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主論文の要約

博士の専攻分野の名称：博士（水産科学）　　Alan Hertz Marin Guerrero

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学位論文題目

Studies on Mitochondrial Genome for Phylogenetic Inference and Species Identification in Pectinidae

（イタヤガイ科貝類の系統推定と種判別に向けた

ミトコンドリアゲノムに関する研究）

Scallops (Bivalvia, Pectinidae) are species of high economic value and comprises 350 extant species within four commonly accepted groups: three subfamilies, Chlamydinae, Palliolinae, Pectininae and one tribe Aequipectini. Scallops display unique genetic characteristics that make them very interesting candidates for evolutionary studies. For instance, all available scallop mitogenomes possess dramatic mitochondrial gene rearrangement as well as the lack of the gene *atp8*. In spite of the abundant fossil record extending from the early Triassic (250 mya: Hautmann 2010), the phylogenetic relationship among members of this family is poorly understood and is still a matter of debate. Thus, in order to obtain further insights into the very complex taxonomy and evolutionary relationships among Pectinidae species, more studies on the mitochondrial genomes of scallop species are needed.

This dissertation has been divided in three chapters. Chapter one explores the characteristics of newly developed scallop mitochondrial genomes from three species: *Argopecten purpuratus*, *Pecten maximus* and *Pecten albicans*. The novel mitogenomes were used to infer the phylogenetic relationships among 9 scallop species with available mitochondrial genome. The complete mitochondrial genome of *A. purpuratus* is 16,266 bp in length. The approximate size of the complete mitogenomes of *P. albicans* and *P. maximus* are 22.6 and 24.8 kb, respectively. Due to the presence of long tandem repeats within the control region of both *Pecten* species, partial mitogenomes containing the complete protein coding regions were obtained in *P. maximus* (17,271 bp) and *P. albicans* (16,680 bp). As a result, the mitochondrial genomes of the three scallop species studied are typical of marine bivalves. They contain 12 protein-coding genes, lack of *atp8*, display a large level of gene order rearrangement, and their complete molecule size vary among species. All Pectinidae mitochondrial genes are encoded in the same strand. By determining the complete protein-coding region of *P. maximus* and *P. albicans*, a novel mitochondrial gene order arrangement from the sub family Pectininae was uncovered. Information obtained from scallop gene order arrangement revealed that their gene order is clearly reflected in their phylogenetic relationship supporting sister subfamilies relationships as well as relationships among members of the same subfamily. Phylogenetic relations were analyzed by using the nucleotide information from all mitochondrial protein coding genes and gene order arrangement. The maximum likelihood phylogentic results based on nucleotide and amino acid information from all 12 protein coding genes, showed clear and consistent evolutionary relationships among the 9 scallop species. The family Pectinidae is

resolved as monophyletic. All congeneric species were clustered in the same monopyletic group with high bootstrap values. The branches containing *Pecten* and *Argopecten* species are closely related and placed in the basal part of the phylogenetic tree. Species from the subfamily Chlamydinae (*Chlamys*, *Mizuhopecten*, and *Mimachlamys*) were placed in branches at the top of the tree, followed by *P. magellanicus* (subfamily Palliolinae). A neighbor joining approach based on the complete mitochondrial 16S gene confirmed the monophyly of Pectinidae, as well as the early divergence of *Argopecten* species (branch placed at the base of the tree) and recent divergence of the subfamily Chlamydinae (branch placed at the top of the tree). Interestingly, a phylogenetic analysis based on the complete 16S gene showed a polyphyletic origin of the genus *Amusium*. *Amusium pleuronectes* is closely related to the *Pecten* group and *A. japonicum* showed a very close phylogenetic relationship with *Annachlamys macassarensis* with high bootsrap value (100 %). My results based on phylogenetic analysis of the complete mitochondrial 16S gene supported the convergence evolution of shells and life habits theory (Mynhardt et al., 2014) between *Amusium pleuronectes* and *Amusium japonicum*. Thus, both *Amusium* species are not congeneric. A reclassification of the *Amusium* genus is suggested.

