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Genetic analysis of disease resistance to *Vibrio harveyi* by challenge test in Chinese tongue sole (*Cynoglossus semilaevis*)

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ABSTRACT

Recently, *Vibrio harveyi* has emerged as an important pathogenic disease with a high mortality rate (50%–70%) in Chinese tongue sole. The aim of this study was to estimate genetic parameters for resistance to *V. harveyi* in Chinese tongue sole and to evaluate the genetic correlation between resistance and growth traits (i.e. body weight and total length) (involving 50 full-sib families and 8547 individuals). Longitudinal linear models and cross-sectional threshold models were fitted by using different trait definitions (binary and categorical). After a 14-day test, the overall challenge test survival was 57.86% (ranging from 9.30% to 94.30% in families). The heritabilities of survival were ranging from 0.11 to 0.28, estimates obtained by linear models were higher than threshold models. The genetic correlations between resistance (binary and categorical traits) and two growth traits were moderately positive (0.27–0.51). Very high Pearson and Spearman correlations (0.99 to 1) of full-sib family EBVs between different models were all close to unity which might reveal the similar predictive ability of the four models. The favourable heritabilities and moderate positive genetic correlations indicate that joint genetic improvement of vibriosis resistance and growth performance would be feasible.

Statement of relevance: This paper offers guidelines to disease-resistance selective breeding strategies in Chinese tongue sole.

1. Introduction

Disease is a common threat for both marine and freshwater aquaculture species. With the development of the indoor intensified aquaculture systems, disease outbreaks occur more frequently, which have reportedly cost the aquaculture industry tens of billion dollars in the last two decades (FAO, 2015; FAO, 2016). Chinese tongue sole (*Cynoglossus semilaevis*) is an important marine flatfish which is widely cultured in coastal areas in China. However, with the rapidly developing, tongue sole cultivation has also suffered enormous losses due to bacterial diseases, especially *Vibrio harveyi* disease in recent years. *Vibrio harveyi* is one of the most prevalent infectious pathogens of marine reared fish, crustaceans and shellfish, being responsible for high mortality rates in commercial industries worldwide (Liu et al., 1996; Austin and Zhang, 2006; Haldar et al., 2010; Li et al., 2011; Woo and Bruno, 2011; Hashem and El-Barbary, 2013; Medina et al., 2015; Stalin and Srinivasan, 2016). *Vibrio harveyi* has emerged as the most important disease in tongue sole in China, an epidemic with a high mortality rate (50%–70%) recently occurred. Natural outbreaks and mortality of *V. harveyi* in tongue sole are usually observed in juvenile fish (body length between 10 cm and 20 cm) at relatively high summer temperatures. Symptoms mainly included surface ulcers, erosion of the tail, eye infection, and septicemia.

Generally, prevention of diseases in intensive fish aquaculture is not only an economical but also an environmental and ethical issue (Antonello et al., 2009). Antibiotics should be discouraged for the problems of residue and resistant pathogens. Furthermore, vaccines and probiotics as alternative methods are widely used to prevent fish pathologies and control bacterial infections (Magnadottir, 2010; Song et al., 2014; Nguyen et al., 2017). These methods, however, cannot obtain a continuous and permanent genetic gain. In aquaculture, the selective breeding of fish has been suggested a more effective approach to enhance disease resistance (Bangera et al., 2014). Further, resistance to specific pathogens, together with other economically important traits (e.g. growth-related traits, body weight or length), should be included in the breeding goal for economic and sustainability purposes (Ødegård

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et al., 2011a; Gjedrem, 2012).

Usually, resistance can be defined as the ability of a host to prevent pathogen entry and to control pathogen life-cycles in a manner to reduce pathogen burden within a host individual (Kause and Ødegård, 2012). In practice, however, the selection of disease resistance, the resistance of individuals or families is commonly achieved by challenge testing for specific pathogens. In this situation, disease resistance is roughly recorded as a binary trait (dead/alive) at the end of the recording period, with no further information on infection status of the survivors (Ødegård et al., 2011a). Also, the cross-sectional binary threshold (BTH) model as a simplest generalized linear mixed model is widely used to obtain estimates of variance components and predictions of family breeding values for survival data (Gitterle et al., 2006; Ødegård et al., 2006). In the BTH model, however, other potentially available information in the data (such as time until death) is not fully used. Time until death reflects the resistance to disease to some extent (the later ones are the more resistant ones). Hence, in this study, in order to improve the accuracy of genetic analysis of survival data, the time until death (in days) is taken into account (assuming that resistance is associated with survival time) and apply this trait in alternative advanced models (longitudinal models).

