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Intraspecific Genetic Diversity in the Marine Shrimp *Penaeus vannamei:* Multiple Polymorphic Elongation Factor-1α Loci Revealed by Intron Sequencing

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Abstract: Intron sequences from the *elongation factor-1*α (*EF1*α) gene from the marine shrimp *Penaeus vannamei* reveal extensive variation even among inbred populations of hatchery-raised shrimp. Among 44 individuals analyzed, we found 13 alleles varying by up to 7.5% sequence differences, and including several allele-diagnostic insertions and deletions. High heterozygosity contrasts with low genetic variation at allozyme loci, but we observed up to four alleles per individual, suggesting that we have identified two separate, polymorphic loci. We partitioned the observed alleles into two groups representing hypothetical duplicated loci. However, the alleles are so similar to one another that a phylogenetic analysis does not cluster them into monophyletic groupings. A possible explanation is that concerted evolution is acting to homogenize genetic variation among these two putative loci.

Key words: EPIC-PCR, nuclear introns, shrimp, concerted evolution.

INTRODUCTION

The development of "universal" polymerase chain reaction (PCR) primers able to amplify a variety of mitochondrial genes from a broad array of taxa has resulted in an explosion of DNA sequence data (Kocher et al., 1989; Palumbi, 1996). One criticism of mitochondrial DNA studies is that the genes are linked and act as a single locus. Sequences of nuclear genes could serve as additional nonlinked genetic markers for population genetic studies and as markers for pedigree analysis. These nuclear markers would differ from mitochondrial markers in their rates of evolution and biparental mode of inheritance. The ideal nuclear marker for intraspecific studies would show relatively high levels of neutral variation. One approach to finding such markers has been to target introns in highly conserved nuclear genes (Bradley and Hillis, 1997; Lessa, 1992). An added benefit of such introns is that "universal" primers can be designed that will anneal to regions in the highly conserved exons flanking noncoding introns. Palumbi and Baker (1994) termed this strategy EPIC-PCR, for exon-primed, intron-crossing PCR.

We have been using EPIC-PCR to study nuclear intron variation in hatchery-raised populations of the marine shrimp *Penaeus vannamei.* Our study began with a survey of several different nuclear loci (see primers in Palumbi, 1996), and we found that intron sequences from *elongation factor-*

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1α (*EF1α*) were highly variable between species. The *EF1α* gene codes for a protein that is involved in the translation of messenger RNA protein, and the amino acid sequence is highly conserved among taxa from plants to animals (Palumbi, 1996). The number of introns in this gene varies among taxa (e.g., Hovemann et al., 1988; Walldorf and Hovemann, 1990), with some taxa apparently lacking introns (e.g., heliothine moths; Cho et al., 1995). The sequence of the coding region of the gene has been used in systematic studies of higher taxa (e.g., Kojima et al., 1993; Cho et al., 1995; Kobayashi et al., 1996; McHugh, 1997; Kojima, 1998).

In this report we will show that *EF1α* introns can be highly variable within populations, and so of use in intraspecific analyses of genetic variation. We will highlight potential problems, and solutions, involved in sequencing nuclear introns, as well as the data required to understand the basis of the variation.

Materials and Methods

Hatchery-raised *Penaeus vannamei* came from a Hawaiian shrimp farm (Aquatic Farms, Ltd.), which was seeded from stock originating off the Pacific Coast of Central America. Wild *P. vannamei* were collected from the Gulf of California, and coastal waters of Panama, Peru, and two locations off Ecuador. Whole *P. vannamei* postlarvae, or pleopods only, were preserved and stored in 95% ethanol. DNA extraction from pleopod muscle followed protocol 1 from Hillis et al. (1996) with the following modifications: pleopods were cut open and briefly rinsed in STE buffer prior to mincing; digestion (200–250 μl volume) and organic extractions were performed in PLG I tubes (5 Prime, 3 Prime, Inc.) for 2 hours to overnight; the initial phenol-only extraction was replaced by a phenol:chloroform:isoamyl alcohol (25:24:1) extraction.

