et al., 1996). The sequence encoding the mtDNA proteins seen in Figures 6, 7, and 8 was translated using the echinoderm mtDNA genetic code. All of the *F. serratissima* protein coding genes begin with the traditional ATG codon except for ND4L, which uses GTG. This use of GTG

as an initiation codon, for the *Florometra* ND4L gene, is an apparently unique feature relative to the other echinoderm ND4L genes encountered thus far. The ND4L genes of three sea urchins, from two different orders, and two sea stars, from different orders all seem to utilize the unorthodox initiation codons of either ATC or ATT (Jacobs et al., 1988a; Cantatore et al., 1989; Smith et al., 1990; Asakawa et al., 1995; and De Giorgi et al., 1996), whereas the brittle star has been seen to use the standard ATG (K. Beckenbach, pers. comm.). However, the use of GTG as an initiation codon, has been seen in other echinoderm mtDNA protein genes. The ATPase8 gene of *Strongylocentrotus purpuratus* (Jacobs et al., 1988a), *Paracentrotus lividus* (Cantatore et al., 1989), and *Arbacia lixula* (De Giorgi et al., 1996), as well as the ND1 and ND5 genes of *Asterina pectinifera* (Asakawa et al., 1995) all utilize GTG as their initiating codons. In addition, the use of the GTG start codon is also seen in vertebrates, *Drosophila,* and *Mytilus edulis*. The chicken COI gene (Desjardins and Morais, 1990), the rat ND1 gene (Gadeleta et al., 1989), the *Drosophila* ND5 gene (Clary and Wolstenholme 1985), and the *Mytilus* ND1 gene (Hoffman et al., 1992) all utilize GTG as initiating codons. It had been suggested earlier that the GTG start codon seen in the *S. purpuratus* ATPase8 gene may reflect a mechanism to ensure that these two overlapping genes, on the same transcriptional unit, are translated at a similar rate. It was hypothesized by Jacobs et al. (1988a) that the inefficiency of internal translation initiation of the ATPase6 gene may be balanced by the use of the rare start codon, GTG, of the ATPase8 gene. Bibb et al. (1981) also suggested that the

heterogeneity of all the potential initiation codons may indicate a translation control at the level of selection of initiator codons. However further data on the use of unorthodox start codons, demonstrate that homologous mtDNA protein genes in different species, within classes or phyla, differ in their use of initiation codons. Therefore, it has been proposed that the unorthodox start codons do not play a role in the regulation of mitochondrial protein gene expression (Wolstenholme, 1992). This hypothesis is supported by the ND4L genes in all echinoderms studied so far, in which different initiation codons are found in four classes and in one case, different orders of the same class. Rather than fulfilling a translation control purpose, the occurrence of unorthodox initiation codons may result when there is no spacer nucleotide separating the initiation of the protein gene from an upstream tRNA gene. The start of translation, from the first base after a tRNA gene, may allow any ATN or GTG to act as initiators where ATG would be required at 5' ends containing upstream sequence (Attardi, 1985; Cantatore et al., 1989). This no-extra base rule is generally seen in all instances when GTG is utilized, except in the COI gene of the chicken (Desjardins and Morais, 1990), but is not as conserved a rule when ATC, ATT, or ATA are used. In *Florometra*, there is no extra nucleotide between the ND4L GTG start codon and the upstream tRNA gene for arginine (Figure 5). Therefore, the start codon GTG for the *Florometra* ND4L gene, conforms to this no-extra base rule seen in other metazoans.

Termination Codons

The termination of the *Florometra* mtDNA protein genes all contain full termination codons except COI, which terminates in TA, and ND1 and ND2 which end with a T, only. In all three cases, the 3' ends of the genes are punctuated with a tRNA gene (see Figure 5). A complete termination codon can be achieved from the sequence data for the ND2 and COI genes, but it would result in the 3' ends of the protein genes overlapping with the 5' ends of their adjacent tRNA gene. Although overlapping genes, transcribed on separate strands are more frequent among metazoan mtDNA genomes, processing overlapping genes on the same transcriptional unit would require extensive mechanisms (Anderson et al., 1982) and therefore less likely to occur as often. Therefore, rather than same-strand overlapping transcriptional units, incomplete termination codons are suggested for these *Florometra* protein genes. A complete termination codon would most likely be created through post-transcriptional polyadenylation, as seen in other organisms (Anderson et al., 1981; Ojala et al., 1981; Clayton, 1991). Most metazoan mtDNA protein genes have demonstrated the presence of incomplete stop codons, such as TA or T, to some degree within their genomes (Attardi, 1985, Wolstenholme, 1992). A TA partial termination codon is present in the ND1 gene of *A. lixula* (De Giorgi et al., 1996), however, the ND1 gene has complete termination codons in the rest of the echinoderms studied thus far. Incomplete termination codons are seen in the ND4L and Cyt b genes of *P. lividus* (Cantatore et al., 1989); the Cyt b, ND4L, ND6, and ND4 genes of *S. purpuratus;*

the Cyt b and COII genes of *A. pectinifera* (Asakawa et al., 1995); the Cyt b, ND4, and COIII genes of *Pisaster ochraceus* (Smith et al, 1990); and the ND2 and ND4L genes of *A. lixula* (De Giorgi et al., 1996).

Transfer RNA Genes

All 22 tRNA genes have been found in the *Florometra* mtDNA genome. The sequences of each tRNA gene are shown in Figure 5 and the transcriptional polarity and position of each, in the mtDNA genome are shown in Figures 5 and 9. Figure 8 illustrates the predicted secondary structures of each tRNA gene. These cloverleaf secondary structures were determined by identifying the anticodon sequence of each tRNA gene and then folding the tRNA gene according to other metazoan mitochondrial tRNAs and utilizing the consensus tRNA form of Sprinzl et al. (1989) as much as possible. In some cases, alternative structures to the ones shown in Figure 8 may be drawn, however, they were generally considered to be less conserved than the ones presented. The standard tRNA structure format includes the amino acyl (AA) stem composed of seven base pairs (bp), the dihydrouridine (D) stem containing four bp, and the anticodon (AC) stem and the T stem both having five bp (Sprinzl et al., 1989). The 3' CCA terminus on the AA stem, found in all tRNAs, has not been found to be encoded in metazoan mitochondrial tRNA genes, but is instead is added post-transcriptionally. In *Florometra,* as well, the 3' CCA terminus is not encoded in the mtDNA tRNA genes. In most cases, the *Florometra* mitochondrial tRNAs follow the standard cloverleaf structure but,

some variations do occur. The most obvious example of variation is seen in the *Florometra* tRNA structure for $serine_{AGN}$ in which the D stem is completely lacking. However, this is a common feature for this tRNA in echinoderms, vertebrates, and *Drosophila.*

The termination of the *Florometra* tRNA genes, at the AA stem, are also debatable in some cases. One example is seen in the tRNA gene for glutamic acid (Figure 8). The seventh nucleotide pair in the AA stem is the unconventional G-U match, seen in tRNA structures. It is unclear if the tRNA gene extends to this seventh nucleotide pair in the AA stem or if it terminates at the sixth pair. Termination at the G-U seventh pair, would result in an overlap of one nucleotide between the 3' end of the glutamic acid tRNA gene and the 5' end of the threonine tRNA gene which are transcribed on the same strand (Figure 5). Sequencing of the tRNA gene would resolve this conflict. However, similar AA stem length discrepancies have been noted elsewhere (Jacobs et al., 1988a; Cantatore et al., 1989; Hoffmann et al., 1992).