In Chinese tongue sole, females can reverse to males (referred to pseudo-males) (Shao et al., 2014; Chen et al., 2014), and there is a higher male ratio (> 90%) in pseudo-males' offspring (Shao et al., 2014), this explains the lower growth performance of males (Ji et al., 2011; Chen et al., 2014; Hu et al., 2014a, 2014b). So in tongue sole breeding schedule, pseudo-males are usually identified by sex-specific molecular markers (Chen et al., 2007, 2008; Liu et al., 2014) and eliminated from the brood stock.

The genetic analysis studies for disease resistance in this species are still limited. The primary objectives of current study therefore were to estimate genetic parameters for resistance to *V. harveyi* in Chinese tongue sole based on survival data obtained from challenge test using longitudinal and cross-sectional models. Besides, the predictive abilities of different models and genetic correlations between resistance and growth traits were also investigated. The findings of the study have significance in planning breeding strategies for controlling this disease by genetic improvement.

2. Materials and methods

2.1. Production of families and fish rearing

The families were produced in Laizhou Mingbo Aquatic Co., Ltd. in March 2014 as described by Chen et al. (2010). All the female brood stocks were collected from Laizhou Mingbo Aquatic Co., Ltd. and the males were collected from four locations (populations) (i.e. Haiyang, Laizhou, Changyi and Yangzi). It is worth mentioning that the Haiyang population is an in-house selected family strains in 2012, Changyi and Yangzi populations are cultured stocks, and Laizhou population is the first generation of wild caught population. At the age of 2.5 years old, the mature fish were used to produce families in the breeding season (late April). All the parental fish were individually tagged with Passive Integrated Transponders (PIT) (Qingdao Starfish Instruments Co., Ltd.) before using, and the pseudo-males were identified by using sex specific AFLP markers and eliminated according to methods as described by Chen et al. (2007). Strip spawning procedures were used to create full-sib families. The mating design was followed: dams were mated with one sire, while each sire was mated with one or two dams. Not all the matings, however, were successful mainly due to mortality in the hatchery.

The fertilized eggs were incubated in hatchery cages, about 6 h before hatch, they were transferred to 2.5m^3 fiberglass-reinforced plastic tanks for hatching and larvae rearing. Finally, each family was cultured in a separate tank, with a similar rearing environment. The tanks were supplied with filtered and sterilized sea water, with a continuous aeration and flowing water exchange (400% per day). Before 30 days post hatch, the water temperature was controlled at 22–23 °C, after that the temperature was not exceeding 24 °C. Salinity was 28–32‰, and dissolved oxygen was 6–8 mg/L. At approximately 90 days post hatch, 1000 fry of each family remained in tanks. Larvae and juvenile fish were feed under a standard procedure. In total, > 100 full-sib families were produced and 50 (50 dams and 46 sires) of them were used in this study.

2.2. Challenge test

When fish reached an average total length of approximately 10 cm, 600 individuals from each family were transferred to an experimental station in Weifang Shandong Provence for challenge testing. The *V. harveyi* strain (as reported by Wei et al., 2018) used for the challenge test in this study was isolated from diseased tongue soles displaying typical disease symptoms in 2014, and subsequently confirmed by means of biochemical characterization and a multiplex PCR-based protocol (Cano-Gomez et al., 2015). The symptoms of the rechallenge experiment were the same to natural outbreak individuals. After a 2-week accommodation, the *V. harveyi* challenge test was conducted using a median lethal dose (LD_{50}) by intraperitoneal injection (0.1 mL per 5 g body weight). The method of determination of LD_{50} was similar to Xiong et al. (2017). The value of LD_{50} was determined as 2.5×10^6 cfu/5 g fish. 150–200 individuals (with few exceptions) were sampled randomly from each family and inoculated fish were kept at separate tanks (2.5m^3) by family. In the latter experiment, tank environments were standardized through a flowing water exchange, and these tanks were thus assumed to be the same common environment. For the limitation of facilities there was no replication. The day of challenge was defined as day 0, and the last day was the day when the last mortality was observed. Mortality was recorded for 14 days, and dead animals were removed three times daily. Body weight (BW) and total length (TL) were measured for all the dead fish and survivors.