Initial PCR amplifications used universal *EF1α* primers EF0 and EF2 (Palumbi, 1996). Subsequently, intron-flanking primers were designed to anneal upstream (EF3s, 5′-GACAAGGCCCTCCGTCTTCC-3′) and downstream (EF4s, 5′-GGGCACTGTTCCAATACCTC-3′) of the single intron found within the EF0–EF2 fragment. Amplifications with intron-flanking primers used the Perkin Elmer GeneAmp XL PCR kit with the following conditions: 50-μl reactions containing XL reaction buffer II (tricine, potassium acetate, glycerol, dimethyl sulfoxide), 1.5 mM $Mg(OAc)_2$, 160 μM of each dNTP, 1 unit of r*Tth* DNA polymerase, XL, and 0.2 μM of each primer. Reactions were run on a PE 9600 thermal cycler (or a PE 480 with appropriate modifications to cycling times) with an initial denaturation of 60 seconds at 94°C, followed by 30 cycles of 15 seconds at 94°C, 15 seconds at 55°C, 15 seconds at 72°C, and finally a 3-minute extension at 72°C. PCR products were visualized on an agarose gel and purified using the QIAgen PCR Purification kit.

Purified PCR products were ligated into an appropriate vector, either pBluescript (Stratagene), pCR2.1 (Invitrogen), or 5′3′ vector (5 Prime, 3 Prime, Inc.), and subsequently transformed with either XL-1 Blue cells (Stratagene), One-Shot INVF′ cells (Invitrogen), or 5′3′ vector (5 Prime, 3 Prime, Inc.), following manufacturer's instructions. White or light-blue colonies were picked and subsequently screened by PCR using vector primers flanking the insert region and the following conditions: 25-μl reactions containing PE PCR reaction buffer II (10 mM Tris-HCl, pH 8.3, 500 mM KCl, 2.5 mM $MgCl_2$), 160 μM of each dNTP, 0.55 units of PE Ampli *Taq* DNA polymerase, and 0.4 μM of each primer. Reactions were run on an MJ Research PTC-100 with an initial denaturation of 60 seconds at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C, and finally a 3-minute extension at 72°C. The entire reaction was loaded and run on a 1.5% SeaKem GTG agarose (FMC) gel in TAE buffer, and products containing inserts of an appropriate size were excised. These products were eluted from the gel using Supelco GenElute columns (Sigma Chemical Co.).

Inserts were cycle sequenced using Ampli *Taq,* FS (Perkin Elmer ABI), following manufacturer's protocols. Initial sequencing employed vector primers and intron-flanking primers in both forward and reverse orientations; once we were confident of the system, we settled on using the M13rev primer (5′-GAATTCAACAGCTATGACCATG-3′). Sequence reactions were run on an ABI fluorescent autosequencer (model 310 or model 377) and edited using Sequence Navigator (PE ABI). Sequence alignments and comparisons were done with SeqApp version 1.9a169 (D.G. Gilbert, 1992; available via anonymous ftp from ftp.bio.indiana.edu/molbio/seqapp). Phylogenetic analyses were performed using a test version of PAUP* version 4.0.0d64 (D.L. Swofford, 1998).

Intron sequences were aligned and clustered into related groups. Allele names were assigned to sequences that shared phylogenetically informative sites. In all but one case these sequences were derived from multiple individuals, i.e., alleles shared among different shrimp; the remaining case involved allele D3, which was found multiple times (inde-

pendent PCR reactions) in a single individual (AFP5-13). Technically, the substitutions defining these alleles would not be considered as phylogenetically informative because they are found in only a single individual; however, they appeared in independent PCR reactions of the same individual, so may be considered rare alleles. Gaps (insertions/deletions, or INDELs) were included as a character used to define alleles.

