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Research paper

# Enrichment of short sequence repeats and development of molecular markers for fisheries and conservation management of the southern brown shrimp *Farfantepenaeus subtilis*

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## Abstract

*Enrichment of short sequence repeats and development of molecular markers for fisheries and conservation management of the southern brown shrimp* Farfantepenaeus subtilis. The southern brown shrimp *Farfantepenaeus subtilis* is a commercially important species along the Atlantic coast of Central and South America. This study presents the first panel of microsatellite markers developed through a double-enrichment hybridization procedure. Twenty loci were isolated and characterized from samples collected in shallow coastal waters off Ceará (Brazil). No linkage disequilibrium was detected. Alleles per locus ranged from 3 to 19. Mean observed and expected heterozygosity were 0.655 and 0.790, respectively. Thirteen loci conformed to Hardy-Weinberg equilibrium. Polymorphic information content ranged from 0.478 to 0.919. These microsatellites are useful for exploring fine-scale genetic structure and connectivity among populations.

**Key words**: Microsatellites, Simple sequence repeats (SSR), PCR-enrichment, Sustainable fisheries, Conservation

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## Introduction

The penaeid shrimp is the most valuable fishery export of tropical coasts internationally, serving as an important source of protein-rich food, and family income for fishermen and aquaculture farmers' worldwide (D'Incao 1995, Santos et al 2020). Among these, the southern brown shrimp *Farfantepenaeus subtilis* Pérez-Farfante, 1967 (Decapoda, Crustacea) is a key commercially exploited species along the Atlantic coast of Central and South America, from the Caribbean Sea (Cuba and the Lesser Antilles) to the coast of Cabo Frio, Rio de Janeiro, Brazil (D'Incao 1995).

Understanding the species' population structure, and distribution area, is paramount for effective fisheries management efforts and the conservation of fish stocks. The assessment of genetic diversity and spatial connectivity across the species' natural geographic range remains insufficient, despite its crucial role in the persistence of local populations (Valles-Jimenez et al 2004, Santos et al 2020).

The study of the genetic variability of a species, even if it exhibits complex life cycles and broad geographic distributions, as seen in shrimp, can be performed using molecular markers. These molecular tools may provide critical insights for the understanding of genetic diversity, gene flow, and population connectivity among fish stocks (Jose et al 2024).

Microsatellites are short DNA sequences tandemly repeated over the entire genome, and their high polymorphism has proven to be a powerful molecular tool for studying the genetic variability within and among populations of many aquatic species (Mangabeira-Silva et al 2024).

To the best of our knowledge, no microsatellite markers have been developed for *F. subtilis*, except for two compound dinucleotides (AY049201 and AY049202; Maggioni and Rogers 2002), which fall far short of sufficiency for any comprehensive genetic study. Therefore, we describe in this paper the first panel of microsatellite markers developed from the *F. subtilis* genome using a new double-enrichment hybridization procedure. These

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markers are intended for future use in conservation genetics and population structure studies of this important fishery resource.

## Material and methods

### Sample collection and DNA isolation

Specimens (n = 25) were collected in mid-April, from artisanal fisheries operating in shallow coastal waters off Ceará, Brazil. According to the fishers, all individuals originated from the same fishing ground routinely exploited by these small-scale fishing operations. Upon landing at the Fortaleza (Ceará) fish market, the shrimp were sampled and transported to the laboratory. Although exact haul coordinates are not provided in local artisanal fisheries, the collection area corresponds to the coastal sector of Ceará within FAO Major Fishing Area 31 (Western Central Atlantic). Morphometric data (body size) were recorded; however, sex could not be reliably determined due to the processing state of the market specimens. All individuals measured were above the minimum legal capture size for *Farfantepenaeus subtilis* (90 mm total length), in accordance with Brazilian fisheries regulations (Portaria SAP/MAPA nº 656/2022). Genomic DNA was isolated individually from each of the 25 *Farfantepenaeus subtilis* specimens using small portions of tail muscle tissue and the Wizard® Genomic DNA Purification Kit (Promega), with minor modifications. High molecular weight DNA was quantified by gel-electrophoresis staining comparison on a 1% agarose gel stained with Ethidium Bromide.