In some instances, *Florometra* tRNA structures contain only three nucleotide pairs in the D stem and/or four in the T stem, which is also seen in some tRNAs of *S. purpuratus* (Jacobs et al., 1988a), *P. lividus* (Cantatore et al., 1989) and *A. pectinifera* (Asakawa et al., 1995) as well as vertebrates (Bibb et al., 1981; Desjardins and Morais, 1990; and Roe et al., 1985). In addition, a few other interesting features of some of the *Florometra* tRNA genes should be addressed. The first nucleotide pair in the AC stem of aspartic acid, is a G-A mispair. This is also seen in the same tRNA gene of *S. purpuratus* (Jacobs et

al., 1988a) and *P. lividus* (Cantatore et al., 1989) in which a G-G and a A-A mispair are seen, respectively. The *Florometra* tRNA structure for alanine has two C-A mispairs on the end of the T stem facing the T-loop (Figure 8). In the other echinoderms the terminal pair is a C-G, however, internal C-A mispairs are seen in the T stems of *A. pectinifera* (Asakawa et al., 1995) and *P. ochraceus* (Smith et al., 1990). In the case of *P. ochraceus,* the C-A pair is the second nucleotide pair in from the loop. The *Florometra* cysteine tRNA gene has two consecutive U-U pairs at the top of the AC stem (Figure 8). However, this condition is identical to that found in *P. lividus* (Cantatore et al., 1989) and similar to that in *A. pectinifera* (Asakawa et al., 1995). Finally, the *Florometra* $leucine_{UUR}$ tRNA structure contains a C-U mispair at the end of the T stem, closest to the AA stem (Figure 8). Although this condition is unusual, its application does follow the five bp T stem length convention for standard tRNA structures (Sprinzl et al., 1989) and its presence is apparently necessary to maintain proper alignment of the AA stem.

Unassigned Sequence

Apart from the small numbers of nucleotides between identified genes (0-18), three regions of unassigned nucleotide sequence (UAS) have been found within the *Florometra* mtDNA genome. The largest UAS sequence between genes is 382 nucleotides and is situated between the tRNA genes for aspartic acid and threonine, and has been denoted UAS I (Figures 5 and 9). A second UAS (UAS II) region is located between the 5' ends of the protein

coding genes of ND1 and COI and is comprised of 77 nucleotides (Figures 5 and 9). Finally, a third UAS region (UAS III) is found between the 5' ends of the protein coding genes for Cyt b and ND6 and is 73 nucleotides in length (Figures 6 and 9). The UAS I region is located in a similar position and generally contains the same flanking sequence as the sea urchin denoted putative origin of replication or D-loop (Jacobs et al., 1988a, 1989). In sea urchins, the D-loop in situated between the threonine tRNA gene (adjacent to the 12S rRNA gene), and the tRNA proline gene (of the echinoderm tRNA cluster) (Figure 10) and is composed of 121 nucleotides in *S. purpuratus* (Jacobs et al., 1988a), 132 nucleotides in *P. lividus* (Cantatore et al., 1989) and 136 nucleotides in *A. lixula* (De Giorgi et al., 1996). In sea stars the putative D-loop is located between the threonine tRNA gene (adjacent to the 12S rRNA gene) and the 16S rRNA gene (Figure 10) and is considerably larger: 337 nucleotides in *P. ochraceus* (Smith et al., 1990), and 486 nucleotides in *A. pectinifera* (Asakawa et al., 1995). The UAS I region of *F. serratissima* is 382 nucleotides, which is almost three times the size of any of the sea urchin D-loop regions, but is comparable to sea stars. In addition, the *Florometra* UAS I region does contain sequence similarities to the D-loop regions in these other echinoderms. After the threonine tRNA gene, there is a stretch of nucleotides that is considerably pyrimidine rich, followed by a string of G nucleotides, which is followed by a high A-T rich region (Figure 5). Similar nucleotide features have been seen in the assigned D-loop region of *S. purpuratus* which has been proposed to be a condensed version of the vertebrate mtDNA replication

Sea Urchin

Cytb-F-12S-E-T-DI-P-Q-N-Lc-A-W-C-V-M-D-Y-G-Lu-ND1-I-ND2-16S
> > > > > > < > > < > > < > < > < > > > > > > >

Crinoid

Cytb-P-Q-N-Lc-A-W-C-V-M-D-DI-T-E-12S-F-Lu-G-16S-Y-ND2-I-ND1
> > < > > < > > < > < < < < < < < < < < < < <

Sea Star

Cytb-F-12S-E-T-DI-16S-ND2-I-ND1-Lu-G-Y-D-M-V-C-W-A-Lc-N-Q-P
> > > > > < < < < < < < > < > < < > < < > <

Vertebrate

E-Cytb-T-P-DI-F-12S-V-16S-Lu-ND1-I-Q-M-ND2-W-A-N-C-Y
< > > < > > > > > > > > < > > > < < < <

Figure 10. The partial mitochondrial gene order arrangements of the basic sea urchin, crinoid, sea star, and vertebrate patterns. Division between genes are indicated by a (-). Arrow heads (< or >) indicate the transcriptional polarity of the individual genes. The 13 tRNA gene cluster is boxed for clarity. The D-loop is denoted by DI and the tRNA genes are represented by their single letter abbreviations.

origin (Jacobs et al., 1988a, 1989). It is therefore likely that UAS I contains the putative control region for the *Florometra* mtDNA genome.

The unassigned nucleotide region between the 5' ends of the protein genes Cyt b and ND6, denoted UAS III, is comprised of only AT sequence and is 73 nucleotides in length. Jacobs et al. (1989), noted that a conserved block of TTATATATAA sequence, more commonly associated with the putative control region, was also found in other echinoderm gene junctions. In *S. purpuratus, A. pectinifera, A. amurensis,* this sequence motif occurs between the 5' ends of the oppositely transcribed ND6 and Cyt b genes. It has been therefore proposed, that this sequence may function as a bi-directional promoter (Elliot and Jacobs 1989; Jacobs et al., 1989). This TTATATATAA motif is present in the *Florometra* UAS III region at the 5 ' end of the ND6 gene (Figure 6). Specific mtDNA genes are transcribed from either the light or heavy DNA strand. This defines a requirement for the presence of promoters on both strands or a bi-directional promoter. Identification of the *Florometra* UAS II region is still indeterminate and somewhat perplexing. Although there are instances in which several intergenic spaces, within mtDNA genomes, contain the TTATATATAA-like motif (Jacobs et al., 1989), UAS II does not seem to include it. However, the UAS II region is AT rich and may contain a more modified motif. In addition, the *Florometra* COI and ND1 genes flanking the UAS II region are transcribed in opposite directions (Figure 5), and a bi-directional promoter has been proposed to occur between the 5' ends of the COI and the proline tRNA genes in sea stars, which are transcribed on opposite strands, (Jacobs et al.,

1989; Smith et al., 1990; and Asakawa et al., 1995). However, the function of this UAS II region is still unknown.

Mitochondrial Gene Orders Among Phyla

As mentioned previously, the mitochondrial gene content is highly conserved within most metazoans. However, the order of the genes within the mtDNA genomes seem to vary from phylum to phylum (Cantatore et al., 1989; Desjardins and Morais, 1990; Pääbo et al., 1991; Okimoto et al., 1992; and Wolstenholme, 1992). Vertebrates, for the most part have a relatively stable mtDNA genome except for the variations seen in birds (Desjardins and Morais, 1990, 1991) and marsupials (Pääbo et al., 1991). However, the completion of several mtDNA genome maps from the sea urchins, *S. purpuratus* (Jacobs et al., 1988a), *P. lividus* (Cantatore et al., 1989), and *A. lixula* (De Giorgi et al., 1996), the sea star *A. pectinifera* (Asakawa et al., 1995) and the brittle star *Ophiopholis aculeata* (K. Beckenbach, pers. comm.) as well as partial sequence data from another sea star *P. ochraceus* (Smith et al., 1990), and gene-junction PCR amplification data from several echinoderms (Smith et al., 1993), have provided evidence that the mtDNA gene arrangement is less stable within the phylum Echinodermata. In addition, this idea is further strengthened by the determined mtDNA gene order for the crinoid, *F. serratissima* shown in Figure 9, which up to this time was not known.