The pre-challenge mortality was excluded. During the challenge test period, fish were fed twice daily according to their appetite, the water temperatures were maintained at 21–22 °C. Salinity was 28–30‰, water exchange rate was 400% per day and dissolved oxygen was 6–8 mg/L. Before injection, the fish were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate) to avoid handling stress. Finally, 8547 individuals were used in the challenge test. More details of the experimental data about the two tests are given in Table 1.

2.3. Trait definition

In this study, challenge test survival was defined using two different trait definitions:

Trait 1: Binary test survival (BTS), which was scored 0 if the fish died before the end of the test day and scored 1 elsewhere;

Trait 2: Categorical test day survival (CTDS), with one record per fish died at the specific test day. The observation was scored n (n = 1–14) if the fish died on the n^{th} test day and scored 15 if the fish

Table 1
Descriptive statistics of the challenge test.

Average sample size	Fish no.	Average body weight (g)	Survivors	Survival rate (%)	Range of each family (%)	Duration (day)
171	8547	12.9	4945	57.86	9.30–94.30	14

was alive after the test. This definition was similar to the paper of Yáñez et al. (2013), but the survived fish were not censored.

2.4. Statistical models

Survival data was analyzed with four different models (univariate models), including two linear models for both traits and two threshold models for Trait 1. All the models using restricted maximum likelihood (REML) algorithm were implemented for the genetic analysis based on the ASReml-R 3.0 software package (Butler et al., 2009). The models were defined below:

(1) Linear animal model (LAM):

$$y_{ik} = \mu + f + a_i + d_k + e_{ik}$$

where y_{ik} was the phenotypic observation for the traits; μ is the overall mean; f was the fixed effect, which was the population of sire; a_i was the random additive effect for the i^{th} individual; d_k was the random effects of maternal and common environment to full-sib k; e_{ik} was random residual. This model was fitted according to the Akaike's Information Criterion (AIC) value (Akaike, 1974) described in ASReml-R reference manual (Butler et al., 2009). The approach is similar to Li et al. (2018).

(2) Linear sire-dam model (LSM):

$$y_{ijk} = \mu + f + a_i + s_j + d_k + c_{jk} + e_{ijk}$$

where s_j was the random additive genetic effect of sire j; d_k was the random additive genetic effect of dam k; c_{jk} was the effect of common full-sib families (including tank effect); the other parameters were as described above.

(3) Threshold (logit) animal model (TAM):

$$Pr(y_{ijk} = 1) = \frac{\exp(\mu + f + a_i + c_{jk})}{1 + \exp(\mu + f + a_i + c_{jk})}$$

where all the parameters were as described in LAM.

(4) Threshold (logit) sire-dam model (TSM):

$$Pr(y_{ijk} = 1) = \frac{\exp(\mu + f + a_i + s_j + d_k + c_{jk})}{1 + \exp(\mu + f + a_i + s_j + d_k + c_{jk})}$$

where all the parameters were as described in LSM. In the preliminary analysis, significance of fixed effect was tested based on Wald F statistics by using the ASReml-R 3.0 software package (Butler et al., 2009). For the both traits, the fixed effect (i.e. population of sire) was not significant ($P > .1$), despite this, it was not excluded from models because it influenced the results to some extent. In this paper, Trait 2 was only applied in LAM and LSM, according to the theories of Ødegård et al. (2011a, 2011b) and Bangera et al. (2014), in this case, the two models belong to longitudinal model (also called cure model) or ordinal model; Trait 1, which is a binary trait, was applied to the 4 models, thus they are cross-sectional models. The heritabilities of BW and TL were estimated based on the Model 1 that replacing maternal effect with common full-sib family effect.

2.5. Heritability

For cross-sectional animal models (LAM and TAM) heritability was calculated as follows:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_d^2 + \sigma_e^2}$$

where $\sigma_a{}^2$ was additive genetic variance; $\sigma_d{}^2$ was maternal and common environmental genetic variance; and $\sigma_e{}^2$ was the residual variance ($\pi^2/3$ for threshold model).

