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Limited evidence for genetic variation for resistance to the white spot syndrome virus in Indian populations of *Penaeus monodon*

Ben J Hayes[1,2], Thomas Gitterle[3,4], Gopalapillay Gopikrishna[5], Chavali Gopal[5], Gopal Krishna[6], Shrivinas Jahageerdar[6], Carlos Lozano[4], Shankar Alavandi[5], Sivagnanam Paulpandi[5], Pitchaiyappan Ravichandran[5] & Morten Rye[4]

[1]Biosciences Research Division, Department of Primary Industries Victoria, Bundoora, Vic., Australia

[2]Nofima, Ås, Norway

[3]CENIACUA, Bogotá, Colombia, South America

[4]Akvaforsk Genetics Center AS, Sunndalsøra, Norway

[5]Central Institute for Brackish Water Aquaculture, Chennai, Tamil Nadu, India

[6]Central Institute for Fisheries Education, Versova, Mumbai, India

Correspondence: B J Hayes, Biosciences Research Division, Department of Primary Industries Victoria, La Trobe R&D Park, 1 Park Drive, Bundoora, Vic. 3083, Australia. E-mail: ben.hayes@dpi.vic.gov.au

Abstract

There has been a highly detrimental impact of the white spot syndrome virus (WSSV) on black tiger shrimp (*Penaeus monodon*) aquaculture in India. Currently, no cost-effective measures are available for controlling the disease. One alternative is to improve WSSV resistance through a selective breeding programme for disease-resistant shrimp, provided that genetic variation exists for this trait. The aim of this study was to evaluate the evidence for genetic variation in resistance to WSSV in *P. monodon* sourced from Indian populations. Post-larval shrimp ($n = 1950$) from 54 full-sibling families were challenged with WSSV using WSSV-infected mince meat. The heritability was estimated using four different statistical models fitted to the resulting time to death data, including two linear models and two Weibull proportional hazard frailty models. None of the estimated heritabilities were significantly different from zero. We suggest three possible explanations for these results: there actually is very little variation between *P. monodon* in WSSV resistance and all individuals are highly susceptible to the disease; there is genetic variation in resistance to WSSV in *P. monodon* but we did not find it in our experiment because the level of challenge in the experiment was too high to allow genetic differences to be expressed; the variation is due to mutations conferring resistance, which are at a low frequency in the population, and we did not sample a broad enough genetic base to capture these mutations.

Keywords: *Penaeus monodon*, white spot syndrome virus, genetic variation

Introduction

The impact of the white spot syndrome virus (WSSV) on black tiger shrimp (*Penaeus monodon*) aquaculture in India has been highly detrimental since it was first reported in 1994 (Karunasagar, Otta & Karunasagar 1997, for a review see Escobedo-bonilla, Alday-sanz, Wille, Sorgeloos, Pensaert & Nauwynck 2008). All age groups and sizes of shrimp are affected by WSSV, and in most kinds of production systems (Karunasagar *et al.* 1997). Transmission of WSSV can occur vertically, from infected broodstock to larvae, or horizontally, through the water column, or from animal–animal contact. The disease is prevalent in commercial hatcheries in India. Uma, Koteeswaran, Karunasagar and Karunsangar (2005) randomly sampled broodstock used in commercial hatcheries along the southeast Indian coast, and found that 39.4% tested positive for WSSV using a PCR test.

Despite a large research effort, currently, there are no cost-effective measures for controlling WSSV. The development of vaccines for example is thought to be prevented by the absence of an acquired immunity system in crustaceans (however, for an alternative viewpoint, see Witteveldt 2006; Johnson, Van Hulten & Barnes 2008). An alternative is to improve WSSV resistance through a selective breeding programme for disease-resistant shrimp, provided that genetic variation exists for this trait.

Limited genetic variation in WSSV resistance has been demonstrated in *Penaeus vannamei* under controlled challenge testing conditions (Gitterle, Salte, Gjerde, Cock, Johansen, Salazar, Lozano & Rye 2005; Gitterle, Gjerde, Cock, Salazar, Rye, Vidal, Lozano, Erazo & Salte 2006). The heritability of WSSV resistance, calculated from survival across full-sibling families under challenge test conditions, ranged from 0.00 to 0.07, depending on whether the shrimp in the challenge test were infected as a result of consuming minced muscle tissue infected with WSSV, given an individual oral infection or infected through waterborne virus particles. Gitterle *et al.* (2006) concluded that the dosage of WSSV was better controlled with oral infection than with other methods, as all animals were exposed to approximately the same risk of infection at the same time, and that this should improve the accuracy of estimating the genetic variance and hence the accuracy of breeding values (BV) for use in the selection programme.