### Microsatellite isolation

Genomic DNA (approx. 150 ng/µL) was digested separately with HaeIII and HincII in the presence of bovine serum albumin (BSA). Genomic DNA digests (300-1000 bp size range) were dephosphorylated at their 5' ends with calf intestinal alkaline phosphatase (CIP) at 37 °C for 2 hours in a thermocycler. Then, they were ligated to double-stranded SNX LINKERS (SNXF 5'–CTAAGGCCTTGCTAGCAGAAGC–3' AND SNXR 5'–PGCTTCTGCTAGCAAGGCCTTAGAAAA–3') with $T_4$ DNA ligase (New England BioLabs) in the presence of XmnI. Linker-ligated inserts were amplified by a polymerase chain reaction (PCR) with SNXF [95 °C for 5 min, 20 × (95 °C for 45 s, 62 °C for 1 min, and 72 °C for 2 min), and 72 °C for 30 min] in a Veriti® 96-well Thermalcycler (Applied Biosystems Inc.). Purified amplicons were used for hybridization with a biotinylated tetranucleotide probe $(GACA)_4$ at 55 °C and complexed with streptavidin-coated magnetic beads (Dynal Biotech). After hybridization, beads were washed with decreasing concentrations of standard saline citrate (SSC), in the presence of 0.5 ng/µL SNXF primer. After the final wash, 30 mL low TE (10 mm Tris, 0.1 mm EDTA, pH 8.0) was added to the beads, and the mixture was incubated at 98 °C for 15 min. The supernatant containing the heat-released single-stranded DNA was transferred to a clean tube. Enriched-DNA recovered from the beads was amplified with SNXF linker [95 °C for 5min, 10 × (95 °C for 45 s, 62 °C for 1 min, and 72 °C for 2 min), 1 × 72 °C for 30 min].

### Transformation of recombinant plasmids and sequencing of positives

Amplified-enriched DNA was cleaned with Qiaquick PCR columns (QIAGEN) and ligated into the pDrive cloning vector (QIAGEN PCR cloning kit), which was used to transform INVITROGEN OneShot®TOP10 chemically competent *E. coli*. Transformed bacteria were initially grown for 1.5 h on sterile liquid media, on a shaking incubator at 37 °C. After the initial growth phase, 100 mL of the culture was spread on culture plates containing 50 µg/mL ampicillin, and incubated overnight at 37 °C. Plasmid inserts were amplified directly from bacterial colonies with M13F and M13R and size fractionated on agarose gels (1.5%). Plasmid DNA was prepared using a Qiaprep spin miniprep kit (QIAGEN) and cycle-sequenced [96 °C for 3 min, 40 × (96 °C for 20 s, 52 °C for 20 s and 60 °C for 4 min] with a universal primer (M13F/M13R or SP6/T7) using the CEQ DTCS - Quick Start kit and sequenced on a CEQ 8000XL DNA Analysis System (BECKMAN COULTER).

### Primer design and optimization

Repeat regions were initially identified using the web-based software WebSat (https://bioinfo.inf.ufg.br/websat/). Primers for microsatellite loci were designed on the unique flanking regions of each microsatellite locus using PRIMER 3 (https://primer3.ut.ee/).

Microsatellites were amplified using the following PCR conditions: (I) 1 × 95 °C for 10 min, 30 × (1 min at 94 °C, 1 min at Ta °C, 1 min at 72 °C) and 5 min at 72 °C. To optimize the PCR reaction for some primers, an alternate touchdown PCR was used based on the following profile (II): 95 °C for 10 min, 10 × (94 °C for 1 min, 65 °C for 30 s with annealing Ta 1 °C per cycle and 72 °C for 30 s), and 25 × (94 °C for 1 min, 55 °C for 45 s, 72 °C for 45 s) and finally 72 °C for 1 min.

