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# Molecular Evolution and Phylogeny of Actin Genes in *Haliotis* Species (Mollusca: Gastropoda)

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**Frank Y.T. Sin, Maxine J. Bryant, and Alice Johnstone (2007)** Molecular evolution and phylogeny of actin genes in *Haliotis* species (Mollusca: Gastropoda). *Zoological Studies* **46**(6): 734-745. Gene conversion and positive selection have been implicated as possible mechanisms involved in the evolution of actin genes in some marine organisms. We analyzed the molecular characteristics of 3 fully sequenced complementary (c)DNA and 3 partially sequenced cDNA of the actin gene family of the abalone, *Haliotis iris* Martyn (1784) (Mollusca: Gastropoda), 3 partially sequenced genomic DNAs of *H. virginea* Gmelin (1791), and actin gene sequences of *H. rufescens* Swainson (1822) and *H. discus hannai* Ino (1952) retrieved from GenBank to determine the possible evolutionary mechanism of this gene family in haliotids. There was no evidence to support either gene conversion or positive selection in the evolution of the actin gene family in haliotids. However, codon usage bias was evident in the actin gene family of these 4 haliotids. Phylogenetic analysis of the actin gene family revealed 2 distinct clades with *H. iris* actins A1, A1a, A1b, and A1c, *H. virginea* A1, A2, and A3, and *H. rufescens* actin in 1 clade and *H. discus* actin and *H. iris* actins A2 and A3 in the 2nd clade. In the expression analysis of actin genes, *H. iris* A1, A2, and A3 showed that these genes were expressed in all muscular structures (mantle musculature, foot, and retractor muscle) and non-muscular structures (gills and gonads) of adult *H. iris*, suggesting that they are all cytoplasmic-type actins. The lack of phylogenetic separation of actin genes into cytoplasmic and muscle actins and the absence of muscle-specific expression of actin genes may reflect the primitive taxonomic status of haliotids. The role of actin genes as genetic markers is discussed. http://zoolstud.sinica.edu.tw/Journals/46.6/734.pdf

**Key words:** Actin, Gene family evolution, Nucleotide bias, Codon usage, *Haliotis*.

Gene conversion and positive selection are often proposed as mechanisms for the evolution of biological molecules. Changes in sequence similarity have been used as evidence for gene conversion (Crain et al., 1987). Evidence for the evolution of the actin gene family by gene conversion in the echinoderms, *Strongylocentrotus purpuratus* and *Pisaster ochraceus*, came from amino acid and DNA sequence analyses (Crain et al. 1987, Kowbel and Smith 1989, White and Crother 2000). Comparison of the cytoplasmic and muscle actin genes of the sea star, *P. ochraceus*, revealed that the N-terminal domains of the genes were apparently conserved, but the C-terminal domains showed higher levels of variation (White and Crother 2000). Muscle-specific amino acids were only found in the C-terminal domain of the *P. ochraceus* muscle actin gene (Kowbel and Smith, 1989). In the sea urchin, *S. purpuratus*, Crain et al. (1987) found that sequence similarity was high between codons 61 and 120 in muscle and cytoplasmic actin genes with 7.9% synonymous substitutions, compared to 43.3% in the rest of the gene. Further evidence for gene conversion in the actin genes of *P. ochraceus* and *S. purpuratus* was based on sequence analyses using the GENECONV program (Drouin 2002). The role of gene conversion in the actin genes of haliotids has not been reported.

Positive selection is inferred when the num-

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ber of non-synonymous substitutions is higher than synonymous substitutions in a sequence. In haliotids, the sperm protein, lysine, which is involved in fertilization, of 7 conspecific Californian abalone has a higher number of non-synonymous substitutions than synonymous substitutions, suggesting that positive selection was responsible for the evolution of lysin (Lee and Vacquier 1992). Further studies showed that positive selection was a general mechanism in the evolution of lysin in different species of abalone from Japan, Taiwan, Australia, New Zealand, South Africa, and Europe (Lee et al. 1995).

However, it is not known whether gene conversion and/or positive selection was involved in the evolution of abalone actin genes. Actin is a ubiquitous eukaryotic structural protein involved in the cytoskeleton and muscle (Kabsch and Vandekerckhove 1992). The existence of multiple actin genes in animals is thought to provide a higher copy number to cope with the demands for actin, and to allow specialization of actin's functions (Rubenstein 1990, Herman 1993). However, whether actins can be distinguished into cytoplasmic and muscle actins is uncertain in mollusks. In species of gastropods of *Biomphalaria* and *Helisoma*, cytoplasmic and muscle actins form distinct phylogenetic groups, supporting the orthology of distinct actin groups that may be functionally specialized (Adema 2002). Orthologous relationships have been detected in cephalopod actins (Carlini et al. 2000). However, the absence of muscle-specific amino acids in the actins of the mollusks *Aplysia*, *Patella vulgata*, and *Pecten* sp. led Mounier et al. (1992) to speculate that distinct actin types do not exist in mollusks.

Despite the important functional roles played by actin in *Haliotis* species (Mollusca: Gastropoda) and the fact that actin is a well-characterized protein in other species, little sequence information is available for the actin gene family in haliotids. The molecular characteristics and evolutionary mechanism of the haliotid actin gene family are not known. Previous phylogenetic analyses of haliotid species were based on ribosomal (r)DNA and mitochondrial (mt)DNA sequences (Coleman and Vacquier 2002, An et al. 2005). Those studies grouped haliotid species on the basis of geographical distributions. Thus characterization of the actin genes of haliotids may provide insights into the processes involved in the evolution and the phylogenetic relationships of the actin gene family of haliotids. In this study, we determined (1) whether gene conversion and/or positive selection were possible mechanisms in the evolution of actin genes of 4 geographically separated species of Pacific abalone: *H. virginea* and *H. iris*, endemic to New Zealand, *H. rufescens*, widely distributed along the California coast, and *H. discus hannai*, commonly found in Korean and Japanese waters; (2) the phylogenetic relationships of the actin family in these species; and (3) whether muscle and cytoplasmic actins can be distinguished by expression analysis and phylogenetic methods as shown in other studies.