Jacobs et al. (1988a) first provided evidence that a novel sea urchin mtDNA gene order, in comparison to that previously seen in vertebrates

(Anderson et al., 1981, 1982; Bibb et al., 1981; Roe et al., 1985) and *Drosophila* (Clary and Wolstenholme, 1985) does occur. One of the most obvious differences between the mtDNA genomes of the vertebrates and the sea urchin, *S. purpuratus,* was the presence of a 13 tRNA gene cluster adjacent to the D-loop. In the echinoderm mtDNA sequences to date, this cluster arrangement of the tRNA genes is generally conserved. In vertebrates, the tRNA genes are found more dispersed throughout the genome. In addition, utilizing protein and rRNA genes, it was proposed that the sea urchin pattern could be related to the vertebrate pattern with only a few rearrangements (Jacobs et al., 1988a; Cantatore et al., 1987a, 1989).

Organization of mtDNA within Echinodermata

The other two complete sea urchin mtDNA maps have been found to be identical to that of *S. purpuratus* (Cantatore et al., 1989; De Giorgi et al., 1996). Of the three sea urchin species, *S. purpuratus* and *P. lividus* are from the order Camarodonta where *A. lixula* belongs to the order Stirodonta. These orders are believed to have diverged between 100-150 million years ago (mya) (De Giorgi et al., 1991), which indicates stability within the class Echinoidea. Smith et al. (1989) demonstrated that the sea stars, class Asteroidea, contained a 4.6 kb inversion in comparison to the mtDNA of the sea urchins (see Figure 10). This inversion includes the tRNA cluster, the 16S rRNA gene, and the ND1 and ND2 protein genes. In addition, this inversion is present in sea stars from the orders Forcipulatida and Valvatida, which are believed to have diverged at

least 225 mya (Blake 1987). Therefore, the mtDNA gene order stability, within the class Asteroidea, also appears to be maintained. Through PCR amplification spanning gene junctions, it was further demonstrated that the sea cucumbers, class Holothuroidea, maintained the basic sea urchin mitochondrial gene orientation, whereas the brittle stars, class Ophiuroidea, displayed the basic sea star pattern (Smith et al., 1993). A subsequent secondary inversion has occurred in the brittle star, but it does maintain the 4.6 kb inversion pattern of sea stars (K. Beckenbach, pers. comm.). In addition, the brittle stars (Smith et al., 1993; K. Beckenbach, pers. comm.) and some sea cucumber species, from the genus *Cucumaria* (Allan Arndt, pers. comm.), exhibit an altered tRNA cluster which contain less than the 13 found in sea urchins and sea stars. However, it is generally considered that mitochondrial tRNA genes may be moving at an accelerated rate, probably by a separate mechanism, in comparison to other mtDNA genes. In addition, they may also be involved in gene rearrangements seen elsewhere in the genome (Jacobs et al., 1989; Cantatore et al., 1987b). Therefore, this original 4.6 kb inversion separates these four echinoderm classes into echinoid/holothuroid and asteroid/ophiuroid lineages.

The five major echinoderm classes: Asteroidea, Echinoidea, Holothuroidea, Ophiuroidea, and Crinoidea, were more than likely distinct by the early Palaeozoic, and probably diverged between 450-550 mya (Smith 1988) (see Figure 1). However, there has been controversy to how these classes are related as there is no single character that defines the

phylogenetic relationships between the classes (Smiley, 1988; Smith, 1992). Although fossil evidence is generally good for echinoderms, it gives no clear indication as to when each class diverged, or how the classes may be related. In addition, there are no fossil intermediates which would link the different lineages (Smith, 1992). Echinoderm class phylogenies based on comparative morphology (Smith, 1988; Smiley, 1988) and larval characteristics (Strathmann, 1988) have produced conflicting relationships. Earlier investigations of mitochondrial DNA suggested that the analysis of mitochondrial gene order could prove to be a useful tool to ascertain inter-relationships of phyla, classes and orders (Brown et al., 1985; Jacobs et al., 1988b). Characters that discriminate the relationship of higher taxa should be homologous and highly stable so that any changes in the characters would be unique events and therefore readily observed (Smith et al., 1993). The 4.6 kb inversion event demonstrated earlier that separated four of the classes into two groupings (Smith et al., 1989, 1990, 1993; Jacobs et al., 1989; Asakawa et al., 1991, 1995) is, by definition, such a unique character and represents a significant phylogenetic marker (Smith, 1992). The Asteroidea and Ophiuroidea classes have been distinct since the Ordovician (Figure 1), and diverged probably between 490-530 mya (Smith 1988). The Echinoidea and Holothuroidea classes, also distinct since the Ordovician, are believed to have diverged around 450-530 mya (Smith 1988). Fossil evidence indicates that the class Crinoidea diverged from the rest of the echinoderms probably before the end of the Lower Cambrian, approximately 550-560 mya (Smith, 1988). Therefore, the

relationship of the class Crinoidea to the other echinoderms, as well as the polarity of this 4.6 kb inversion could be possibly defined through the determination of *F. serratissima* mtDNA genome arrangement, seen in Figure 9, which previously was not known.

Florometra serratissima Mitochondrial Gene Order Features

This *F. serratissima* resolved mtDNA genome arrangement presented in Figure 9 demonstrates a novel gene order in relation to the other echinoderm classes. The region of the genome spanning from the COI gene through to the ND6 gene is conserved across all of the major five echinoderm classes, including Crinoidea, with the notable exception of a tRNA gene duplication in the sea cucumber *Cucumaria* genus (Allan Arndt pers. comm.). The major gene inversion event that separates the four echinoderm classes into the echinoid/holothuroid and ophiuroid/asteroid lineages appears to have happened in the crinoids. However, the presence of novel rearrangements that have occurred within the genome, make it difficult to align it to either lineage distinctly.

An initial restriction map had been created earlier (Figure 2), which located some of the specific *Florometra* mtDNA genes to regions of the genome, yet did not reveal any significant gene order determinations. Sequence data obtained from the mtDNA clone, FSHD8, first indicated the presence of a novel gene order within *Florometra.* The sequence from this clone demonstrated that the ribosomal genes 16S and 12S were transcribed

on the same strand, with the 3' end of the 16S rRNA gene directed towards the 5' end of the 12S rRNA gene, with three tRNA genes, glycine, leucine$_{UUR}$, and phenylalanine, separating them (Figures 5 and 9). All five genes have the same transcriptional polarity. The presence of the phenylalanine tRNA gene at the 5' end of the 12S gene appears to be a conserved feature throughout the echinoderms and vertebrates studied thus far. However, the genes for the glycine and leucine$_{UUR}$ tRNAs have been usually located within the 13 tRNA gene cluster found in sea urchins, sea stars, and in some sea cucumbers (Allan Arndt, pers. comm.). In *Florometra*, the terminal three tRNA genes seen in the 13 tRNA cluster after aspartic acid- leucine$_{UUR}$, glycine, and tyrosine, have moved. The leucine$_{UUR}$ and glycine tRNA genes apparently moved as a single unit to between the ribosomal genes and the tyrosine tRNA gene to between the 3' end of the ND2 gene and the 5' end of the 16S gene (Figure 9). This location of the tyrosine tRNA gene is not seen in any of the other echinoderms studied so far.