Amplifications were carried out in 10-µL reaction volume containing 20-50 ng DNA, 1.5-2.5 mM $MgCl_2$, 50 µM each dNTP, 0.5-1.0 U HotStar Taq polimerase (QIAGEN), 0.3-0.5 µM of primer, and 1 × PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl) in a Veriti® 96-well Thermalcycler. Amplified PCR products and size markers were screened by silver nitrate detection on denatured 6% polyacrylamide gels. Allele sizes were initially determined against a 10-bp DNA ladder (INVITROGEN) and by comparison with the expected size of the cloned fragment and then scored manually.

### Data analysis

Numbers of alleles, observed ($H_O$) and expected heterozygosity ($H_E$), and the test for departure from Hardy-Weinberg expectations were performed using GENEPOP (Raymond and Rousset 1995). Allelic richness, defined as the number of alleles per locus, and linkage disequilibrium between loci were determined with the FSTAT v.2.3 software (Goudet 2009). Polymorphic information content (PIC) and observed fixation coefficient ($F_{IS}$), were calculated with CERVUS 3.0 (Kalinowski et al 2007). MICROCHECKER v2.2.3 (1,000 randomizations; van

Oosterhout et al 2004) was employed to detect the presence of null alleles, large allele dropout, and scoring errors. Bonferroni-corrected *p*-values were applied to evaluate significance ($P < 0.05$). Pairwise relatedness was estimated from the microsatellite data using the Queller and Goodnight (1989) and Wang (2002) estimators implemented in the related package in R. Allele frequencies were calculated from the dataset, and relatedness values were evaluated against null distributions generated from 10,000 permutations. All analyses were performed in R (version 4.3.2; R Core Team 2023) using the default settings of the coancestry function. Linkage disequilibrium (LD) among loci was evaluated using exact tests with 10,000 permutations across all 190 pairwise comparisons. A Bonferroni-corrected threshold ($\alpha = 0.000263$) was applied to account for multiple testing. No locus pair remained significant after correction, indicating that all loci segregated independently and were suitable for subsequent analyses.

## Results and discussion

Across the relatedness estimators tested, pairwise relatedness values were consistently low and centered around zero, indicating no evidence of close kinship among sampled individuals. This pattern suggests that the dataset represents a largely unrelated population, with no pairs showing relatedness values consistent with full-siblings, half-siblings, or parent-offspring relationships.

All microsatellite loci were polymorphic in *Farfantepenaeus subtilis* across the entire data set. Details of the twenty polymorphic microsatellite loci for the southern brown shrimp, including their corresponding GenBank accession numbers (MH469833-MH469850, MH549205, and MH549206), are presented in table 1.

According to the results, a total of 176 alleles were mapped to 20 loci. The fragment sizes ranged from 92 (*Fsu*19) to 269 bp (*Fsu*11), with an *allele richness* varying from 3 (*Fsu*7 and *Fsu*10) to 19 (*Fsu*16), and an average of 8.8 ± 5.3 alleles/locus (fig. 1). No evidence of linkage disequilibrium (LD) was observed among the microsatellite loci. All 190 pairwise comparisons were non-significant after applying the Bonferroni-corrected threshold ($\alpha = 0.000263$), and all *P*-values remained well above this criterion. The absence of significant LD indicates that the loci segregate independently, supporting their suitability for population-genetic inference and confirming that the dataset is free from non-random allelic associations that could bias downstream analyses.

Microchecker analyses indicated evidence of null alleles at six of the 20 microsatellite loci (*Fsu*2, *Fsu*4, *Fsu*5, *Fsu*6, *Fsu*10, and *Fsu*20), with no sign of stuttering or large-allele dropout. The presence of null alleles is widely documented in marine invertebrates, and crustaceans in particular often exhibit comparatively high null-allele frequencies (e.g., Boyle et al 2013, Nakajima et al 2018). The highest estimated null-allele frequency in our dataset (0.1711) was moderate and remained below the commonly applied exclusion thresholds in population genetic studies (0.20-0.25). Although this value warrants some caution, it falls within an acceptable range for microsatellite analyses, as frequencies below 0.20 are generally considered unlikely to strongly bias estimates of population differentiation (Dakin and Avise 2004).