## MATERIALS AND METHODS

### Actin genes

Isolation of the complete actin genes of *H. iris*, A1, A2, and A3, was described previously (Bryant et al. 2006). The partial gene sequences of *H. iris* A1a, A1b, and A1c were isolated as byproducts during *H. iris* A1 sequence analysis, by polymerase chain reaction (PCR) amplification of genomic DNA using the *H. iris* A1 primer pairs of 5'-agagctgtcttcccctccat-3' (forward) and 5'-caagggcgatgtagcaggc-3'(reverse) (Bryant 2004). Actin genes from *H. virginea* were isolated from sperm DNA by PCR using the primers of 5'-agagctgtcttcccctccat-3' (forward) and 5'-gctctgcta catgccccttg-3' (reverse). The primer sequences used for *H. virginea* were based on the sequence information obtained by cycle sequencing (Bryant, 2004). The GenBank accession numbers for these sequences are given in table 1.

### Nucleotide and codon biases

Eleven actin sequences from 4 species of haliotids were analyzed (Table 1). The base compositions were estimated using MEGA 2.1 (Kumar et al. 2001). The base frequencies for the 1st, 2nd, and 3rd positions and the overall frequency for each codon were determined. Codon bias was determined by the relative synonymous codon usage (RSCU) statistic (Sharp and Li 1988, Nei and Kumar 2000). Methionine and tryptophan were not considered in the codon bias analysis as they are only encoded by a single codon. Stop codons were not analyzed. An RSCU value of 1.0 indicates no bias, with increasing deviation from this expectation indicating codon bias (Sharp and Li 1988). In this study, RSCU values exceeding 1.5 were considered biased, as this reflects 50% greater use than would be expected if all codons

were used equally. Regression analysis was used to examine the relationship between RSCU and the frequencies of amino acids in the actin protein.

### Gene conversion

Gene conversion in the actin gene family of *H. iris* was assessed using GENECONV vers. 1.81 (Sawyer 1999). The coding sequence silent option, which compares polymorphic codon sites, was used for the analysis. The silent option is more conservative for coding sequences, as the silent sites within a codon are expected to be correlated. This option ignores amino acid polymorphisms, which may incorrectly increase significance values. A G-scale of 0 was used, which sets the program to ignore internal mismatches, as there were no mismatches in the sequences. *Haliotis iris* coding sequences were truncated to the same length. The program analyses aligns sequences and detects possible gene conversion events as regions with high alignment scores. As the number of aligned sequences increases, the probability of finding an erroneously significant result by pair-wise comparison increases, therefore a global comparison is preferred as it is more conservative. The significance of the possible gene conversion event is calculated as a permutation *p* value or a Karlin-Altschul (KA) *p* value. Permutation and KA *p* values were corrected for the number of sequences compared and the length of the sequence, and for KA values, a Bonferroni correction was used (BC-KA).

### Positive selection

The number of synonymous substitutions per synonymous site ($d_S$), the number of non-synonymous substitutions per non-synonymous site ($d_N$) and the $d_N/d_S$ ratio ($\omega$) were calculated for the 11 actin gene sequences using the approximate method of Yang and Nielsen (2000), implemented in the yn00 program available in PAML vers. 3.12 (Yang 1997).

### Phylogenetic analysis

Maximum parsimony (MP) trees were constructed using MEGA 2.1 (Kumar et al. 2001). Gaps were handled by pairwise deletions. First, 2nd and 3rd codon position data were used. CNI searching was used. Statistical support for branching was estimated by bootstrapping 500 times. The 11 haliotid sequences were analyzed with actins from *Aplysia californica* as an outgroup.

### Actin gene expression

To determine whether *H. iris* A1, A2, and A3 are expressed in adult tissues, RNA was extracted from gonads, muscular foot, gills, mantle, and retractor muscle of 3 adult females using TriReagent (Molecular Research Center, Progenz Limited, Auckland, New Zealand). Expression was detected by reverse transcription (RT), followed by PCR as described previously (Bryant et al. 2006).

**Table 1.** Actin sequences used for the comparative analyses

| Species | Abbreviation | Nt coding[a] | PCR fragment (nt) | Accession no. | Reference |
|---|---|---|---|---|---|
| *Haliotis. iris* | *H. iris* A1 | 1128 | 1302+ | AY921237 | Bryant et al. 2006 |
| | *H. iris* A1a | 583 | 812 | AY961954 | Bryant 2004 |
| | *H. iris* A1b | 583 | 865 | AY961955 | Bryant 2004 |
| | *H. iris* A1c | 583 | 884 | AY961956 | Bryant 2004 |
| | *H. iris* A2 | 1128 | 2206+ | AY921238 | Bryant et al. 2006 |
| | *H. iris* A3 | 1128 | 1709+ | AY921239 | Bryant et al. 2006 |
| *H. virginea* | *H. virginea* A1 | 583 | 1008 | AY959325 | Bryant 2004 |
| | *H. virginea* A2 | 583 | 798 | AY959326 | Bryant 2004 |
| | *H. virginea* A3 | 583 | 747 | AY959327 | Bryant 2004 |
| *H. rufescens* | - | 497 | na | AF032125 | Gomez-Chiarri et al. 1999 |
| *H. discus hannai* | - | 1131 | na | AY380809 | Ma et al. (unpublished data) |

[a]Sequences aligned with *H. iris* A1 cDNA; na, not available; +, complete gene sequence.

## RESULTS

### Nucleotide bias

The nucleotide frequencies of *Haliotis* actins are given in table 2. Nucleotide frequency for the overall codon favored G+C, but showed no strong bias. The G+C content ranged 51%-55%, with an average of 54%.