Genetic variation for WSSV resistance in *P. monodon* has not been investigated previously. The aim of this study was to evaluate the evidence for genetic variation in resistance to WSSV in *P. monodon* sourced from Indian populations.

Material and methods

Collection of broodstock and establishment of shrimp families

With the goal of establishing a breeding programme in *P. monodon* mainly focused on growth, survival under commercial conditions and resistance to WSSV, gravid females were collected from three different Indian states (Tamil Nadu, Andhra Pradesh and Andaman and Nicobar Islands) to ensure genetic variability of the base population (Fig. 1). These females were spawned in commercial hatcheries and families were reared from postlarvae (PL) to tagging size in individual tanks placed at two different research stations: CIBA's research station in Muttukadu and CIFE's station in Kakinada. A total of 54 full-sibling families were produced, and 10 of these families (four from Tamil

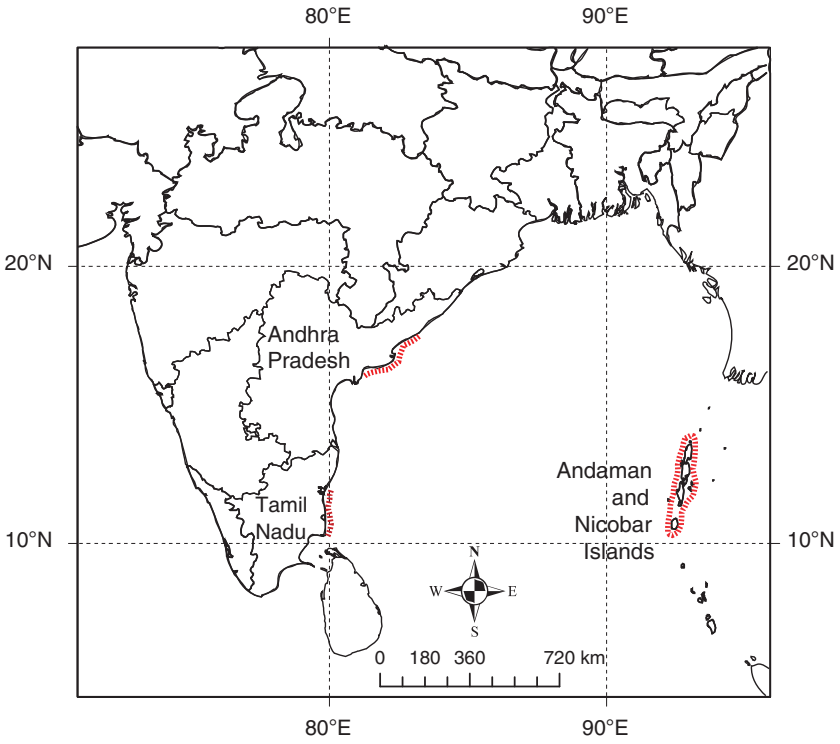

Figure 1 Location of the sampled populations are indicated in red.

Nadu and six from Andhra Pradesh) were reared in both research stations so that family and location effects could be jointly estimated. At Muttudaku, a total of 37 individually tagged families were reared (26 from Tamil Nadu, 10 from Andhra Pradesh and one from the Andaman Islands). These families were produced between 1st of July and 13th of August 2006 in commercial hatcheries and were stocked in CIBA's station at an average stage of PL 15 over August and September 2006. At the CIFE station in Kakinada, a total of 27 families (23 from Andhra Pradesh and four from Tamil Nadu) were reared. Families were produced in August and stocked in individual tanks on September 2006 in order to reach the tagging weight.

On average, 219 individuals from each of the families reared at CIFE and 198 animals from the families reared at CIBA were individually tagged using visible implant fluorescent elastomers (Godin, Carr, Hagino, Segura, Sweeney & Blankenship 1995).

Challenge test for resistance against the WSSV

In December 2006, approximately 30 tagged individuals per family were transferred to CIBA, Santhome Challenge test facilities in order to evaluate the family resistance to WSSV. Families were placed in two 4 tonne tanks for experimental infections (15 animals per tank per family). WSSV-infected minced muscle tissue was administered once at 16:00 hours on 20 December 2006. A total of 1950 animals were infected. The test was terminated when all animals died.