Observed heterozygosity ($H_O$) ranged from 0.050 (*Fsu*6 and *Fsu*10) to 1.000 (*Fsu*16 and *Fsu*19), while expected heterozygosity ($H_E$) ranged from 0.523 (*Fsu*15) to 0.947 (*Fsu*16) (table 2). The average $H_O$ and $H_E$ values were 0.655 ± 0.303 and 0.790 ± 0.122, respectively, indicating a global heterozygote deficit. Deviations from Hardy-Weinberg equilibrium (HWE) were detected at loci *Fsu*2, *Fsu*5, *Fsu*6, *Fsu*13, *Fsu*14, *Fsu*19, and *Fsu*20. Among these, *Fsu*13, *Fsu*14, and *Fsu*19 did not show evidence of null alleles, suggesting that all observed HWE deviations could be attributed to the presence of a small sample size, or the Wahlund effect (Waples 2015). The latter may have resulted from sampling different subpopulations along the Brazilian coast, which could exhibit some degree of reproductive isolation and genetic divergence. Although artisanal fishers reported that all specimens originated from a single fishing ground, slight mixing among adjacent micro-habitats cannot be entirely ruled out. Such subtle mixing may contribute to the heterozygote deficits and HWE deviations observed at some loci, consistent with a mild Wahlund effect in penaeid shrimp (Wagner et al 2017). Both the Wahlund effect and null alleles increase $F_{IS}$, leading to apparent heterozygote deficits (De Meeûs 2018). A global $F_{IS}$ value of 0.175 also suggested a heterozygosity deficit in the *F. subtilis* samples over all loci.

It is also important to note that microsatellite null alleles are widespread among marine invertebrates and that the impact of null alleles can be more pronounced in smaller sample sizes or populations with low genetic diversity, as the relative effect of each allele is more significant (Chapuis and Estoup 2007). Moreover, heterozygosity deficiency is not unique to this genus, as similar findings have been reported in multiple shrimp species, including *F. notialis* (Atencia-Galindo et al 2021), *F. paulensis* (Maggioni and Rogers 2002), and *F. brasiliensis* (Francisco et al 2009).

The PIC values for these *F. subtilis* microsatellites ranged from 0.919 (*Fsu*16) to 0.478 (*Fsu*15). In this study, 90% (18 loci) of the loci exhibited high information content (PIC > 0.5), while the remaining two loci had moderate information content (0.5 < PIC < 0.25). The polymorphism found in these *loci* is consistent with the levels observed in populations of related coastal shrimp species (Valles-Jimenez et al 2004, Francisco et al 2009).

## Conclusions

The new microsatellite markers, first panel isolated and characterized for *F. subtilis*, will be used to reveal fine-scale genetic structure and genetic connectivity amongst populations along the Western Atlantic Ocean coast. As local populations may be facing various threats, continued monitoring and research are crucial to ensure the long-term sustainability of these shrimp populations. This genotyping information generated by larger sample sizes is anticipated to serve as an efficient tool for guiding management decisions aimed at the future protection of

**Table 1.** Primer sequences, repeat motifs, PCR conditions, annealing temperature (Ta), allele size (bp) and GenBank accession numbers for the microsatellite loci isolated from *Farfantepenaeus subtilis*. ‡ PCR conditions described on the Material and Methods section; (I): 1 × 95 °C for 10 min, 30 × (1 min at 94 °C, 1 min at Ta °C, 1 min at 72 °C) and 5 min at 72 °C; (II): 95 °C for 10 min, 10 × (94 °C for 1 min, 65 °C for 30 s with Ta decreasing 1 °C per cycle and 72 °C for 30 s), and 25 × (94 °C for 1 min, 55 °C for 45 s, 72 °C for 45 s) and 72 °C for 1 min.