At the position there was a G+C bias, with the G+C content ranging 52%-60% and an average of 56%. At this position the G composition was 34%-39%. At the 2nd codon position, there was an A+T bias, with an average G+C content of 40%. At the 3rd codon position, there was a strong G+C bias, with the G+C content reaching 66%. The average G+C content at the 3rd position was 66%, with a C composition of 37%-47%.

*Haliotis iris* actin introns had very similar nucleotide frequencies with an average G+C content of 32% (Table 3).

**Table 2.** Nucleotide frequency of *Haliotis* actins

| Codon Position | Nucleotide | *H. iris* A1 | *H. iris* A1a | *H. iris* A1b | *H. iris* A1c | *H. iris* A2 | *H. iris* A3 |
|---|---|---|---|---|---|---|---|
| Overall | A | 0.23 | 0.23 | 0.23 | 0.23 | 0.24 | 0.23 |
| | T | 0.23 | 0.22 | 0.23 | 0.23 | 0.25 | 0.24 |
| | G | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 |
| | C | 0.30 | 0.31 | 0.30 | 0.30 | 0.27 | 0.29 |
| 1st | A | 0.29 | 0.30 | 0.29 | 0.29 | 0.29 | 0.29 |
| | T | 0.19 | 0.14 | 0.14 | 0.14 | 0.18 | 0.17 |
| | G | 0.34 | 0.34 | 0.35 | 0.35 | 0.34 | 0.35 |
| | C | 0.18 | 0.23 | 0.22 | 0.22 | 0.19 | 0.20 |
| 2nd | A | 0.30 | 0.31 | 0.31 | 0.31 | 0.31 | 0.31 |
| | T | 0.28 | 0.29 | 0.29 | 0.30 | 0.29 | 0.29 |
| | G | 0.16 | 0.16 | 0.16 | 0.16 | 0.15 | 0.15 |
| | C | 0.26 | 0.24 | 0.24 | 0.23 | 0.26 | 0.26 |
| 3rd | A | 0.11 | 0.09 | 0.09 | 0.09 | 0.13 | 0.10 |
| | T | 0.22 | 0.23 | 0.26 | 0.24 | 0.27 | 0.26 |
| | G | 0.21 | 0.23 | 0.22 | 0.22 | 0.23 | 0.23 |
| | C | 0.47 | 0.45 | 0.44 | 0.45 | 0.37 | 0.40 |

| Codon Position | Nucleotide | *H. virginea* A1a | *H. virginea* A1b | *H. virginea* A1c | *H. rufescens* | *H. discus* | *Haliotis* | A+T/G+C |
|---|---|---|---|---|---|---|---|---|
| Overall | A | 0.23 | 0.23 | 0.24 | 0.23 | 0.21 | 0.23 ± 0.01 | 0.46 |
| | T | 0.22 | 0.23 | 0.24 | 0.22 | 0.24 | 0.23 ± 0.01 | |
| | G | 0.25 | 0.25 | 0.24 | 0.25 | 0.26 | 0.24 ± 0.01 | 0.54 |
| | C | 0.30 | 0.30 | 0.29 | 0.30 | 0.29 | 0.30 ± 0.01 | |
| 1st | A | 0.30 | 0.30 | 0.30 | 0.29 | 0.27 | 0.29 ± 0.01 | 0.44 |
| | T | 0.14 | 0.14 | 0.14 | 0.17 | 0.13 | 0.15 ± 0.02 | |
| | G | 0.34 | 0.34 | 0.34 | 0.35 | 0.39 | 0.35 ± 0.02 | 0.56 |
| | C | 0.23 | 0.22 | 0.22 | 0.19 | 0.21 | 0.21 ± 0.02 | |
| 2nd | A | 0.31 | 0.30 | 0.30 | 0.31 | 0.30 | 0.31 ± 0.00 | 0.60 |
| | T | 0.29 | 0.30 | 0.30 | 0.28 | 0.28 | 0.29 ± 0.01 | |
| | G | 0.16 | 0.16 | 0.16 | 0.16 | 0.17 | 0.16 ± 0.01 | 0.40 |
| | C | 0.24 | 0.24 | 0.24 | 0.26 | 0.25 | 0.24 ± 0.01 | |
| 3rd | A | 0.09 | 0.10 | 0.10 | 0.11 | 0.07 | 0.10 ± 0.02 | 0.35 |
| | T | 0.23 | 0.24 | 0.27 | 0.22 | 0.30 | 0.25 ± 0.03 | |
| | G | 0.24 | 0.23 | 0.21 | 0.24 | 0.23 | 0.23 ± 0.01 | 0.66 |
| | C | 0.44 | 0.43 | 0.42 | 0.44 | 0.41 | 0.43 ± 0.03 | |

For each species, the nucleotide frequency for the overall codon and the nucleotide frequencies at each codon position are given. The nucleotide frequency (mean ± standard error of the mean (SE)) for *Haliotis* species are shown. (Random substitutions predict equal nucleotide frequencies, i.e., 0.25.)

### Codon usage bias

In *Haliotis*, RSCU values for all of the amino acids studied ranged between 0.00 and 3.30 (Table 4). The highest biases were found in serine (with an RSCU value of up to 3.13), proline (with an RSCU value of up to 3.23), and arginine (with an RSCU value of up to 3.33). Some amino acids showed a slight codon bias between their alternative codons, and in some cases, the codon bias was consistent across all of the haliotids examined. In the 6-fold degenerate amino acids, leucine, serine, and arginine, only two of the possible 6 codons were favored. The only amino acids in which the favored codon differed among haliotids were valine and alanine.

Regression analysis revealed a significant relationship between amino acids showing a codon bias and the frequency of that amino acid in the actin protein, with *p* values of 0.010, 0.032. 0.016, and 0.045 for *H. iris*, *H. virginea*, *H. discus hannai*, and *H. rufescens*, respectively (Fig. 1).