Results

Sequencing of PCR fragments generated with *EF1α* primers EF0 and EF2 revealed a single intron in this region in *Penaeus vannamei*. The intron was identified by alignment to other coding sequences in GenBank and by characteristic intron sequence motifs at the 5′ (GT) and 3′ (AG) ends. New primers, designed to flank the intron, amplified a fragment of approximately 260 bp. This fragment includes 27 bp of coding sequence at the 5′ end and 36 bp at the 3′ end (including primers), and an intron ranging in length from 191 to 200 bp. Electrophoretic patterns on agarose gel typically revealed a closely spaced pair of bands. We interpreted the fainter, upper band as an artifact of heteroduplex formation, and not a second gene locus. We cloned PCR products and cycle-sequenced 1 to 15 positive (insert-bearing) clones per PCR reaction.

Sequencing revealed a great deal of intraspecific variation. Thirteen identified alleles were discovered among 44 hatchery-raised individuals. These differed by substitutions at 24 nucleotide positions, as well as five different multibase INDELs (gaps in sequence alignment) (Figure 1). Only one site (site 25) showed multiple substitutions. Multibase INDELs at sites 24 to 30 (A), 37 to 38 (D4) and 189 to 190 (D1) were found in single alleles, whereas INDELs at 98 to 102 (alleles F, G, H, and I) were shared among alleles. Note that all the INDELs appear in a region displaying some form of repeat motif: allele A deletes one AAATGT repeat; D1 adds a CT repeat; D4 loses a GA repeat; and alleles F, G, H, and I share a deletion of an imperfect 5-bp repeat. Substitutions among alleles were divided into 13 transitions and 11 transversions, and one site with both. The average genetic distance (Kimura 2-parameter) among alleles is 2.9%, with a maximum of 7.5% (Table 1). Note that alleles A and B1 show a distance of 0 between them. The allele sequences are identical except for two INDELs, including the 5-bp deletion characteristic of the A allele.

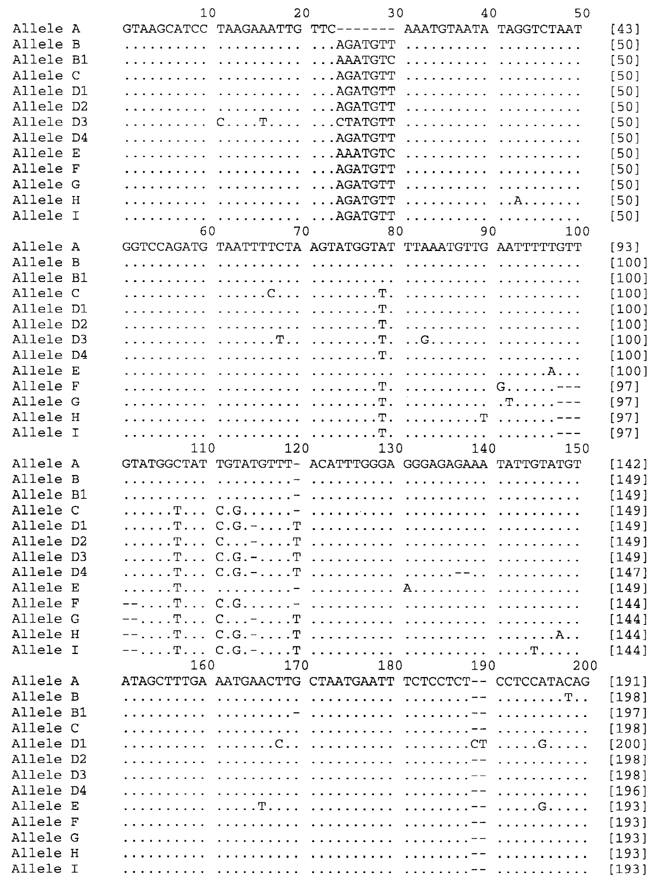

Figure 1. Alignment of *Penaeus vannamei EF1α* intron allele sequences.

Within an allele class, a number of sequences that were otherwise identical showed unique substitutions at various sites. This may represent polymerase error early in a PCR reaction, or rare alleles. In some cases, variants within allele classes were observed among multiple clones from a single individual, but not shared between individuals. For example, in the sequences of allele F derived from nine clones from two independent PCR reactions of individual AFP5-13, two substitutions are observed, as well as 15 ambiguous bases. These unique variants were interpreted as resulting from polymerase error and thus not considered to represent alternative alleles.