| SSR loci | Primer sequence (5'-3') | Repeat motif | PCR Profile‡ | $MgCl_2$ (nM) | Primer (µM) | Ta (°C) | Allele size | GenBank accession |
|---|---|---|---|---|---|---|---|---|
| *Fsu*1 | TCGATTCAGAACAGGAGGCA<br>ACGTAAATACATATGACAGACAGAC | $(TGTC)_{18}$ | I | 1.5 | 0.3 | 51 | 182 | MH469833 |
| *Fsu*2 | AGAGAGTGAGAGAGAGCGAGAGA<br>CTCCCTTTGCTTGTTTCTGTTT | $(GACA)_{12}$ | I | 1.5 | 0.3 | 52 | 143 | MH469834 |
| *Fsu*3 | TGCCTGTCTGTTTATCTTTGTCTG<br>CGAATGGCGGAAGACGTA | $(GTCT)_{13}$ | I | 1.5 | 0.3 | 53 | 200 | MH469835 |
| *Fsu*4 | GTGCCAAGACGCTGAAATA<br>CAGAAGGAGACAGACAAACAGAG | $(CT)_8(GTCT)_3CT(GTCT)_2(CTGT)_2(TGTC)_6$ | II | 2.0 | 0.3 | 51 | 190 | MH469836 |
| *Fsu*5 | TCCTCCTTCTAGCTTTGGGT<br>GAACAGACAGACAGAGACAGACA | $(TGTC)_{11}$ | I | 1.25 | 0.5 | 52 | 126 | MH469837 |
| *Fsu*6 | CCAACAGGTTAAGGTGAGAGAGA<br>GTGAGAGAGAGCGAGAGACAGA | $(GTCT)_{12}$ | I | 1.5 | 0.5 | 52 | 195 | MH469838 |
| *Fsu*7 | TCGCAGTTACTAAGAGAGAGAGG<br>CTAGCTCTGTCTCCGTCTCTG | $(AGAC)_{10}AGG(CAGA)_6$ | II | 2.0 | 0.3 | 52 | 150 | MH469839 |
| *Fsu*8 | TTTACCTTGCTACTGGAGGGG<br>GCTTGTAGGGCACAGTCTTTCT | $(GGCA)_4N(AGAC)_8GGA(CAGA)_4$ | II | 2.0 | 0.3 | 53 | 144 | MH469840 |
| *Fsu*9 | ATATGGATGGATGGCAGATCAC<br>TCTGTCTCTTTCTCTGTCTCTCTCTG | $(ACAG)_{13}$ | I | 1.5 | 0.3 | 53 | 193 | MH469841 |
| *Fsu*10 | ACTGCATGAAAACTGACGAAGA<br>CGGGACCTAAAAGTAGGAGCAT | $(GA)_7C(AGAC)_6N_{10}(ACAG)_7$ | II | 2.0 | 0.5 | 53 | 221 | MH469842 |
| *Fsu*11 | TTCTAGTTTGTTCGCTGTTCCA<br>TGTGTGTGTATTTGTCTGTCGC | $(CA)_{10}N_6(TA)_5N_7(TAGA)_9N_{14}(GACA)_6$ | II | 2.0 | 0.3 | 51 | 269 | MH469843 |
| *Fsu*12 | CTAGCTAGCAGAATCACGTGA<br>TATCAGCGTCTTAGTCTTCCGC | $(AG)_6N_4(ACAG)_{10}N_4(ACAG)_6$ | II | 2.0 | 0.3 | 51 | 168 | MH469844 |
| *Fsu*13 | TGCACTCATTCACTCACACG<br>TCTCTAATGGAAGAGTGAGTTTGTGT | $(AC)_{21}G(CA)_{40}N_8(AC)_6$ | II | 2.0 | 0.3 | 52 | 278 | MH469845 |
| *Fsu*14 | TGGTTCACGCACGCACTC<br>AAAGGGTGACAGACAGACAGCT | $(AC)_{31}$ | I | 1.5 | 0.3 | 53 | 125 | MH469846 |
| *Fsu*15 | ACACACATACACTCACGCACAG<br>TGTCTTTCGCTCCTTTCGTTAT | $(AC)_{14}AT(AC)_{12}$ | I | 1.5 | 0.3 | 53 | 124 | MH469847 |
| *Fsu*16 | AACTAACGAGAACAGACTTTGCG<br>TTCTTTGTGTGTGTATGTGTGTC | $(AG)_{15}$ | I | 1.5 | 0.3 | 53 | 175 | MH469848 |
| *Fsu*17 | CACATAGATATAGGAATGCGTG<br>GCGATTGTCATGTTCTGTTTC | $(GA)_{26}$ | I | 1.5 | 0.3 | 50 | 180 | MH469849 |
| *Fsu*18 | CTAGCAGAATCACGTAGTCCTTTA<br>CTATCTGTCTGTCTCTCTTACTCCG | $(AG)_{19}$ | I | 1.5 | 0.3 | 50 | 193 | MH469850 |
| *Fsu*19 | GACGGGGAGAAATTAAGGAAAA<br>TGTCTGTCTGTCTGTCTCTGTCTG | $(GA)_7N_{10}(GA)_{12}AG(GA)_{13}$ | II | 2.0 | 0.3 | 53 | 136 | MH549205 |
| *Fsu*20 | AGTCCAGACACAGCTACCCG<br>CTAGCACGAAATCCCTCAACTC | $(AC)_{10}G(CA)_{19}T(AC)_{11}$ | II | 2.0 | 0.3 | 53 | 137 | MH549206 |