### Positive selection

Of the 27 possible pairwise comparisons for *Haliotis* actins, none showed a $d_N$ value greater than the $d_S$ value. The $d_N/d_S$ ratios, $\omega$, were all below 0.08 (Appendix 1). Regression analysis showed that the 2 substitution types were positively correlated, i.e., as $d_N$ increased, $d_S$ also increased (*p* value = 0.009) (Fig. 2).

**Table 3.** Nucleotide frequency in *Haliotis iris* actin introns

| Nt | *H. iris* A1 | *H. iris* A1a | *H. iris* A1b | *H. iris* A1c |
|---|---|---|---|---|
| A | 0.30 | 0.31 | 0.34 | 0.35 |
| T | 0.35 | 0.35 | 0.38 | 0.36 |
| G | 0.22 | 0.19 | 0.16 | 0.18 |
| C | 0.13 | 0.16 | 0.12 | 0.12 |

| Nt | *H. iris* A2 | *H. iris* A3 | *H. iris* | A+T/G+C |
|---|---|---|---|---|
| A | 0.34 | 0.32 | 0.33 ± 0.01 | 0.68 |
| T | 0.32 | 0.37 | 0.35 ± 0.01 | |
| G | 0.19 | 0.17 | 0.18 ± 0.01 | 0.32 |
| C | 0.15 | 0.14 | 0.14 ± 0.01 | |

The mean ( ± SE) nucleotide frequency for *H. iris* introns is shown.

### Gene conversion

GENECONV detected 1 gene conversion event between *H. iris* actins A2 and A3 at nt 250-326, corresponding to aa 83-109. This conversion was supported by a significant permutation *p* value (0.0326), but not by a significant BC-KA *p* value (0.21813).

When the gene conversion analysis was repeated using the conservative silent sites option for coding sequences, the analysis detected no gene conversion events.

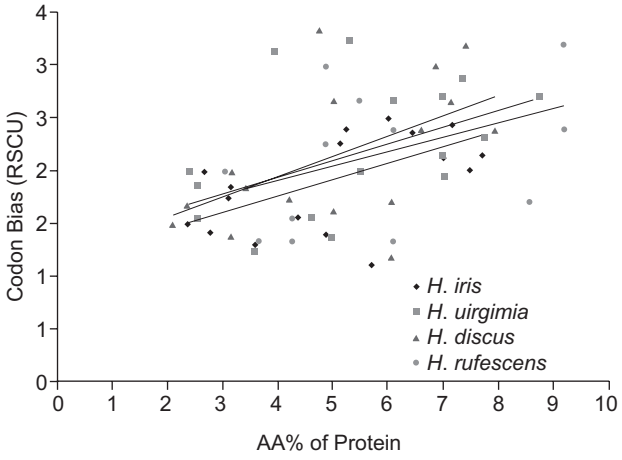


**Fig. 1.** Relationships among amino acids showing a codon bias and the frequency of that amino acid in the actin protein. The occurrence of an amino acid in the actin protein (AA% of protein) is plotted against the codon showing the highest bias for that amino acid.

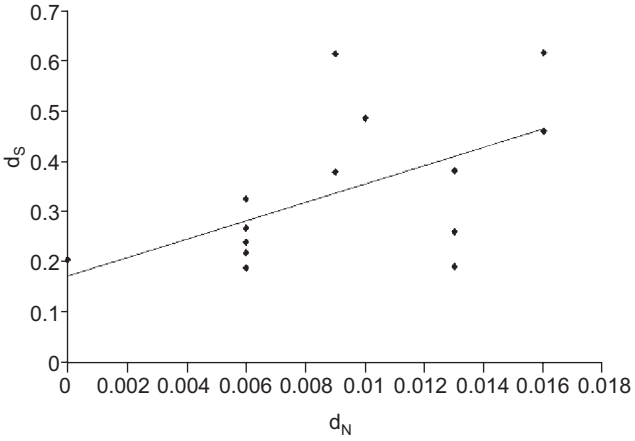


**Fig. 2.** Relationship between non-synonymous substitutions per non-synonymous site ($d_N$) and synonymous substitutions per synonymous site ($d_S$).