The transcriptional and positional arrangement of the ribosomal genes in *Florometra* also differs from any of the other echinoderms as well as the vertebrates. In the basic sea urchin/sea cucumber gene arrangement, the ribosomal genes are transcribed on the same strand but the genes are separated by the putative control region, the tRNA gene cluster, and the ND1 and ND2 protein genes (Figure 10). In addition, the 3' end of the 12S gene is directed towards the 5' end of the 16S gene. In the sea star pattern the 16S and 12S rRNA genes are transcribed on opposite strands with the 3' ends of

the genes facing each other with the putative control region and two tRNA genes separating them, as a result of the 4.6 kb inversion (Figure 10). In vertebrates, the 12S and 16S genes are tandem with the tRNA gene for valine between them but, the 3' end of the 12S gene faces the 5' end of the 16S gene, which is opposite to that in *Florometra* (Figure 9). In addition to the novel ribosomal RNA gene arrangements, or perhaps as a result of it, there is a unique arrangement of the remaining *Florometra* tRNA cluster, putative D-loop (UAS I) and the Cyt b gene (Figure 9). Although this arrangement appears to be more similar to the sea urchin lineage than to the sea star (Figure 10), obvious differences can be noted. The *Florometra* Cyt b gene is directly beside this modified tRNA cluster and the putative *Florometra* D-loop (UAS I) is situated on the opposite side of the cluster. In sea urchins, the 12S gene and D-loop separate the original tRNA cluster (Figure 10).

The 4.6 kb inversion, with respect to the sea urchin lineage, seen in the sea star mtDNA pattern, places the 13 tRNA gene cluster adjacent to the COI gene, and the 16S gene adjacent to the putative D-loop (Figure 10). A similar inversion appears to have taken place in *Florometra* (Figure 9), however the novel rearrangements between the Cyt b gene and the rRNA genes, including the modified tRNA cluster and putative D-loop, suggest that this inversion happened after these modifications. The original 4.6 kb inversion involves the region spanning the 16S rRNA gene through to the proline tRNA gene of the tRNA cluster (Figure 10). In comparison, the inversion seen in the *Florometra* mtDNA genome includes only the 16S rRNA gene through to the ND1 gene. In

this case the tRNA cluster, is not involved in the inversion. As the class Crinoidea is the oldest of the extant echinoderms according to fossil records (Smith and Paul, 1984; Smith 1988), it was believed that they may exhibit the more ancestral mtDNA genome pattern or at least allow the determination to what the primitive pattern may have been.

Mitochondrial Gene Rearrangements

There is no evidence for intermolecular recombination between mtDNA molecules within animals (Jacobs et al., 1988b), however, intramolecular recombination has not been ruled out. In addition, there is no direct evidence of transpositions occurring within mtDNA genomes. Therefore, protein and rRNA genes rearrangements between mtDNA genomes are often thought to be the result of intramolecular gene inversions (Smith et al., 1993) or intramolecular gene duplications (Moritz and Brown, 1987). As tRNA genes are generally thought to move by a different mechanism (Cantatore et al., 1987b; Jacobs et al., 1989) than that of the rRNA and protein genes, they are generally omitted from the calculation of rearrangement steps required to obtain different gene orders seen in mtDNA genomes. However, in the case of the crinoid, two tRNA genes appear to have moved as a group and therefore will be treated the same as other changes. Figure 11 is a schematic drawing with the minimal number of changes it would require to change from the sea urchin pattern to either the sea star or crinoid pattern form while in Figure 12, the pathways of such changes are illustrated.

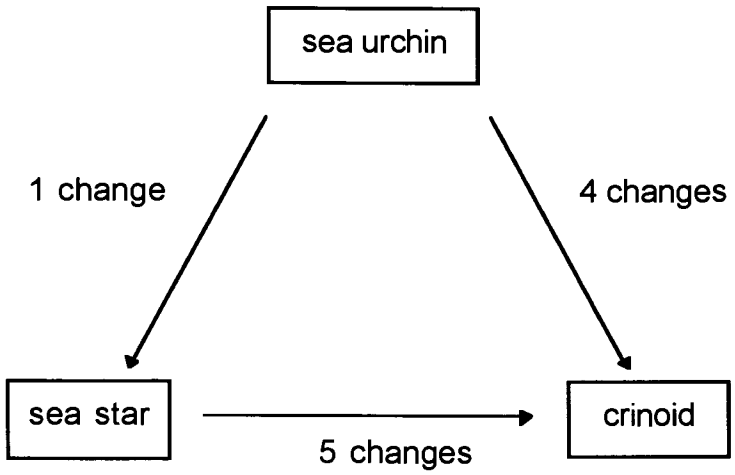


Figure 11. Minimum number of changes required to achieve specific mitochondrial gene patterns.

Generally, the number of steps it would require to achieve either the sea urchin or the sea star pattern from the crinoid pattern is greater than that seen if the sea urchin orientation was original. There is only one change, the 4.6 kb inversion, between the sea urchin and sea star patterns, which hypothetically means that the ancestral polarity of the inversion could be either orientation. However, Jacobs et al. (1988) noted that the sea urchin pattern and the vertebrate pattern could be related with a few protein and rRNA gene

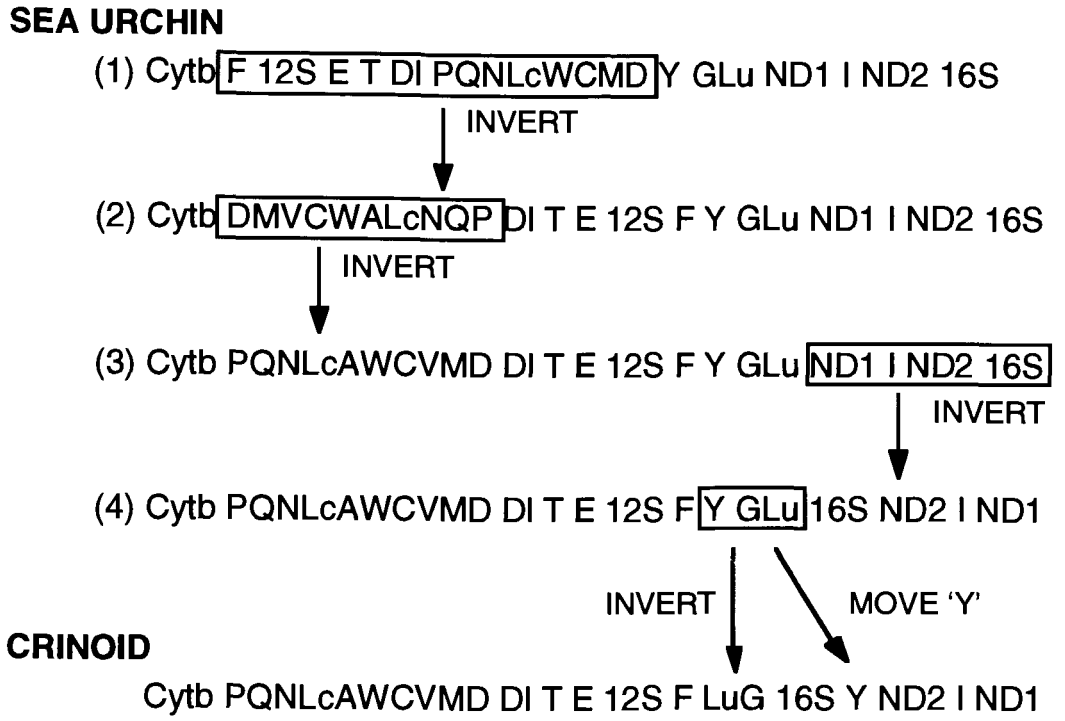


Figure 12. Outline of the minimum number of steps to achieve the crinoid mtDNA gene order from the sea urchin pattern. Inversion blocks are boxed for clarity. tRNA genes are represented by their single letter abbreviations. Rearrangement steps assume an independent movement of the tyrosine (Y) tRNA gene.