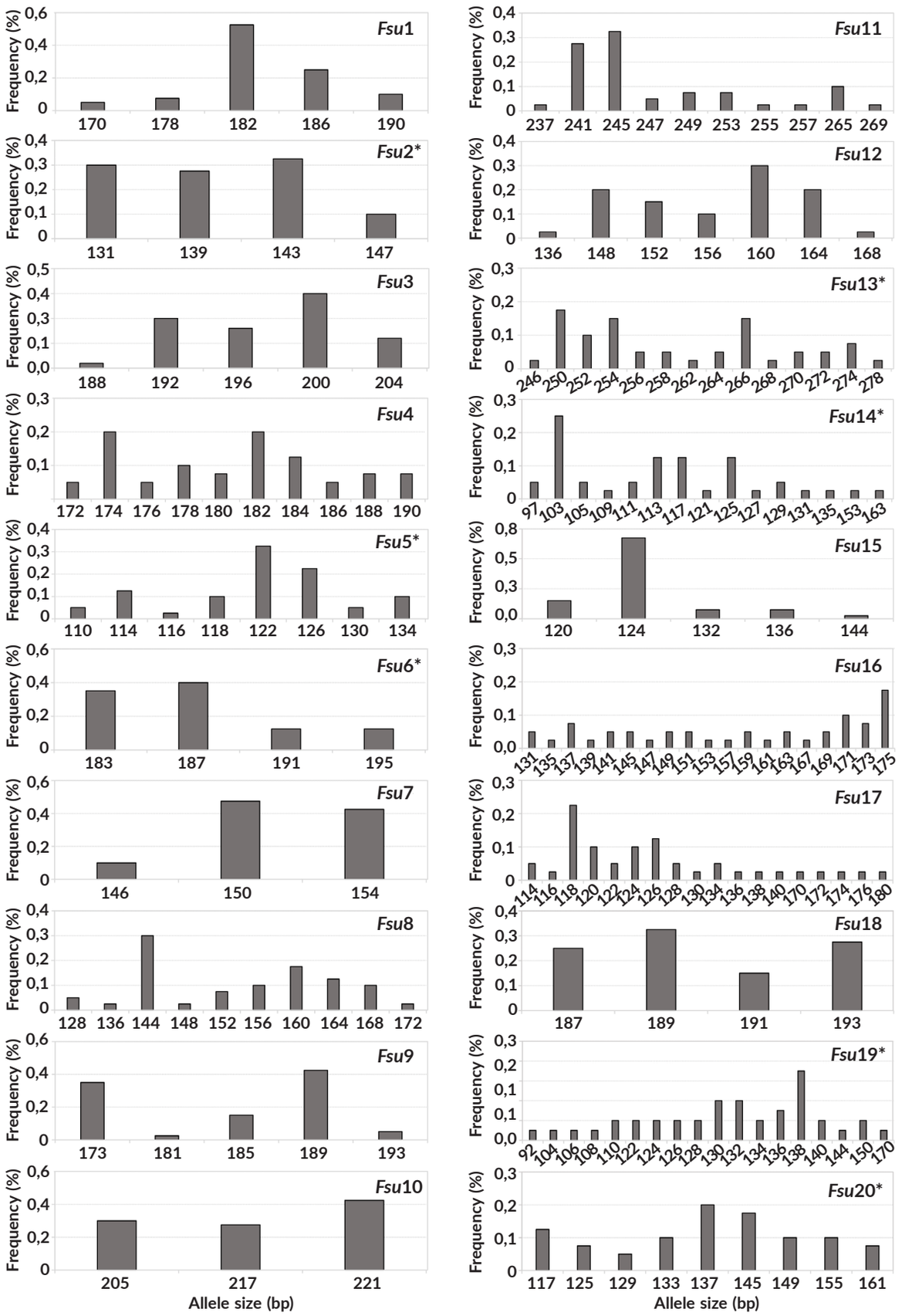


**Fig 1.** Distribution of allele frequencies at the 20 polymorphic microsatellite loci in *Farfantepenaeus subtilis*.

**Table 2.** Characterization of 20 primer pairs that amplify short tandem repeats from *Farfantepenaeus subtilis*. *Locus that deviated significantly from *HWE* after Bonferroni correction (adjusted critical $P < 0.0025$). $A_R$, number of alleles per locus; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; $P_{HWE}$, probabilities of departure from Hardy-Weinberg Equilibrium; PIC, polymorphic information content.

| SSR loci | Allele size range (bp) | $A_R$ | $H_O$ | $H_E$ | $F_{IS}$ | $P_{HWE}$ | PIC | Null allele frequency |
|---|---|---|---|---|---|---|---|---|
| *Fsu*1 | 170-190 | 5 | 0.800 | 0.660 | -0.2184 | 0.0234 | 0.597 | -0.1856 |
| *Fsu*2* | 131-147 | 4 | 0.300 | 0.737 | 0.5993 | **0.0000** | 0.665 | 0.1711 |
| *Fsu*3 | 188-204 | 5 | 0.650 | 0.753 | 0.1394 | 0.0451 | 0.689 | 0.0604 |
| *Fsu*4 | 172-190 | 10 | 0.600 | 0.892 | 0.3333 | 0.0031 | 0.857 | 0.1522 |
| *Fsu*5* | 110-134 | 8 | 0.250 | 0.823 | 0.7017 | **0.0000** | 0.778 | 0.1297 |