We also surveyed *EF1α* intron variation in 12 *Penaeus vannamei* individuals collected from four locations along the Central and South American coast. Among the 21 clones analyzed from these shrimp, 17 unique sequences were identified (Figure 2); none were identical to the alleles found in the Hawaiian hatchery-raised shrimp, although one differed by only four INDELs from allele D4. The range of variation among the sequences was the same as in the

Table 1. Kimura 2-Parameter Distances between *EF1*α Intron Alleles*

	A	B	B1	C	D1	D2	D3	D4	E	F	G	H
Allele A												
Allele B	0.0053											
Allele B1	0.0000	0.0155										
Allele C	0.0267	0.0310	0.0365									
Allele D1	0.0324	0.0365	0.0422	0.0155								
Allele D2	0.0160	0.0206	0.0261	0.0102	0.0153							
Allele D3	0.0435	0.0582	0.0585	0.0365	0.0417	0.0362						
Allele D4	0.0216	0.0261	0.0317	0.0051	0.0103	0.0051	0.0313					
Allele E	0.0267	0.0418	0.0259	0.0524	0.0472	0.0419	0.0748	0.0480				
Allele F	0.0331	0.0373	0.0431	0.0158	0.0105	0.0158	0.0430	0.0106	0.0482			
Allele G	0.0220	0.0265	0.0322	0.0158	0.0210	0.0052	0.0426	0.0106	0.0485	0.0211		
Allele H	0.0388	0.0429	0.0489	0.0211	0.0264	0.0210	0.0482	0.0159	0.0653	0.0265	0.0264	
Allele I	0.0275	0.0319	0.0376	0.0105	0.0157	0.0104	0.0372	0.0053	0.0540	0.0158	0.0157	0.0210

*Note that alleles A and B1 are identical except for a 7-bp INDEL at positions 24–30 and a single INDEL at position 170.

previously identified alleles, and maximum genetic distance (Kimura 2-parameter) between intron sequences from South American and Hawaiian hatchery-raised shrimp was 8.0%. Only one sequence was shared by individuals collected from multiple locations: sequence Peru-1a was also found in a shrimp collected off coastal Ecuador.

Polymerase Errors

To estimate polymerase error rate, we compared the sequences of a cloned PCR product to those generated by amplifying and recloning this insert. After sequencing and identifying a particular cloned insert, that clone was used as a template in a new amplification of the *EF1*α intron using PCR primers EF3s and EF4s. Two PCR experiments were conducted, one using *Taq* polymerase (USB) and the other using r*Tth*/Vent polymerase (PE GeneAmp XL), which includes proofreading capability. The resultant PCR products were cloned, and two complete copies were sequenced from each PCR experiment. Because the template in the initial PCR was a single clone, variation in the resultant sequences can be attributed to polymerase error. Among four clones derived from a reamplified insert, we observed two substitutions, one from each polymerase, accounting for a low error rate of 0.19%. This error rate is too low to be solely responsible for the high levels of variation observed in the intron, and thus we believe polymerase error is not generating the observed alleles. However, the error rate per clone (50%) is high enough to suggest that single unique substitutions within allele sequences (singletons *sensu* Villablanca et al., 1998) may often be due to polymerase errors during genomic amplifications.

More Than Two Alleles

We initially analyzed 34 shrimp to determine their intron genotypes. We sequenced up to 10 clones per individual and assigned sequences to nominal alleles (Figure 3). All shrimp but one (AFP4-1) with more than two clones sequenced showed heterozygous genotypes. However, many individuals had more than two alleles (e.g., AFP4–5 and AFP5–8). Most individuals for which we sequenced more than five clones showed three or more *EF1*α alleles (Figure 3). Because in vitro recombination is known to generate artifactual sequences during PCR amplification (Pääbo et al., 1990), we repeated the initial intron amplification in several individuals and conducted new ligation, cloning, screening, and sequencing steps to generate replicate sets of allelic sequences. In all cases the alleles identified in the second round were the same as those found initially. This result rules out random recombination generating allele sequences, and suggests the multiple alleles found within these individuals are present in the genomes of these shrimp.