**Table 4.** Codon bias in haliotids

| Amino Acid | Codon | *H. iris* | *H. virginea* | *H. rufescens* | *H. discus* |
|---|---|---|---|---|---|
| Phe | UUU | 0.15 | 0.25 | 0.40 | 0.00 |
| | UUC | **1.85** | **1.75** | **1.60** | **2.00** |
| Leu | UUA | 0.14 | 0.00 | 0.00 | 0.00 |
| | UUG | 0.95 | 0.71 | 0.67 | 0.89 |
| | CUU | 1.41 | 1.18 | 0.00 | 0.89 |
| | CUC | **2.14** | **2.71** | **2.67** | **2.67** |
| | CUA | 0.00 | 0.12 | 0.00 | 0.00 |
| | CUG | 0.36 | 1.29 | **2.67** | **1.56** |
| Ile | AUU | 0.51 | 0.29 | 0.33 | 0.33 |
| | AUC | **2.44** | **2.71** | **2.67** | **2.67** |
| | AUA | 0.05 | 0.00 | 0.00 | 0.00 |
| Val | GUU | 1.02 | 0.88 | **1.71** | **1.71** |
| | GUC | **2.36** | **1.95** | 1.43 | 1.43 |
| | GUA | 0.04 | 0.10 | 0.00 | 0.00 |
| | GUG | 0.58 | 1.07 | 0.86 | 0.86 |
| Ser | UCU | **2.27** | **3.13** | **3.00** | **3.00** |
| | UCC | **2.50** | **2.09** | **2.25** | **2.25** |
| | UCA | 0.35 | 0.00 | 0.00 | 0.00 |
| | UCG | 0.12 | 0.00 | 0.00 | 0.00 |
| | AGU | 0.06 | 0.00 | 0.00 | 0.00 |
| | AGC | 0.70 | 0.78 | 0.75 | 0.75 |
| Pro | CCU | 0.41 | 0.13 | 0.44 | 0.44 |
| | CCC | **2.27** | **3.23** | **2.67** | **2.67** |
| | CCA | 1.27 | 0.65 | 0.89 | 0.89 |
| | CCG | 0.05 | 0.00 | 0.00 | 0.00 |
| Thr | ACU | 0.90 | 0.80 | 0.80 | 0.80 |
| | ACC | **2.13** | **2.31** | **2.40** | **2.40** |
| | ACA | 0.67 | 0.71 | 0.40 | 0.40 |
| | ACG | 0.30 | 0.18 | 0.40 | 0.40 |
| Amino acid | Codon | *H. iris* | *H. virginea* | *H. rufescens* | *H. discus* |
| Ala | GCU | 2.02 | 2.15 | 2.40 | 2.40 |
| | GCC | 1.64 | 1.56 | 1.33 | 1.33 |
| | GCA | 0.28 | 0.29 | 0.27 | 0.27 |
| | GCG | 0.06 | 0.00 | 0.00 | 0.00 |
| Tyr | UAU | 0.43 | 0.62 | 0.50 | 0.25 |
| | UAC | **1.57** | 1.38 | **1.50** | **1.75** |
| His | CAU | 0.58 | 0.76 | 0.67 | 0.50 |
| | CAC | 1.42 | 1.24 | 1.33 | **1.50** |
| Gln | CAA | 0.26 | 0.13 | 0.00 | 0.15 |
| | CAG | **1.74** | **1.87** | **2.00** | **1.85** |
| Asn | AAU | 0.00 | 0.00 | 0.00 | 0.00 |
| | AAC | **2.00** | **2.00** | **2.00** | **2.00** |
| Lys | AAA | 0.50 | 0.44 | 0.29 | 0.32 |
| | AAG | **1.50** | **1.56** | **1.71** | **1.68** |
| Asp | GAU | 0.70 | 0.76 | 0.67 | 0.61 |
| | GAC | 1.30 | 1.24 | 1.33 | 1.39 |
| Glu | GAA | 0.60 | 0.43 | 0.44 | 0.37 |
| | GAG | 1.40 | **1.57** | **1.56** | **1.63** |
| Cys | UGU | 0.89 | 0.00 | 1.33 | 1.20 |
| | UGC | 1.11 | **2.00** | 0.67 | 0.80 |
| Arg | CGU | **2.40** | **2.67** | **2.25** | **3.33** |
| | CGC | 0.40 | 0.17 | 0.00 | 0.00 |
| | CGA | 0.47 | 0.17 | 0.75 | 0.00 |
| | CGG | 0.00 | 0.00 | 0.75 | 0.00 |
| | AGA | **1.93** | **2.50** | **2.25** | **1.67** |
| | AGG | 0.80 | 1.00 | 0.00 | 1.00 |
| Gly | GGU | **2.86** | **2.88** | **3.20** | **3.20** |
| | GGC | 0.76 | 0.47 | 0.80 | 0.80 |
| | GGA | 0.25 | 0.65 | 0.00 | 0.00 |

For each species, the bias given is the average for actin sequences available. The relative synonymous codon usage (RSCU) values above 1.5 are shown in bold.

### Phylogenetic relationship

The MP tree, using *Aplysia* as an outgroup, grouped haliotid actins into 2 clades: clade 1 contained *H. iris* A2, *H. iris* A3, and *H. discus hannai*, and clade 2 contained all the other haliotid actins identified in this study (Fig. 3). There was 100% bootstrap support for this division. Within each clade, actins were grouped by species; in clade 1 *H. iris* actin A1 was grouped with *H. virginea* and *H. rufescens*. In clade 2 *H. iris* actin A2 and A3 were grouped with the *H. discus hannai* actin. *Haliotis rufescens* actin was the most divergent lineage in clade 1.

### Expression

All 3 fully sequenced actin genes, *H. iris* A1, A2 and A3, tested were expressed in muscular and non-muscular organs (Fig. 4).

## DISCUSSION

### Nucleotide bias

The nucleotide frequencies were very similar in the 4 *Haliotis* species. The nucleotide frequencies at each codon position showed some degree of bias. The phenomenon of nucleotide bias has been well documented in prokaryotes and eukaryotes and is thought to be caused by differences in the forward and backward mutation rates between nucleotides (Sueoka 1962). In *Drosophila*, the bias is towards G+C, predominantly at the 3rd codon position (Shields et al. 1988). In *Bacillus subtilis*, the bias is towards A+T (Shields and Sharp 1987). The degree of nucleotide bias is often strongest in the 3rd codon position, as observed in *Haliotis* and *Drosophila*, due to the redundancy in the genetic code permitting changes in the 3rd codon site and functional constraints preventing changes in the 1st and 2nd codon sites. If substitutions at a codon position do not affect the amino acid encoded, the substitutions that occur at that site may be influenced by differences in mutation rates, resulting in nucleotide biases.

### Codon bias

Codon usage bias is evident in haliotid actin genes as in other species such as *Escherichia coli* and *Drosophila* (Sharp and Li 1988, Shields et al. 1988). In *E. coli*, codon usage bias increases in genes that are expressed at higher levels (Sharp and Li 1987). This pattern of codon usage is consistent with selection for translational efficiency, as