| *Fsu*6* | 183-195 | 4 | 0.050 | 0.704 | 0.9307 | **0.0000** | 0.629 | 0.1247 |
| *Fsu*7 | 146-154 | 3 | 0.800 | 0.599 | -0.3481 | 0.0034 | 0.494 | -0.2183 |
| *Fsu*8 | 128-172 | 10 | 0.850 | 0.855 | 0.0062 | 0.0053 | 0.816 | -0.0175 |
| *Fsu*9 | 173-193 | 5 | 0.650 | 0.688 | 0.0573 | 0.0135 | 0.611 | 0.0079 |
| *Fsu*10 | 205-221 | 3 | 0.050 | 0.671 | 0.9272 | 0.0277 | 0.580 | 0.1149 |
| *Fsu*11 | 237-269 | 10 | 0.900 | 0.813 | -0.1104 | 0.0038 | 0.766 | -0.0976 |
| *Fsu*12 | 136-168 | 7 | 0.750 | 0.817 | 0.0836 | 0.0056 | 0.767 | 0.0304 |
| *Fsu*13* | 246-178 | 14 | 0.750 | 0.917 | 0.1857 | **0.0000** | 0.885 | 0.0808 |
| *Fsu*14* | 97-163 | 15 | 0.950 | 0.899 | -0.0587 | **0.0000** | 0.866 | -0.0428 |
| *Fsu*15 | 120-144 | 5 | 0.500 | 0.523 | 0.0452 | 0.0249 | 0.478 | 0.0425 |
| *Fsu*16 | 131-175 | 19 | 1.000 | 0.947 | -0.0570 | 0.0117 | 0.919 | -0.0432 |
| *Fsu*17 | 114-180 | 18 | 0.900 | 0.921 | 0.0229 | 0.0122 | 0.890 | 0.0026 |
| *Fsu*18 | 187-193 | 4 | 0.950 | 0.753 | -0.2711 | 0.0106 | 0.684 | -0.1593 |
| *Fsu*19* | 92-170 | 18 | 1.000 | 0.944 | -0.0615 | **0.0000** | 0.915 | -0.0454 |
| *Fsu*20* | 117-161 | 9 | 0.400 | 0.892 | 0.5581 | **0.0000** | 0.856 | 0.1612 |