Genetic analysis

The fixed effects used in all the models were origin [geographical origin of the family, three levels: Tamil Nadu Coast, Andhra Pradesh Coast and Andaman Islands (only one family)], the rearing place (two levels: CIBA research station or CIFE research station) and the infection tank (two levels: tank 1 and tank 2). Because no half-sibling families were produced, only one random effect was considered (actually a family effect). Note that this means that family and tank of rearing are completely confounded in all models.

We used four different approaches to estimate the heritability of white spot resistance. These were:

(a) A linear animal model where disease resistance was defined based on whether or not the animal was alive when the population reached 50% total mortality (model LAM). The 'animals' here are the challenged individuals.
(b) A linear animal model based on time to death [hours post-infection (pi)]. This model does not take censored observations into account; however, as no animal survived the challenge, the model is appropriate (LTM).
(c) A Weibull proportional hazard frailty model based on time until death (days pi) and taking censored observations into account (WHD).
(d) A Weibull proportional hazard frailty model based on time until death as above, but with hours pi rather than days (WHH). This model was fitted to determine whether there was an advantage in assessing survival hourly or whether daily records were sufficient.

Fixed effects were equal in all models, while random effects varied depending on whether it was an animal model (LAM and LTM) or a family model (WHD and WHH). A preliminary analysis was performed to estimate the relative effect of the different fixed effects over the mortality for the linear models and to check the assumption on proportionality in the hazards models.

The four models are now described in more detail.

LAM: A linear model was applied to the observed binary variable y (0 = dead, 1 = alive) truncated at 50% overall mortality:

$$y_j = F_i + a_j + e_j \qquad (1)$$

where F_i is the fixed effect of the ith origin by rearing place by tank, a_j is the random effect of animal j assumed to be multivariate normal distributed with mean vector 0 and covariance matrix $A\sigma_a^2$, where A is the additive genetic relationship matrix. For example, for a pair of full-siblings k and l, A_{kl} will be 0.5. e_j is the random residual for animal j, where e_j is assumed to be normally distributed with the covariance matrix $I\sigma_e^2$.

LTM: A linear model was applied to the observed death times (hours of the animals), where y is now the time to death. Fixed and random effects are as in the previous model.

WHD: A sire–dam proportional hazard model was assumed for days to death (t):

$$h_{ijl}(t) = h_0(t)\exp(F_i + f_j)$$

where $h_{ijl}(t)$ is the hazard function for animal l at time t, $h_0(t)$ is the baseline hazard function that follows a Weibull distribution (i.e. $\lambda\rho(\lambda t)^{\rho-1}$), where ρ and λ are the parameters of the Weibull distribution, F_i is the fixed effects of the ith origin by rearing place by tank and f_j is the random effect of family j, assumed to be multivariate normal distributed with mean vec-

tor 0 and covariance matrix $A\sigma_f^2$, where A is the additive genetic relationship matrix.

Traditionally, f has been assumed to follow a log-gamma distribution because of its flexibility and mathematical convenience. The gamma distribution tends to show a log-normal distribution as the parameters of the gamma distribution of random effects become larger (Kalbfleisch & Prentice 1980, p. 26) and then f can be regarded as (at least approximately) normally distributed. Therefore, it has been suggested to account for the genetic relationship between animals by assuming a multivariate normal distribution (Ducrocq 1987).

WHH: A proportional hazard model was assumed for hours to death (t): The model and parameters are the same as in the WHD model.

For the linear models, the heritability for disease resistance was calculated as

$$h^2 = \sigma_a^2(\sigma_a^2 + \sigma_e^2)$$

where $\sigma_a{}^2$ is the additive genetic variance and $\sigma_e{}^2$ is the residual variance and for the sire–dam proportional hazard models as:

$$h^2 = \frac{2\sigma_f^2}{\sigma_f^2 + \sigma_e^2}$$

where $\sigma_f{}^2$ is the sire–dam variance, $\sigma_e{}^2$ is the residual variance and is $\pi^2/6$ in the Weibull frailty models (Ducrocq & Casella 1996).