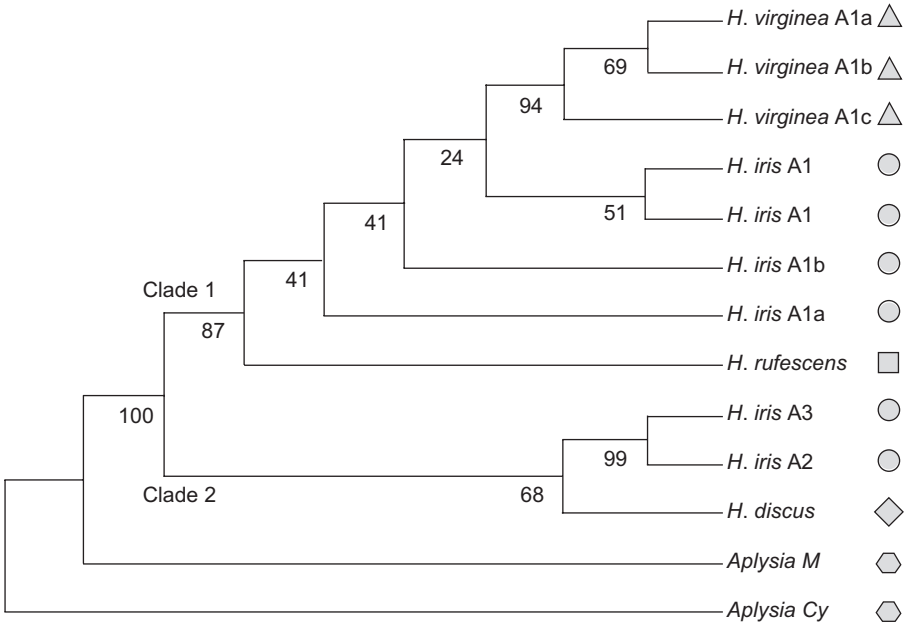


**Fig. 3.** Phylogenetic relationship of haliotid actin genes. The data were bootstrapped 500 times, and the bootstrap values are indicated on each branch.

synonymous substitutions lead to a bias for codons that bind abundant (t)RNA molecules (Ikemura and Ozeki, 1982). In *Drosophila*, a codon bias also appears to be driven by translational efficiency: codon bias is higher in genes which are highly or rapidly expressed and favors codons that bind the most-abundant tRNA molecules (Powell and Moriyama 1997). In unicellular organisms, codons used by highly expressed genes are related to the optimal expression of those genes (Kanaya et al. 1999).

In haliotid actin genes, the degree of codon usage bias for an amino acid is correlated with the frequency of the amino acid within the actin protein. This may reflect selection of codons in relation to tRNA abundance and hence increasing translational efficiency (Ikemura and Ozeki 1982). The frequencies of haliotid tRNA molecules are unknown, so a correlation between the favored codons and the abundances of their tRNAs cannot be established. A possible cause for selection towards increased translational efficiency could be the high demand for actin proteins caused by actin's multitude of structural and functional roles in eukaryotes. In haliotids, the large muscular foot forms the bulk of the animal's mass. As actin is a major muscle protein, it is not surprising that actin gene expression is in demand during development and growth, and for locomotion.

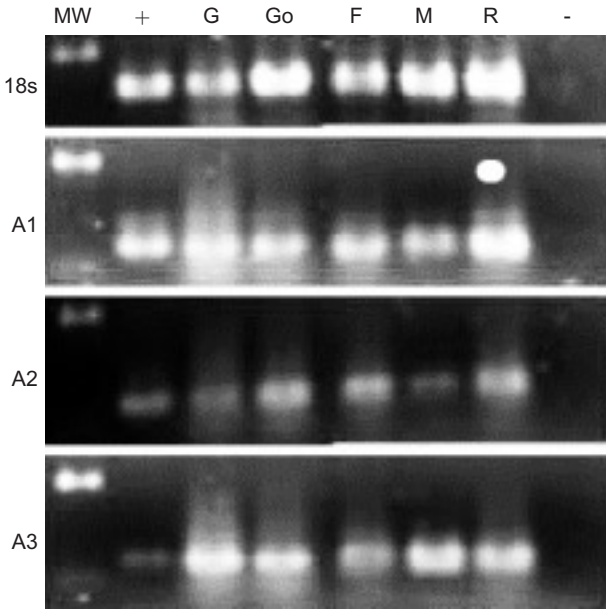


**Fig. 4.** Expression of *Haliotis iris* A1, A2, and A3 actin genes in adult organs detected by RT-PCR. The molecular marker (MW) was lambda DNA digested with *Eco* R1 and *Hind* III. Only the 564-bp fragment is shown in the figure. Abalone DNA was used as a positive (+) PCR control; the adult tissues included gills (G), gonads (Go), foot muscle (F), mantle musculature (M), and retractor muscle (Rm), and water was used as a negative (-) PCR control. All samples were obtained from a single adult female abalone.

## Gene conversion

In the present study, the GENECONV program found evidence of gene conversion when nucleotides were compared, but not when codons were compared. Codon analysis is more conservative, as nucleotide similarity may be the result of a mutational bias; therefore the use of a codon analysis is more valid. The codon analysis suggested that gene conversion has not been involved in the evolution of the *Haliotis* actin gene family.

The high sequence similarity between nt 250-325 of *H. iris* A2 and *H. iris* A3 detected by GENECONV may be the result of functional constraints, nucleotide biases, or a shared evolutionary history, rather than homogenization by gene conversion. He and Haymer (1995) attributed the high sequence similarity in *Drosophila* actin genes to nucleotide and codon biases rather than gene conversions.

## Positive selection

Comparison of the non-synonymous and synonymous substitution rates showed that there is no evidence for positive selection in the *H. iris* actin gene family. Synonymous substitutions are positively correlated with non-synonymous substitutions in *Haliotis*. Similar correlations have also been found in *Drosophila*, bacteria, and mammals (Sharp and Li 1987, Wolfe et al. 1989, Akashi 1994). In *Drosophila*, the degree of codon bias increased as amino acids diverged, suggesting that an increase in synonymous substitutions may act as a mechanism to increase translational efficiency (Akashi 1994).

Positive selection has been detected in sperm lysin of abalone, the sperm protein which facilitates species-specific fertilization (Lee and Vacquier 1992). Lee and Vacquier (1992) compared the amino acid sequences of lysin of 7 conspecific Californian abalone. They found that in 20 of 21 pairwise comparisons possible for the 7 species, the number of non-synonymous substitutions exceeded the number of synonymous substitutions, suggesting the presence of positive selection. Later studies confirmed that positive selection was a general phenomenon in abalone lysin, and non-synonymous substitutions exceeded syn-

onymous substitutions in 20 lysin sequences in abalone from Japan, Taiwan, Australia, New Zealand, South Africa, and Europe (Lee et al. 1995).