the species. Extended geographic sampling and cross-species amplification assays are planned for future studies pending funding availability.

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**MT Kamimura**, investigation and writing of the original draft. **KR da Silva Carvalho,** investigation and writing of the original draft. **F Mendonça Diniz,** conceptualisation, resources, writing, reviewing, and editing.

**Conflicts of interest**
The authors declare no competing interests.

**Data availability**
The data presented in this study are openly available in the NCBI Genbank under accession numbers: MH469833-MH469850, MH549205, and MH549206.

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## Supplementary material

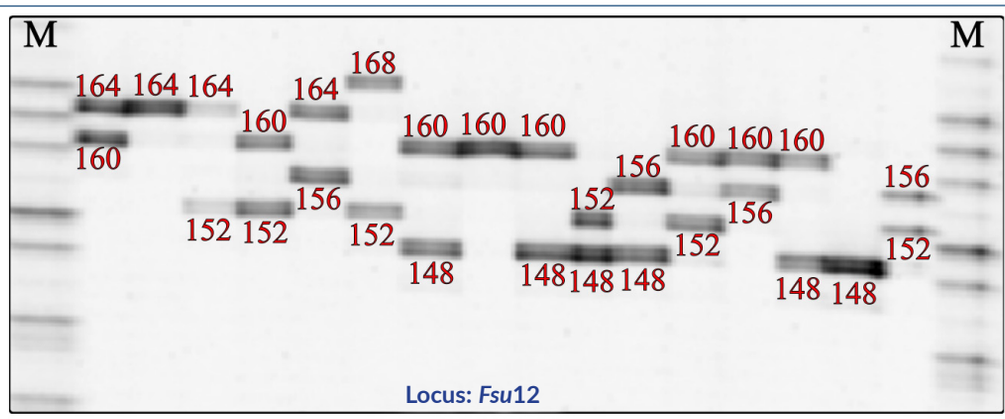


**Fig. S1.** Representative polyacrylamide gel (PAGE) for locus Fsu12, illustrating amplicon quality, allele calling, and the binning rule used for the genotyping of *Farfantepenaeus subtilis*. The figure shows the expected allele size range (152-168 bp) and the consistent banding patterns obtained under the optimized PCR conditions for this locus, as described in table 1. Allele bins were defined based on the repeat motif and empirical fragment-size clustering, and the same scoring criteria were applied across all individuals and loci. M, corresponding molecular marker.