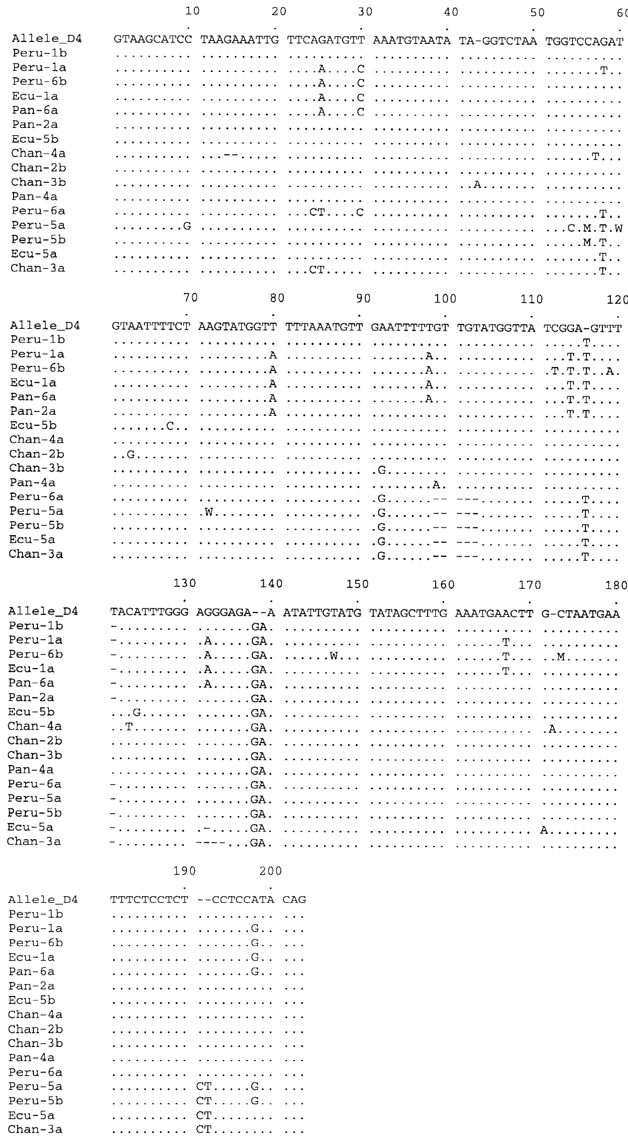

Figure 2. Alignment of *Penaeus vannamei EF1α* intron sequences derived from 21 clones from individuals collected off coastal Panama (Pan-), Ecuador (Ecu-, Chan-), and Peru. Allele D4 is presented for comparison with identified alleles.

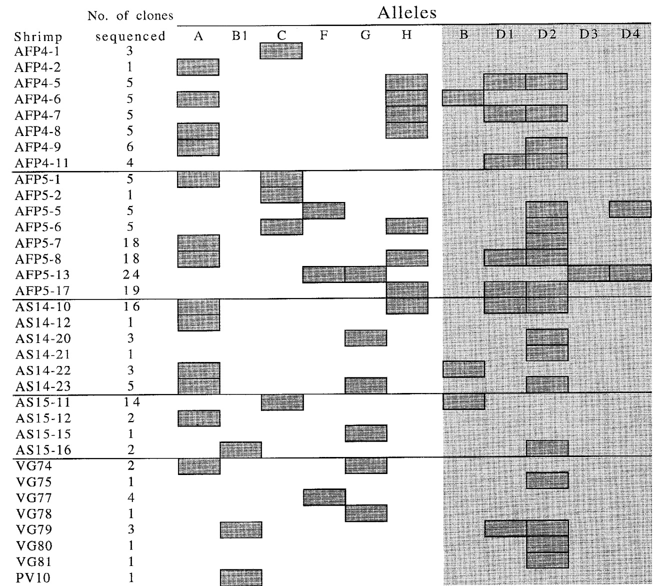

Figure 3. Distribution of *EF1α* intron genotypes in assayed *Penaeus vannamei* individuals. AFP4 and AFP5 prefixes indicate shrimp from two 1996 hatchery ponds; AS14 and AS15 are from two 1995 hatchery ponds. The VG- and PV-labeled shrimp were caught wild in the Gulf of California. Countershading of alleles B and D1–D4 highlights hypothesized second locus (see text).