Positive selection of lysin is thought to be driven by the evolution of the vitelline envelope receptor for lysin (VERL). The VERL contains repetitive protein motifs which are homogenized by concerted evolution, but the VERL protein as a whole has evolved by neutral drift. Neutral drift in the VERL could drive adaptive sweeps in lysin to maintain the fertilization potential (Swanson and Vacquier 1998, Swanson et al. 2001).

Nielson and Yang (1998) used a maximum likelihood analysis to test for the presence of positive selection of amino acids within HIV-I envelope proteins, rather than testing for positive selection for the entire protein as performed in the present study. That approach may be more revealing, as positive selection may drive the evolution of selected functionally important amino acids within a protein. Yang et al. (2000) showed that when using the maximum likelihood method, selection pressure varied at different sites in lysin.

**Phylogenetic relationship of haliotid actin genes**

The groupings produced by haliotid actin genes identified 2 sets of potentially orthologous genes; the 1st set contained *H. iris* A2, *H. iris* A3, and *H. discus hannai* actin genes, and the 2nd set contained the remaining *H. iris* actin genes, the *H. virginea* actin genes, and the *H. rufescens* actin gene. Within the *H. iris*/ *H. virginea*/ *H. rufescens* actin gene clade, the relationships among actin genes were poorly resolved. However, this grouping within the clade suggests that paralogy may exist between *H. iris* A1 and *H. iris* A1c and within the *H. virginea* actin genes, although only the *H. virginea* paralogies were consistently formed and well supported by bootstrapping.

He and Haymer (1995) used distance methods to show orthology between dipteran actin genes. Carlini et al. (2000) used MP methods to show orthology between cephalopod actin genes. In the present study, putative orthologous actin genes were consistently found within haliotids. The inferred phylogenetic relationships among haliotid actins reflect the evolutionary history of actin genes rather than the evolutionary history of haliotids.

The reliability of the haliotid orthologies should be supported by other evidence, such as similar functions of actin genes within orthologous groups, or conserved sequences, as observed in dipterans. In dipterans, orthologous actin genes display similar expression patterns and share intron patterns (Fyrberg et al. 1983, Fyrberg 1984). The ability of introns to support orthologous genes in haliotids is limited by the lack of intron variability in mollusks. However, the expression patterns of the *H. iris* A1, A2, and A3 actin genes were similar in different embryonic stages (Bryant et al. 2006) and in adult muscular and non-muscular tissues.

Phylogenetic analyses of haliotids using rRNA sequences (Coleman and Vacquier 2002), mtDNA, and lysin gene sequences (Lee and Vacquier 1992, Lee et al. 1995, Metz et al. 1998) showed that haliotid species were divided into groups on the basis of geographic distribution. Of the 18 haliotids representing the North Pacific, Caribbean, Europe, South Africa, Australia, and Taiwan with the exception of a New Zealand species, 3 distinct subclades were identified: North Pacific/Caribbean, European, and Australian subclades. However, *H. iris* was excluded from all of these groupings. Of the haliotid species used in the present study, *H. iris* and *H. virginea* would group in the New Zealand clade and *H. rufescens* and *H. discus hannai* would group within the Californian/North Pacific clade. These clades were not evident in the actin phylogenies. Thus phylogenetic relationships derived from actin genes support the conclusion that haliotids contain orthologous actin genes: an ancestral *H. iris* A2/ *H. iris* A3/ *H. discus hannai* actin gene and an ancestral *H. iris* A1-type/ *H. virginea*/ *H. rufescens* actin gene must have been present in the haliotid common ancestor.

The existence of orthologous actin genes in haliotids indicates that the use of actin genes for phylogenetic analysis of haliotids and other mollusks is problematic unless this orthology is taken into account. Using the full sequence of actin genes from species of interest is required to allow identification of orthologues. This would then allow comparisons of homologous genes for valid phylogenetic analyses.

Previous molecular characterizations of haliotid actin genes examined in this study identified an important feature of actin genes, the variable intron lengths (Bryant 2004, Bryant et al. 2006), which can be used as markers for species identification, particularly when external morphological characteristics are similar in different species (An et al. 2005). Intron length variations (Bryant et al. 2006) can be used to identify interspecific hybrids in natural populations in addition to mitochondrial

and rRNA markers (Klinbunga et al. 2003, An et al. 2005). However, as discussed above, the existence of actin gene orthology requires that the introns of homologous genes be analyzed to form valid conclusions.

### Mollusk cytoplasmic and muscle type actins

Cytoplasmic and muscle actin genes in some mollusks, such as *Patella vulgate*, are distinguished by their expression patterns in different tissues. Ubiquitous expression of actin is considered an indicator of a cytoplasmic-type actin (DesGroseillers et al. 1990 1994). The ubiquitous expression of the actin genes, *H. iris* A1, A2, and A3, in muscular and non-muscular structures suggests that these are likely to be cytoplasmic actins. This study suggests that haliotid actins have not differentiated into functionally specialized cytoplasmic and muscle types and that the 2 actin clades might not represent distinct functional groups, as in insects (Mounier et al. 1992, He and Haymer 1995).

In evolutionary more-advanced invertebrates, such as echinoderms and insects, muscle and cytoplasmic actins exhibit temporal and spatial expressions (Cox et al. 1986, Fyrberg 1984, He and Haymer 1995). However, functional analysis is required to confirm the functional nature of actin genes in *Haliotis*. The lack of a clear distinction between cytoplasmic and muscle actins in haliotids may reflect the primitive status of haliotids in the evolutionary tree. It is tempting to speculate that cytoplasmic actin is the primitive form from which muscle actin has evolved. Phylogenetic analysis of arthropod actins demonstrated that the muscle actin clade was rooted within arthropod cytoplasmic actins (Mounier et al. 1992). A more-detailed study of actin genes and actin expression patterns in a range of mollusks would help elucidate and confirm whether a mollusk muscle-type actin is present in more-highly evolved mollusks, and in which group(s) a mollusk muscle actin appears.