rearrangements, which would suggest that the sea star inversion had derived from the sea urchin orientation. Between the sea urchin and the crinoid pattern there are essentially 4 changes. The first is an inversion from the phenylalanine (F) tRNA gene to the end of the aspartic acid (D) tRNA gene of the tRNA gene cluster in the sea urchin pattern (Figure 12). Next, the modified tRNA cluster, missing the terminal 3 tRNA genes for tyrosine, glycine and leucine$_{UUR}$, inverts again. The ND1 to 16S region then inverts, followed by an inversion of the glycine and leucine$_{UUR}$ tRNA gene doublet. Finally, the tyrosine tRNA which has moved to between the ND2 and 16S genes (Figures 10 and 12), probably occurred by an independent movement. In order to get the crinoid pattern from sea star pattern, it would require 5 steps. The sea star pattern would have to invert to the sea urchin pattern first, adding one more event. Alternative models that involve only inversion events for the modification of the sea urchin to crinoid (Figure 13) or the sea star to crinoid (Figure 14) mtDNA gene order patterns also result in fewer steps for the sea urchin / crinoid conversion. Therefore, it seems that the sea urchin pattern is the more ancestral-like state for echinoderm mitochondrial genomes.

Phylogenetic Implications

This accumulated data for the mtDNA gene orders can be applied to the fossil evidence and times of divergence of these five classes of echinoderms. The initial 4.6 kb inversion that separated the echinoid/holothuroid and the asteroid/ophiuroid lineages most likely occurred after the split of the

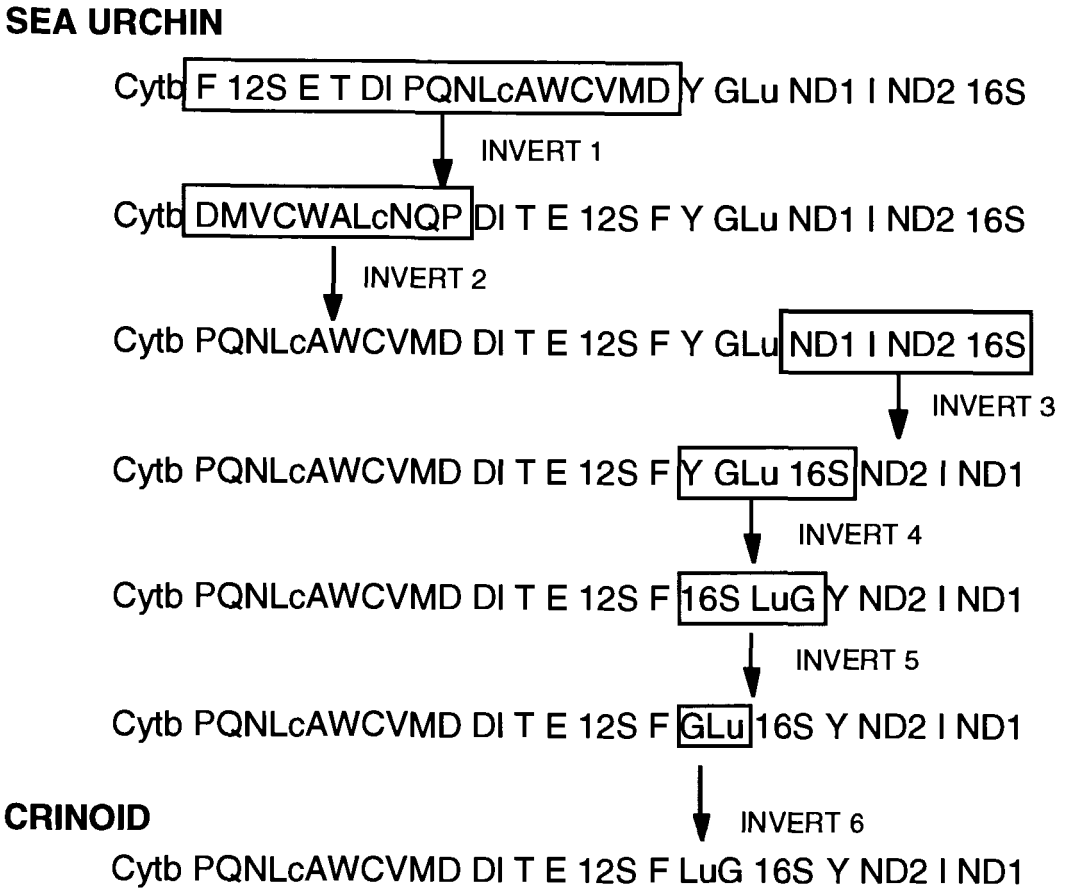


Figure 13. Outline of the 6 inversion steps required to achieve the crinoid mtDNA gene order from the sea urchin arrangement. Inversion blocks are boxed for clarity. tRNA genes are represented by their single letter abbreviations.

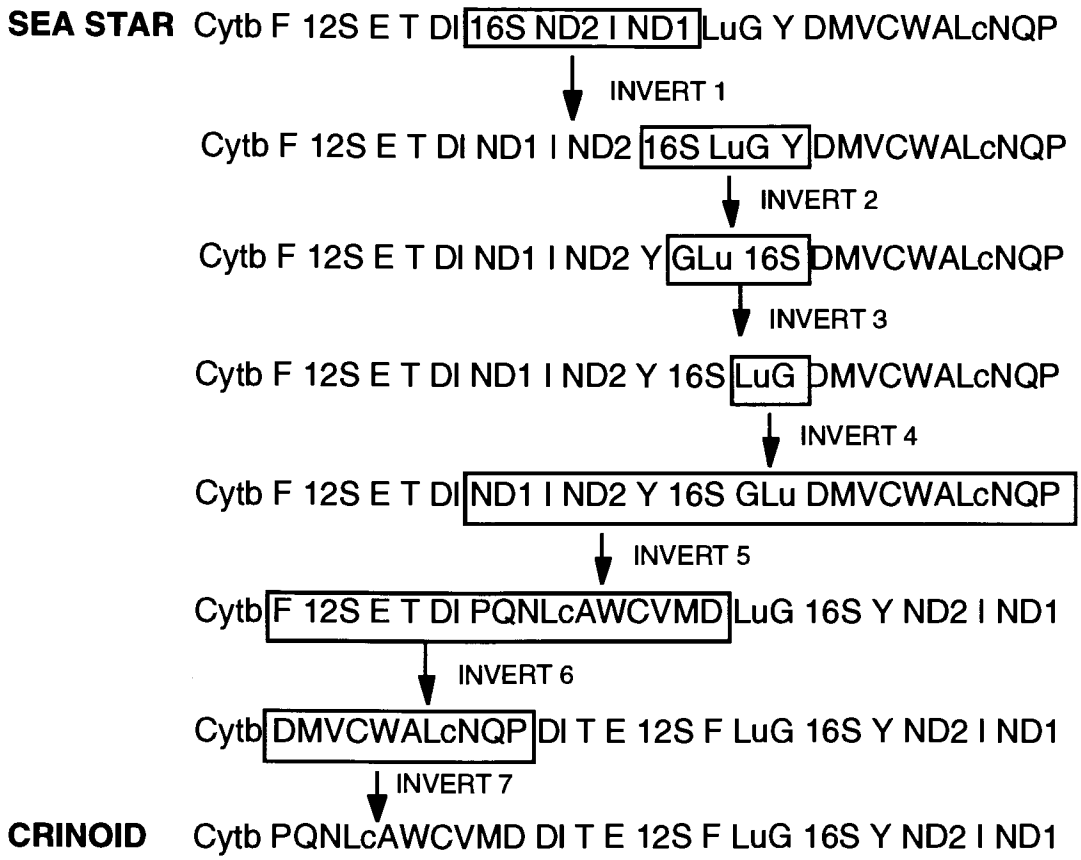


Figure 14. Outline of the 7 inversion steps required to achieve the crinoid mtDNA gene order from the sea star pattern. Inversion blocks are boxed for clarity. tRNA genes are represented by their single letter abbreviations.