Discussion

Results from intron amplification and sequencing show a large amount of nuclear genetic heterozygosity in both aquaculture and wild populations of *Penaeus vannamei*. This is in contrast to results from shrimp allozyme studies, in which observed heterozygosity is 5% or less (Mulley and Latter, 1980; Benzie et al., 1992), lower than seen in crustaceans and invertebrates in general (Hedgecock et al., 1982; Solé-Cava and Thorpe, 1991). Allozyme surveys of *P. vannamei* also show low heterozygosity ($\overline{H}$ = 0.017), with only 3 of 26 assayed loci shown to be polymorphic (5% level) across the range of this species (Sunden and Davis, 1991). Heterozygosity in aquaculture populations is even lower (Sunden and Davis, 1991; Garcia et al., 1994).

Our assumption at the start of the project was that the *EF1α* primers would amplify an intron from a single gene locus and that *Penaeus vannamei* has 2N copies of each gene (i.e., diploid system). Under these assumptions we should recover a maximum of two alleles from a heterozygous individual. However, extensive sequencing of multiple cloned PCR products from individuals often revealed more than two allelic sequences. Experiments show that these alleles are too different to have been the result of polymerase errors. Random crossing-over of incomplete PCR extension products in a heterogeneous template mixture can lead to novel recombination sequences (Pääbo et al., 1990; Bradley and Hillis, 1997). However, replicate PCR amplifications gave identical results, suggesting in vitro recombination of alleles during PCR cannot explain the large number of alleles. In addition, from the initial 34 shrimp, a total of only 11 alleles were found among 129 clones, suggesting that if random recombination is occurring, it is relatively

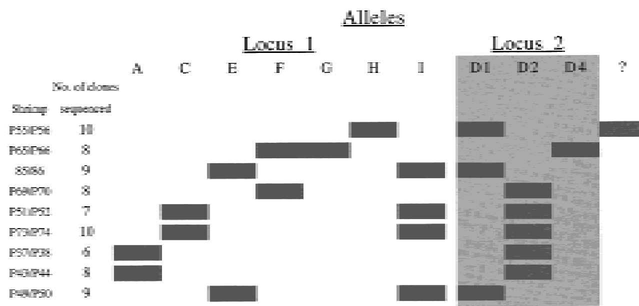

Figure 4. Distribution of *EF1*α intron genotypes from *Penaeus vannamei* individuals from 1997 hatchery pond. The unique intron sequence found in individual P55/P56 was found in only a single PCR reaction and thus has not yet been assigned allele status.

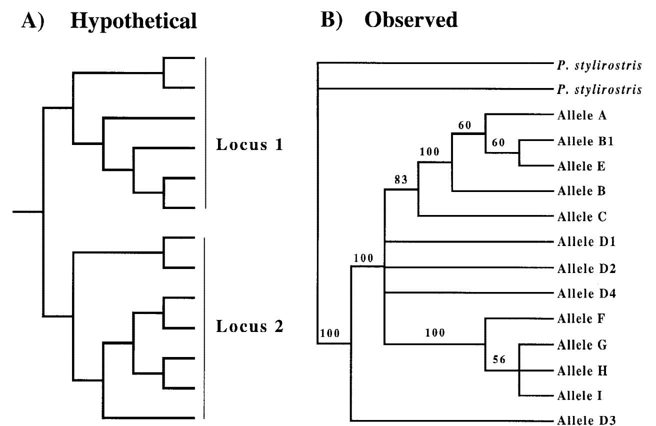

Figure 5. A: Hypothetical phylogenetic tree showing reciprocal monophyletic clades expected if two loci have diverged at some distant time in the past. **B:** Observed 50% majority-rule consensus of 90 equally parsimonious trees of *EF1*α intron alleles from hatchery-raised *Penaeus vannamei* and *P. stylirostris* outgroups. To compute the maximum parsimony trees, gaps were coded as "newstate" and a branch-and-bound algorithm was used. Numbers on internal branches indicate the proportion of the 90 most-parsimonious trees in which the clade was supported.