Correlations between estimated breeding values (EBVs) of full-sibling families from each model were calculated to assess the agreement between genetic predictions of the different methods. Then, to evaluate the accuracy of each method, family full-sibling BV were independently predicted for the replicated tanks using the variance components estimated from all data as input parameters. The Pearson correlation coefficients between the resulting EBV (r_{EBV}) from each tank are closely related to the accuracy of selection (r_τ) (Gitterle *et al.* 2006).

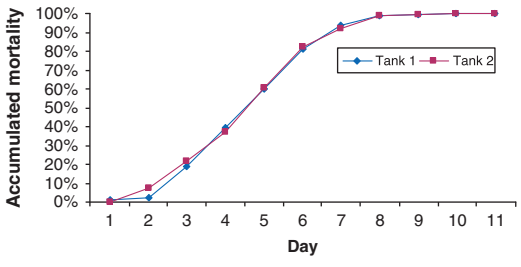

Figure 2 Cumulative proportion of shrimp dead versus days after challenge.

Results

On day 11 of the challenge test, mortality reached 100% (Fig. 2). Mortality data were registered only for 1555 animals, which corresponds to 80% of the infected animals. Thus, 395 animals died, and information could not be retrieved from them (date and hour of death). This was probably due to cannibalism of the recently diseased animals. In our experiment, animals started dying at day 3 pi before reaching 100% mortality at day 11 pi. The high and rapid mortality reflects a very strong infection. As shown in Fig. 3a, no differences in the mortality rate were observed among the tanks, and the risk of dying increased over time (Fig. 3b). This is an atypical situation in experimental infections (Gitterle *et al.* 2006) and also reflects possible cannibalism leading to increased dosages over time.

In order to fulfil the proportional hazard assumption in the Weibull model, the hazard ratio of the

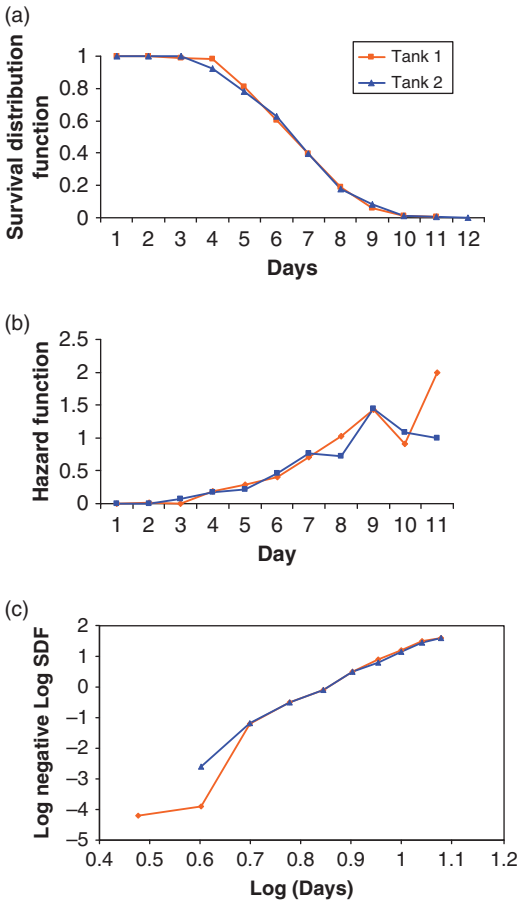

Figure 3 (a) Survival distribution function (SDF) against time from tanks 1 and 2, (b) hazard function from tanks 1 and 2 and c) $\log(-\log(\hat{S}_{0,n}(t)))$ from tanks 1 and 2 against log t in days.

different levels (or strata) from the covariates in the model has to be constant over time. This can be checked by plotting the values of the $\log(-\log(\hat{S}_{0,n}(t)))$ against $\log t$, where $(\hat{S}_{0,n}(t))$ is the baseline survival function from each stratum. Parallel lines indicate that the proportional hazard assumption holds. Moreover, if the lines are straight, the baseline hazard function is assumed to follow a Weibull distribution. Figure 3c shows the plot of the $\log(-\log(\hat{S}_{0,n}(t)))$ against $\log t$, from tanks 1 and 2. We can see straight, parallel lines, indicating that the proportional hazard assumption holds. Similar results were obtained when the proportional hazard assumption was checked for origin and rearing place. Therefore, it was not necessary to stratify for any fixed effect in either of the two hazard models.

The heritability of resistance to white spot infection was not significantly different from zero for any of the statistical models used (Table 1). In all cases, the lack of heritability was due to the lack of genetic variance rather than due to a high environment variance.