Pelmatazoa, to which the crinoids belong, and the Eleutherozoa, from which the other four classes belong. These two groups are believed to have diverged approximately 550 mya (Smith and Paul, 1984; Smith, 1988) (Figure 1). The echinoderm ancestor to these groups most likely had a mtDNA gene order very similar to present day sea urchins, considering the relationship of the sea urchin mtDNA pattern to the crinoids. Shortly after the divergence of the Subphyla, Pelmatozoa and Eleutherozoa, the split between the asteroid/holothuroid and echinoid/ophiuroid lineages happened (Smith and Paul, 1984; Smith, 1988). This event either preceded or was perhaps marked by the 4.6 kb inversion event of the sea star/sea cucumber lineage. The crinoids, although beginning with the ancestral mtDNA gene order state, accumulated changes over the course of their evolution. The apparent inversion event in the *Florometra* ND1 to 16S genes probably happened in the separate crinoid lineage, independent of the original inversion seen in the sea star and brittle star classes. However, it is unclear if this mtDNA arrangement within *F. serratissima* is standard for all crinoids or if there is variation within the class Crinoidea. Obtaining the mtDNA gene order from different feather star species, would establish that the mtDNA gene order within the crinoids is stable. In addition, analysis from the stalked forms of the class Crinoidea, may indicate a different mtDNA order as some believe that the stalked and free-living conditions of the articulate crinoids may have evolved separately (Macurda and Meyer, 1983; Sprinkle, 1987; Simms, 1988). However, the accumulated mitochondrial gene order data to date, implies that the sea urchin

mtDNA arrangement of genes is more like the primitive echinoderm ancestor than any of the other classes. In addition, as the mtDNA gene order appears to be relatively stable within echinoderm classes, it seems reasonable that similar phylogenetic analyses, using rearrangements between classes, can be applied to other systems.

REFERENCES

Anderson S., A.T. Banker, B.G. Barrel, M.H.L. deBruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden, I.G. Young. 1981. Sequence and organization of the human mitochondrial genome. Nature. **290**:457-465.

Anderson S., M.H.L. deBruijn, A.R. Coulson, I.C. Eperon, F. Sanger, I.G. Young. 1982. The complete sequence of bovine mtDNA: conserved features of the mammalian mitochondrial genome. J. Mol. Biol. **156**:683-717.

Asakawa, S., Y. Kumazawa, T. Araki, H. Himeno, K. Miura, K. Watanabe. 1991. Strand-specific bias nucleotide composition bias in echinoderm and vertebrate mitochondrial genomes. J. Mol. Evol. **32**:511-520.

Asakawa, S., H. Himeno, K. Miura, and K. Watanabe. 1995. Nucleotide sequence and gene organization of the starfish *Asterina pectinifera* mitochondrial genome. Genetics. **140**:1047-1060.

Attardi, G. 1985. Animal mitochondrial DNA: an extreme example of genetic economy. Int. Rev. Cytol. **93**:93-145.

Baker, A.N., F.W.E. Rowe, and H.E.S. Clark. 1986. A new class of Echinodermata from New Zealand. Nature **321**:862-864.

Barrell, B.G., A.T. Bankier, and J. Drouin. 1979. A different genetic code in human mitochondria. Nature. **282**:189-194.

Barrell, B.G., S. Anderson, A.T. Bakier, M.H.L. deBruijn, E. Chen, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden, and I.G. Young. 1980. Different pattern of codon recognition by mammalian mitochondrial tRNAs. Proc. Natl. Acad. Sci. USA. **77**:3164-3166.

Bermington, E., T. Lamb, and J.C. Avise. 1986. Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates. J. Hered. **77**:249-252.

Bibb, M.J., R.A. Etten, C.T. Wright, M.W. Walberg, D.A. Clayton. 1981. Sequence and gene organization of mouse mtDNA. Cell. **26**:167-180.

Blake, D.B. 1987. A classification and phylogeny of post-Palaeozoic sea stars (Asteroidea:Echinodermata). J. Nat. Hist. **21**:481-528.

Breimer, A. 1978. In: Treatise on Invertebrate Paleontology (R.C. Moore and C. Teichert, eds.), Part T, Echinodermata 2, Vol. 1, pp 9-10.

Brown. W.M. 1985. The mitochondrial genomes of animals. In: Molecular Evolutionary Genetics (R.J. MacIntyre, ed.), Plenum, New York, pp. 95-130.

Brown, G.G., G. Gadaleta, G. Pepe, C. Saccone, and E. Sbisà. 1986. Structural conservation and variation in the D-loop-containing region of vertebrate mitochondrial DNA. J. Mol. Evol. **192**:503-511.

Buroker, N.E., J.R. Brown, T.A. Gilbert, P.J. O'Hara, A.T. Beckenbach, W.K., Thomas, and M.J. Smith. 1990. Length heteroplasmy of sturgeon mtDNA: an illegitimate elongation model. Genetics. **124**:157-163.

Cabot, E.L. and A.T. Beckenbach. 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. CABIOS. **5**:233-234.

Cantatore, P., M. Roberti, P. Morisco, G. Rainaldi, M. Gadaleta, and C. Saccone. 1987a. A novel gene order in the *Paracentrotus lividus* mitochondrial genome. Gene. **53**:41-54.

Cantatore, P., M. Gadaleta, M. Roberti, C. Saccone, and W.C. Wilson. 1987b. Duplication and remolding of transfer RNA genes during the evolutionary rearrangement of mitochondrial genomes. Nature. **32**:853-855.

Cantatore, P., M. Roberti, G. Rainaldi, M. Gadaleta, and C. Saccone. 1989. The complete nucleotide sequence, gene organization, and genetic code of the mitochondrial genome of *Paracentrotus lividus*. J. Biol. Chem. **264**:10965-10975.

Cantatore, P., M. Roberti, P.L. Polosa, A. Mustich, and M.N. Gadaleta. 1990. Mapping and characterization of *Paracentrotus lividus* transcripts: multiple and overlapping transcriptional units. Curr. Genet. **17**:235-245.

Clary, D.O. and D.R. Wolstenholme. 1985. The mtDNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. **22**:252-271.

Clark, A.H. and A.M. Clark. 1967. A monograph of the existing crinoids. Bull. U.S. Nat. Mus. 82, Vol. 1, Pt. 5, pp. 1-860.

Clayton, D.A. 1982. Replication of animal mitochondrial DNA. Cell. **28**:693-705.

Clayton, D.A. 1984. Transcription of the mammalian mitochondrial genome. Ann. Rev. Biochem. **53**:573-594.

Clayton, D.A. 1991. Replication and transcription of vertebrate mitochondrial DNA. Ann. Rev. Cell Biol. **7**:453-78.

Dan, K. and J. Dan. 1941. Early development of *Comanthus japonicus*. Jap. J. Zool. **9**:565-574.

De Giorgi, C., C. Lanave, M.D. Musci, and C. Saccone. Mitochondrial DNA in the sea urchin *Arbacia lixula*: evolutionary inferences from nucleotide sequence analysis. Mol. Biol. Evol. **8**:515-529.