rare. The *EF1*α intron primers were designed to target a short amplification fragment and thus require short extension time. This, in addition to the stable polymerase mixture used, makes it unlikely that fragment extension is not completed in each cycle, and therefore less likely for partial amplification products to act as templates in succeeding cycles. Another possibility for the greater-than-expected allelic variation is polyploidy, which has been reported in decapods (Lécher et al., 1995). However, previous studies of chromosome structure in *P. vannamei* revealed a diploid genome (Chow et al., 1990).

An alternative explanation is that there are multiple *EF1*α loci in *P. vannamei* and that these multiple loci are amplified by primers EF3s and EF4s. Among taxa for which data are available, *EF1*α is apparently a single-copy gene in the moths (Cho et al., 1995), and was likewise reported so for vertebrates such as chickens, humans, and rabbits, although more recently multiple loci have been found in humans (e.g., Lund et al., 1996). *Drosophila* (Hovemann et al., 1988) and honey bees (Danforth and Ji, 1998) have two divergent copies of *EF1*α with different intron arrangements, while frogs have three copies (Dje et al., 1990) and *Artemia* may have as many as four copies and four introns (Lenstra et al., 1986). Amplification of two paralagous loci could account for up to four alleles in doubly heterozygous individuals, with a minimum of two alleles in homozygotes.

We have examined allele frequencies among shrimp for patterns that would be consistent with a two-locus gene structure. The division of alleles presented in Figure 3 is in agreement with a diploid two-locus system, and thus we proposed the hypothesis that in *P. vannamei,* sequences A, B1, C, F, G, and H are alleles at locus *EF1*α-1, and B, D1, D2, D3, and D4 are alleles at locus *EF1*α-2. After formulating this hypothesis, we amplified and sequenced *EF1*α introns from 10 additional *P. vannamei* obtained from a new hatchery stock. The observed genotypes were consistent with the hypothesized locus structure (Figure 4). Note that novel alleles (E and I) were discovered in this new hatchery stock. We had previously noted a pattern of novel alleles appearing in independent hatchery stocks. For example, alleles D3, D4, and H were first seen in shrimp from the 1996 brood stock; alleles B1 and F1, which were present in shrimp from the 1995 brood stock, were not found among individuals from 1996. The "novel" alleles H and I were not rare when found and suggest a small number of parents in the crosses establishing the stocks.

We found no evidence of heterogeneity in coding sequence downstream of the intron among several individuals, and sequence variation between species is also small at silent sites in coding regions (N. Tachino and S.R. Palumbi, unpublished results), suggesting low divergence between these putative loci. If the two *EF1*α loci arose from a duplication at some time in the past, then one would expect to see reciprocally monophyletic clades of intron alleles representing each locus. A phylogenetic analysis of the intron sequences did not segregate the alleles into two distinct clades (Figure 5). In addition, *P. vannamei* intron alleles are more closely related to each other (7.6% max. difference)

than any are to introns from its sister species *P. stylirostris* (14.2%–22.3%) or the more distantly related *P. monodon* (T.F. Duda and S.R. Palumbi, manuscript in preparation). This suggests that if a gene duplication has occurred, it has probably occurred since the divergence of these species. An alternative is that concerted evolution, driven by unequal recombination between these similar loci or some other process of gene conversion, is homogenizing genetic variation among these two loci, resulting in allelic variants that do not necessarily cluster phylogenetically. Further evidence is needed on the nature of alleleic variation at these two loci, through pedigree studies or genomic cloning, and on whether or not other penaeid shrimp have similar loci.

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