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**Appendix I:** Synonymous substitutions per synonymous site ($d_S$) and non-synonymous substitutions per non-synonymous site ($d_N$) statistics for haliotid actins

| Species compared | $d_N$ | $d_S$ | $\omega = d_N/d_S$ |
|---|---|---|---|
| *H. iris* A1 x *H. iris* A1a | 0.000 | 0.202 | 0.000 |
| *H. iris* A1 x *H. iris* A1b | 0.006 | 0.218 | 0.028 |
| *H. iris* A1 x *H. iris* A1c | 0.006 | 0.218 | 0.028 |
| *H. iris* A1 x *H. iris* A2 | 0.009 | 0.613 | 0.015 |
| *H. iris* A1 x *H. iris* A3 | 0.006 | 0.325 | 0.019 |
| *H. iris* A1 x *H. virginea* A1a | 0.006 | 0.271 | 0.023 |
| *H. iris* A1 x *H. virginea* A1b | 0.000 | 0.409 | 0.000 |
| *H. iris* A1 x *H. virginea* A1c | 0.003 | 0.288 | 0.011 |
| *H. iris* A1 x *H. discus* | 0.006 | 0.334 | 0.018 |
| *H. iris* A1 x *H. rufescens* | 0.009 | 0.274 | 0.034 |
| *H. iris* A2 x *H. iris* A1a | 0.010 | 0.486 | 0.020 |
| *H. iris* A1 x *H. iris* A1b | 0.016 | 0.460 | 0.035 |
| *H. iris* A1 x *H. iris* A1c | 0.016 | 0.615 | 0.026 |
| *H. iris* A2 x *H. iris* A3 | 0.009 | 0.377 | 0.025 |
| *H. iris* A1 x *H. virginea* A1a | 0.016 | 0.581 | 0.027 |
| *H. iris* A1 x *H. virginea* A1b | 0.010 | 0.627 | 0.015 |
| *H. iris* A1 x *H. virginea* A1c | 0.013 | 0.604 | 0.021 |
| *H. iris* A2 x *H. discus* | 0.013 | 0.452 | 0.028 |
| *H. iris* A2 x *H. rufescens* | 0.016 | 0.420 | 0.038 |
| *H. iris* A3 x *H. iris* A1a | 0.006 | 0.265 | 0.023 |
| *H. iris* A1 x *H. iris* A1b | 0.013 | 0.260 | 0.048 |
| *H. iris* A1 x *H. iris* A1c | 0.013 | 0.381 | 0.033 |
| *H. iris* A1 x *H. virginea* A1a | 0.123 | 0.427 | 0.029 |
| *H. iris* A1 x *H. virginea* A1b | 0.006 | 0.438 | 0.014 |
| *H. iris* A1 x *H. virginea* A1c | 0.009 | 0.419 | 0.022 |
| *H. iris* A3 x *H. discus* | 0.003 | 0.194 | 0.016 |
| *H. iris* A3 x *H. rufescens* | 0.123 | 0.301 | 0.041 |
| *H. iris* A1a x *H. iris* A1b | 0.006 | 0.187 | 0.033 |
| *H. iris* A1a x *H. iris* A1c | 0.006 | 0.237 | 0.026 |
| *H. iris* A1a x *H. virginea* A1a | 0.006 | 0.258 | 0.024 |
| *H. iris* A1a x *H. virginea* A1b | 0.000 | 0.380 | 0.000 |
| *H. iris* A1a x *H. virginea* A1c | 0.003 | 0.324 | 0.010 |
| *H. iris* A1a x *H. discus* | 0.006 | 0.253 | 0.024 |
| *H. iris* A1a x *H. rufescens* | 0.009 | 0.160 | 0.059 |
| *H. iris* A1b x *H. iris* A1c | 0.013 | 0.188 | 0.067 |
| *H. iris* A1a x *H. virginea* A1a | 0.013 | 0.285 | 0.044 |
| *H. iris* A1a x *H. virginea* A1b | 0.006 | 0.315 | 0.020 |
| *H. iris* A1a x *H. virginea* A1c | 0.009 | 0.230 | 0.041 |
| *H. iris* A1a x *H. discus* | 0.012 | 0.265 | 0.047 |
| *H. iris* A1a x *H. rufescens* | 0.016 | 0.205 | 0.077 |
| *H. iris* A1c x *H. virginea* A1a | 0.013 | 0.324 | 0.039 |
| *H. iris* A1a x *H. virginea* A1b | 0.006 | 0.396 | 0.016 |
| *H. iris* A1a x *H. virginea* A1c | 0.009 | 0.283 | 0.033 |
| *H. iris* A1a x *H. discus* | 0.012 | 0.390 | 0.032 |
| *H. iris* A1a x *H. rufescens* | 0.016 | 0.275 | 0.057 |
| *H. virginea* A1a x *H. virginea* A1b | 0.006 | 0.157 | 0.040 |
| *H. virginea* A1a x *H. virginea* A1c | 0.009 | 0.202 | 0.046 |
| *H. virginea* A1a x *H. discus* | 0.012 | 0.393 | 0.031 |
| *H. virginea* A1a x *H. rufescens* | 0.016 | 0.305 | 0.051 |
| *H. virginea* A1b x *H. virginea* A1c | 0.003 | 0.232 | 0.014 |
| *H. virginea* A1a x *H. discus* | 0.006 | 0.450 | 0.014 |
| *H. virginea* A1a x *H. rufescens* | 0.009 | 0.417 | 0.023 |
| *H. virginea* A1c x *H. discus* | 0.009 | 0.428 | 0.022 |
| *H. virginea* A1a x *H. rufescens* | 0.009 | 0.359 | 0.026 |
| *H. discus* x *H. rufescens* | 0.016 | 0.271 | 0.057 |