De Giorgi, C., A. Martiradonna, C. Lanave, and C. Saccone. 1996. Complete sequence of the mitochondrial DNA in the sea urchin *Arbacia lixula*: conserved features of the Echinoid Mitochondrial genome. Mol. Phyl. Evol. **5**:323-332.

Desjardins P. and R. Morais. 1990. Sequence and gene organization of the chicken mitochondrial genome. A novel gene order in higher vertebrates. J. Mol. Biol. **212**:599-634.

Desjardins P. and R. Morais. 1991. Nucleotide sequence and evolution of coding and noncoding regions of a quail mitochondrial genome. J. Mol. Evol. **32**:153-161.

Donavon, S.K. 1986. Pelmatozoan columnals from the Ordovician of the British Isles. Part 1. Palaeontographical Society Monographs 1-68.

Donovan, S.K. 1988. The early evolution of the Crinoidea. In: Echinoderm Phylogeny and Evolutionary Biology. (C.R.C. Paul and A.B. Smith eds.), pp. 235-244. Oxford Univ. Press, Oxford.

Elliot, D.J. and H.T. Jacobs. 1989. Mutually exclusive synthetic pathways for sea urchin mitochondrial rRNA and mRNA. Mol. Cell. Bio. **9**:1069-1082.

Fearnley, I.M. and J.E. Walker. 1986. Two overlapping genes in bovine mitochondrial DNA components of ATP synthase. EMBO J. **5**:2003-2008.

Fearnley, I.M. and J.E. Walker. 1987. Initiation codons in mammalian mitochondria: differences in the genetic code in the organelle. Biochemistry. **26**:8247-8251.

Gadaleta, G., G. Pepe, G. DeCandia, C. Quagliariello, E. Sbisà, and C. Saccone. 1989. The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome: cryptic signals revealed by comparative analysis between vertebrates. J. Mol. Evol. **28**:497-516.

Hickman, C.D. 1967. Biology of the Invertebrates. pp 594-603. C.V. Mosby Company, Saint Louis.

Himeno, H., H. Masaki, T. Kawai, T. Ohta, I. Kumagai, K. Miura, and K. Watanabe. 1987. Gene. **56**:219-230.

Hoffmann, R.J., J.L. Boore, and W.M. Brown. 1992. A novel mitochondrial genome organization for the blue mussel, *Mytilus edulis*. Genetics. **131**:397-412.

Holland, N.D. 1991. Echinodermata: Crinoidea. In: Reproduction of Marine Invertebrates, Volume VI: Echinoderms and Lophophorates. (A.C. Giese, J.S. Pearse, and V.B. Pearse eds), pp. 247-299. The Boxwood Press, California.

Hyman, L.H. 1955. The Invertebrates: Echinodermata, Vol. IV, McGraw-Hill, New York.

Jacobs, H.T. and B. Grimes. 1986. Complete nucleotide sequences of the nuclear pseudogenes for cytochrome oxidase subunit I and the large ribosomal RNA in the sea urchin *Strongylocentrotus purpuratus*. J. Mol. Biol. **187**:509-527.

Jacobs, H.T., D.J. Elliot, V.B. Math, A. Farquharson. 1988a. Nucleotide sequence and gene organization of sea urchin mtDNA. J. Mol. Biol. **201**:185-217.

Jacobs, H.T., P. Balfe, B. Cohen, A. Farquharson, and L. Comito. 1988b. Phylogenetic implications of genome rearrangement and sequence evolution in echinoderm mitochondrial DNA. In: Echinoderm Phylogeny and Evolutionary Biology. (C.R.C. Paul and A.B. Smith eds.), pp 121-137. Oxford Univ. Press, Oxford.

Jacobs, H.T., S. Asakawa, T. Araki, K. Mikura, M.J. Smith, and K. Watanabe. 1989. Conserved tRNA gene cluster in starfish mtDNA. Curr. Genet. **15**:193-206.

Janke, A., G. Feldmaier-Fuchs, W.K. Thomas, A. von Haeseler, and S. Pääbo. 1994. The marsupial mitochondrial genome and the evolution of placental mammals. Genetics. **137**:243-256.

Jukes, T.H. and S. Osawa. 1990. The genetic code in mitochondria and chloroplasts. Experientia. **46**:1117-1126.

Kumazawa, Y., T. Yokogawa, E. Hasegawa, K. Miura, and K. Watanabe. 1989. The aminoacylation of structurally variant Phenylalanine tRNAs from mitochondria and various nonmitochondrial sources by bovine mitochondrial Phenylalanyl-tRNA synthetase. J. Biol. Chem. **264**:13005-13011.

Lagerkvist, U. 1981. Unorthodox codon reading and evolution of the genetic code. Cell. **23**:305-306.

Macurda, D.B., Jr., and D.L. Meyer. 1983. Sea lilies and feather stars. Amer. Scien. **71**:354-365

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A Laboratory Manual. Cold Spring Harbour Laboratory.

Marchuk, D., M. Drum, A. Saulino, and F.S. Collins. 1990. Construction of T-vector, a rapid and general system for direct cloning of unmodified PCR products. Nucl. Acids Res. **19**:1154.

Meyer, D.L. and D.B. Macurda Jr. 1977. Adaptive radiation of the comatulid crinoids. Paleobiology. **3**:74-82

Meyer, D.L., C.G. Messing, and D.B. Macurda, Jr. 1978. Zoogeography of tropical western Atlantic Crinoidea Echinodermata. Bull. Mar. Sci. **28**:412-441.

Mladenov, P.V. and F.-S. Chia. 1983. Development, settling behaviour, metamorphosis and pentacrinoid feeding and growth of the feather star *Florometra serratissima*. Mar. Biol. **73**:309-323.

Mladenov, P.V. 1986. Reproductive biology of the feather star *Florometra serratissima*: gonadal structure, breeding pattern and the periodicity of ovulation. Can. J. Zool. **64**:1642-1651.

Mladenov, P.V. 1987. Phylum Echinodermata, Class Crinoidea. In: Reproduction and Development of Marine Invertebrates of the North Pacific Coast. (M.F. Strathmann ed.), pp. 597-606.

Montoya, J., T. Christianson, D. Levens, M. Rabinowitz, and G. Attardi. 1982. Identification of initiation sites for heavy-strand and light strand transcription in human mitochondrial DNA. Proc. Natl. Acad. Sci. USA. **79**:7195-7199.

Montoya, J., G.L. Gaines, and G. Attardi. 1983. The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcriptional units. Cell. **34**:151-159.

Moore, R.C. and R.M. Jeffords. 1968. Classification and nomenclature of fossil crinoids based on studies of dissected parts of their columns. Univ. Kans. Paleontol. Contrib. Artic. **9**:1-86

Moore, R.C. and C. Teichert. 1978. In: Treatise on Invertebrate Paleontology (R.C. Moore and C. Teichert, eds.), Part T, Echinodermata 2, Vol. 1, pp 7-9.

Moritz, C. 1991. Evolutionary dynamics of mitochondrial DNA duplications in parthenogenetic geckos, *Heteronotia binoei*. Genetics. **129**:221-230.

Moritz, C. and W.M. Brown. 1986. Tandem duplication of D-loop and ribosomal RNA sequences in lizard mitochondrial DNA. Science. **233**:1425-1427.

Moritz, C. and W.M. Brown. 1987. Tandem duplication in animal mtDNAs: variations in incidence and gene content among lizards. Proc. Natl. Acad. Sci. USA. **84**:7183-7187.

Moritz, C., T.E. Dowling, and W.M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu. Rev. Ecol. Syst. **18**:269-292.