Chapter two describes the development of a DNA barcoding assay based on the 5' end of the mitochondrial 16S gene for scallop species identification. A partial region at the 5' end of the mitochondrial COI gene, known as the "Folmer region", has been proposed as the most suitable DNA barcoding marker. However, Folmer primers have failed to amplify PCR products in different organisms, including scallops. Searching for an alternative barcoding gene

region, I analyzed the complete mitochondrial 16S rRNA gene in 15 scallop species. My results showed that the interspecific variation at the 5' end is twice as high as that of the 3' end. Based on that evidence, novel Pectinidae family-specific primer set was designed, aiming to amplify a partial region at the 5' end of the 16S rRNA gene, and tested its suitability as barcoding tool. A neighbor-joining analysis identified correctly 100% of the scallop specimens analyzed, with high bootstrap support. The 14 species-specific clades spanned four subfamilies. Within the subfamily Chlamydinae, five species were recovered: *Mizuhopecten yessoensis*; *Chlamys hastata*; *Chlamys farreri*; *Mimachlamys sanguinea*; *Mimachlamys nobilis*. The monospecific genus *Placopecten* was included in the subfamily Palliolinae clade. Within the Pectininae subfamily, six species were recovered: *Pecten maximus*; *Pecten albicans*; *Amusium japonicum*; *Annachlamys macassarensis*; *Decatopecten radula*; *Bractechlamys vexillum*. Finally, from the basal clade belonging to the Aequipectini tribe, two species were recovered: *A. irradians* and *A. purpuratus*. The intraspecific 16S gene nucleotide variation ranged from 0 to 1% (mean 0.19%) and the interspecific variation ranged from from 2.16 to 56.42% (mean 35.4%). Mean K2P interspecific genetic distance was 186-fold higher than the mean intraspecific variation. The clear separation between mean intraspecific and interspecific divergences, and between the maximum intraspecific and minimum interspecific genetic divergence, indicates the presence of the so-called "barcoding gap". The new primers are well suited for DNA barcoding analysis and may contribute to scallop food industry as well as routine taxonomic surveys.

Chapter three was focused on the application of the mitochondrial 16S rRNA gene for the designing of scallop species-specific primers and multiplex PCR assay for scallop species identification. Two different multiplex PCR protocols were designed based in both extremes of the 16S gene. However, the low interspecific variability at the 3' end of this gene has limited its utility and only a few scallop species were assessed. The highly variable 5' end of the 16S gene allowed the development of a novel decaplex PCR assay that enabled a fast and accurate identification of 9 commercially important scallop species in a single PCR reaction: *Mizuhopecten yessoensis*, *Argopecten irradians*, *A. purpuratus*, *Pecten maximus, Chlamys farreri*, *Placopecten magellanicus*, *Mimachlamys nobilis, Bractechlamys vexillum,* and *Annachlamys macassarensis*. The decaplex PCR amplification resulted in one species-specific band for each species, ranging from 113 in *M. yessoensis* to 918 bp in *A. macassarensis*, plus the positive control product that ranged from 600 to 700 bp, depending on species. To enhance the utility of this assay, the PCR product amplified by the family-specific primer set that was utilized as positive control was also used for the identification of unknown (non-target) scallop species by DNA sequencing analysis.

Additionally, an RFLP assay was developed for the discrimination between the congeneric species *P. maximus* and *P. albicans*. In *P. maximus* and *P. albicans*, the primers Pect16BCF and Pect16BCR amplified a fragment of 654 and 655 bp, respectively. After DNA sequence analysis, a unique restriction site was identified only for *P. albicans* in the position 369 bp. Thus, a digestion reaction by *Hae*III yielded two diagnostic bands of 286 and 369 bp in *P. albicans*. The 654-bp

amplicon of *P. maximus* remained uncut, which allowed a clear discrimination between these *Pecten* species by agarose gel visualization. In its present form, this multiplex PCR method can be of great utility for different kinds of studies involving scallop species and for research institutes and governmental agencies that regulate seafood authentication around the world.