When the correlation between full-sibling mean BV was investigated, there were higher correlations among the BV from models that used time to death (LTM, WHH and WHD) than between LAM and the other models (Table 2).

Table 1 Heritabilities and their standard errors from four statistical models used to analyse data from a white spot challenge test

Model	h^2	SE
LAM	0.000010	0.0129
LTM	0.000798	1.1187
WHD	0.000079	
WHH	0.000231	

LAM, linear model with 0 (died) or 1 (survive) as the independent variable; LTM, linear model with time to death (hours) as the independent variable; WHD, a Weibull proportional hazard frailty model based on time until death (days post-infection) and taking censored observations into account; WHH, a Weibull proportional hazard frailty model based on time until death (hours post-infection) and taking censored observations into account.

Table 2 Rank correlations among full-sibling mean breeding values among the different models (see text or Table 1 for an explanation of the models)

	LAM	LTM	WHH
LTM	0.71		
WHH	0.59	0.88	
WHD	0.56	0.86	0.99

All correlations were significant $P < 0.0001$.

Table 3 Pearson correlations of full-sibling family mean breeding values among tanks

Model	r_{EBV}
LAM	0.093
LTM	0.089
WHH	0.026
WHD	0.023

LAM, linear model with 0 (died) or 1 (survive) as the independent variable; LTM, linear model with time to death (hours) as the independent variable; WHD, a Weibull proportional hazard frailty model based on time until death (days post-infection) and taking censored observations into account; WHH, a Weibull proportional hazard frailty model based on time until death (hours post-infection).

As expected, with the lack of genetic variance, the correlations between full-sibling mean BV between tanks were very low in all the models and none of them was significant (Table 3).

Discussion

The major limitation of the current study is the inability to separate PL into tagging tank effects and genetic effects, as each full-sibling family was reared in a separate tank at this stage. Thus, the PL to tagging tank variation will be included in our full-sibling means, biasing the estimates of genetic variation and heritability upwards. In spite of this, we found little or no evidence for genetic variation in resistance to WSSV infection. There are two possible explanations for this. One possibility is that there actually is very little variation between *P. monodon* in WSSV resistance and all individuals are highly susceptible to the disease. As the virus was only first documented in 1992 (Chou, Huang, Wang, Chiang & Lo 1995), it is possible that it has become highly pathogenic to shrimp only very recently. If this is the case, there would not have sufficient time for genetic mutations to confer resistance either to emerge in the population, or if such mutations do exist, to reach moderate frequencies in the population. Gitterle *et al.* (2005, 2006) also found either very low or zero heritabilities for WSSV resistance in *P. vannamei*.

The second possibility is that there is genetic variation in resistance to WSSV in *P. monodon*, but we have not demonstrated such a variation in our experiment. This may occur if either we have not sampled a wide enough genetic base with 54 full-sibling families or the level of challenge in the experiment was too high

to allow genetic differences to be expressed. The stocking densities used in this experiment were high, and higher mortalities are found at higher densities (Wu, Namikoshi1, Nishizawa1, Mushiake, Teruya & Muroga 2001). Another difficulty with the challenge test is that *P. monodon* is highly cannibalistic, such that shrimp that survive beyond a few hours are likely to consume the dead shrimp, thus exposing themselves to much higher virus loads (Fig. 3). Refinement of the challenge test protocol and constant removal of dead shrimp may result in more observed genetic variation for WSSV resistance. Further, infection protocols other than the feeding with WSSV-infected meat used here could allow better control of the infection. Gitterle *et al.* (2006) suggested that the dosage of WSSV in a challenge test could be better controlled with oral infection than with other methods, as all animals were exposed to approximately the same risk on infection at the same time, and that this should improve the accuracy of estimating the genetic variance and hence the accuracy of BV for use in the selection programme. Despite this, the levels of genetic variation for WSSV resistance in their study were low.

If genetic mutations conferring resistance to WSSV do exist, but their frequencies are very low, then a very broad genetic base would have to be sampled in order to capture these mutations in the breeding programme. One option would be to screen an extremely large number of individuals from a large number of subpopulations, either by breeding from the survivors of natural WSSV outbreaks or by performing a natural challenge, as suggested by Cock, Gitterle, Salazari and Rye (2009). The founder population for the breeding programme could then include these individuals.

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