Nichols, D. 1966. Echinoderms (second edition). Hutchinson University Library, London.

ÓFoighil, D. and M.J. Smith. 1995. Evolution of asexuality in the cosmopolitan marine clam *Lasaea*. Evolution. **49**:140-150.

Ojala, D., J. Montoya, and G. Attardi. 1981. tRNA punctuation model of RNA processing in human mitochondria. Nature. **290**:470-474.

Okimoto, R., J. Macfarlane, D. Clary, and D. Wolstenholme. 1992. The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum.* Genetics. **130**:471-498.

Osawa, S., T. Ohama, T.H. Jukes, K. Watanabe. 1989. Evolution of the mitochondrial genetic code I. Origin of AGR Serine and stop codons in metazoan mitochondria. J. Mol. Evol. **29**:202-207.

Osawa, S., T.H. Jukes, K. Watanabe, and A. Muto. 1992. Recent evidence for evolution of the genetic code. Micro. Rev. **56**:229-264.

Pääbo, S., W.K. Thomas, K.M. Whitfield, Y. Kumazawa, and A.C. Wilson. 1991. Rearrangements of mitochondrial transfer RNA genes in marsupials. J. Mol. Evol. **33**:426-430.

Palumbi, S.R., A. Martin, S. Romano, W.O. McMillan, L. Stice, and G. Grabowski. 1991. The simple fool's guide to PCR, Version 2. University of Hawaii Zoology Department, Honolulu, Hawaii.

Paul, C.R.C and A.B. Smith. 1984. The early radiation and phylogeny of echinoderms. Biol. Rev. 59:443-481.

Rand, D.M. and R.G. Harrison. 1989. Molecular population genetics of mtDNA size variation in crickets. Genetics. **121**:551-569.

Roberts, J.W., J.W. Grula, J.W. Posakony, R. Hudspeth, E.H. Davidson, and R.J. Britten. 1983. Comparison of sea urchin and human mtDNA: evolutionary rearrangements. Proc. Natl. Acad. Sci. USA. **80**:4614-4618.

Roe, B., D-P. Ma, R. Wilson, J. Wong. 1985. The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J. Biol. Chem. **260**:9759-9774.

Saccone, C. and E. Sbisà. 1994. The evolution of the mitochondrial genome. In: Principles of Medical Biology, Volume 1B: Evolutionary Biology. (E.E Bittar and N. Bittar, eds.), pp. 39-72. JAI Press Ltd., England.

Sanger, F., S. Nicklen, A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. **74**:5463-5467.

Simms, M.J. 1988. The phylogeny of post-Palaeozoic crinoids. In Echinoderm Phylogeny and Evolutionary Biology. (C.R.C. Paul and A.B. Smith, eds.), pp. 269-284. Oxford Univ. Press, Oxford.

Smiley, S. 1988. The phylogenetic relationship of holothurians: a cladistic analysis of the extant echinoderm classes. In: Echinoderm Phylogeny and Evolutionary Biology. (C.R.C. Paul and A.B. Smith eds.), pp. 69-84.

Smith, A. B. 1988. Fossil evidence for the relationships of extant echinoderm classes and their times of divergence. In: Echinoderm Phylogeny and Evolutionary Biology. (C.R.C. Paul and A.B. Smith eds.), pp. 85-97. Oxford Univ. Press, Oxford.

Smith, A.B. 1992. Echinoderm phylogeny: morphology and molecules approach accord. TREE. **7**:224-229.

Smith, M.J., D.K. Banfield, K. Doteval, S. Gorski, and D. Kowbel. 1989. Gene arrangement in sea star mtDNA demonstrates a major inversion event during echinoderm evolution. Gene. **76**:181-185.

Smith, M.J., D.K. Banfield, K. Doteval, S. Gorski, and D.J. Kowbel. 1990. Nucleotide sequence of nine protein-coding genes and 22 tRNAs in the mtDNA of the sea star *Pisaster ochraceus*. J. Mol. Evol. **31**:195-204.

Smith, M.J., A. Arndt, S. Gorski, and E. Fajber. 1993. The phylogeny of echinoderm classes based on mitochondrial gene arrangements. J. Mol. Evol. **36**:545-554.

Sprinkle, J. 1973. Morphology and evolution of blastozoan echinoderms. Special Publication. The Museum of Comparative Zoology, Harvard University, pp 1-284.

Sprinkle, J. 1987. Phylum Echinodermata: Part II. Additional Concepts. In: Fossil Invertebrates. (R.S. Boardman, A.H. Cheetham and A.J Rowell, eds.), pp. 572-580.

Sprinzl, M., T. Hartmann, J. Webwe, J. Blank, and R. Zeidler. 1989. Compilation of tRNA sequences and sequences of tRNA genes. Nuc.Acids Res. **17**:r1-r172.

Sprinzl, M.T., N. Dank, S. Nock, and A. Schon. 1991. Compilation of tRNA sequences and sequences of tRNA genes. Nuc. Acids. Res. **19**:2127-2171.

Strathmann, R. 1988. Larvae, phylogeny and von Baer's law. In: Echinoderm Phylogeny and Evolutionary Biology. (C.R.C. Paul and A.B. Smith eds.), pp. 53-68.

Ueda, T., T. Ohta, and K. Watanabe. 1985. Large scale isolation and some properties of AGY-specific Serine tRNA from bovine heart mitochondria. J. Biochem. **98**:1275-1284.

Vail, L. 1987. Reproduction in five species of crinoids at Lizard Island, Great Barrier Reef. Mar. Biol. **95**:431-446.

Van Sant, J.F., and N.G. Lane. 1964. Crawfordsville (Indiana) crinoid studies. Univ. Kans. Paleontol. Contrib. Artic. **7**:1-136.

Wakita, K. Y. Watanabe, T. Yokogawa, Y. Kumazawa, S. Nakamura, T. Ueda, K. Watanabe, and K. Nishikawa. 1994. Higher-order structure of bovine mitochondrial $tRNA^{PHE}$ lacking the 'conserved' GG and TΨCG sequences as inferred by enzymatic and chemical probing. Nuc. Acids Res. **22**:347-353.

Walberg, M.W. and D.A. Clayton. 1981. Sequence and properties of the human KB cell and mouse L cell D-loop regions of mitochondrial DNA. Nuc. Acids Res. **9**:5411-5421.

Wallis, G.P. 1987. Mitochondrial DNA insertion polymorphism and germ line heteroplasmy in the Triturus cristatins complex. Heredity. **58**:229-238.

Wolstenholme, D.R., J.L. Macfarlane, R. Okimoto, D.O. Clary, and J.A. Wahleithner. 1987. Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. Proc. Natl. Acad. Sci. USA. **84**:1324-1328.

Wolstenholme, D.R. 1992. Animal mitochondrial DNA: structure and evolution. Int. Rev. Cytol. **141**:173-215.

Wong, J.F.H., D.P. Ma., R.K. Wilson, and B.A. Roe. 1983. DNA sequence of the *Xenopus laevis* mitochondrial heavy and light strand replication origins and flanking tRNA genes. Nuc. Acids Res. **11**:4977-4995.

Yokogawa, T., Y. Kumazawa, K. Miura, and K. Watanabe. 1989. Purification and characterization of two Serine isoreceptor tRNAs from bovine mitochondria by using a hybridization assay method. Nuc. Acids Res. **17**:2623-2637.

Zevering, C.E., C. Moritz, A. Heideman, and R.A. Strum. 1991. Parallel origins of duplications and the formation of pseudogenes in mitochondrial DNA from parthenogenetic lizards (*Heteronotia binoei*; Gekkonidae). J. Mol. Evol. **33**:431-441.