For cross-sectional sire-dam models (LSM and TSM) heritability was calculated as follows:

$$h^2 = \frac{2 \times (\sigma_s^2 + \sigma_d^2)}{\sigma_s^2 + \sigma_d^2 + \sigma_c^2 + \sigma_e^2}$$

where $\sigma_s{}^2$ was sire genetic variance; $\sigma_d{}^2$ was dam genetic variance; $\sigma_c{}^2$ was the common full-sib genetic variance, the other parameters were as described above.

2.6. Genetic correlations

In order to estimate the genetic (r_g) and phenotypic (r_p) correlations of survival in challenge test and growth traits (i.e. BW and TL), bivariate analyses (with a full dataset) were carried out based on LAM by using the two trait definitions. The correlations were calculated as follow (Falconer and Mackay, 1996):

$$r_{p/g(mn)} = \frac{\delta_{p/g(mn)}}{\delta_{p/g(m)}\delta_{p/g(n)}}$$

where $r_{p/g(mn)}$ was the phenotypic and genetic covariance between two traits (m and n), and $\delta_{p/g(m)}$ and $\delta_{p/g(n)}$ were the phenotypic and genetic variance of trait m and n, respectively.

2.7. Model comparison

The models use different trait definitions of survival, so it is not possible to directly compare them based on heritability estimates or fit statistics. Therefore, comparisons of the models were made with respect to their ability to predict the accuracy of selection, which was determined through the correlations among full-sib families estimated breeding values (EBVs) based on Trait 1. Full-sib family EBVs were obtained as $1/2(u_s + u_d)$, where u_s and u_d represent the EBV of the sire and dam of each family, respectively (Li et al., 2018). In addition, the rank correlation (Pearson and Spearman) coefficient among the full-sib family EBVs from each model was calculated to determine the agreement among genetic predictions of the different statistical methods.

3. Results

3.1. Descriptive statistics

The number of observations, overall survival rate, range of survival rate and the duration for challenge test are given in Table 1. The overall survival rate for the challenge test is 57.86%. The range of challenge test survival of each family has a wide-ranging (9.3–94.3%) which implies an ideal variable status for challenge test that would be benefit for selective breeding. The accumulated daily mortality bar charts and Kaplan–Meier survival curves for all the full-sib families in the challenge test are showed in Fig. 1. In the test, the accumulated daily mortalities appeared as a standardized normal distribution, and peaked at the day 8. There was a significant peak period from day 7 to 9.

3.2. Heritabilities

The estimated variance components and heritabilities for challenge test are shown in Table 2. In LAM and LSM, the heritabilities of survival were in a moderate level (0.19–0.24). The estimates by LAM are associated with relatively large standard errors. In the both models, heritabilities estimated by Trait 2 were slightly higher than Trait 1 which might suggest that Trait 2 was a more favourable definition. In the threshold models (TAM and TSM), however, the heritabilities were much lower (0.16 and 0.11 respectively). Nevertheless, due to differences in trait definitions and modeling, as expected, the heritabilities were not comparable between each other (Ødegård et al., 2006, 2007; Bangera et al., 2014). The estimates for heritability of BW and TL were

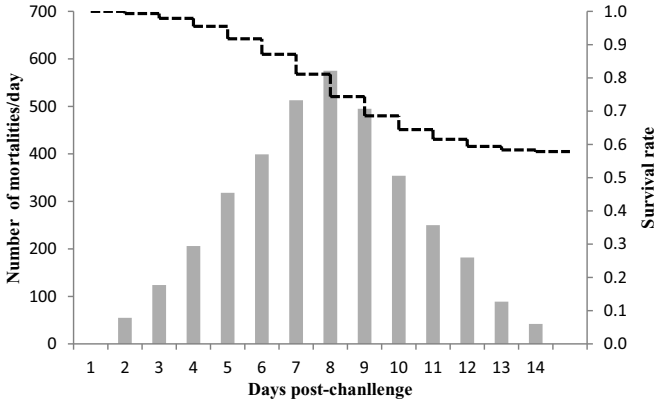

Fig. 1. The observed accumulated daily mortalities and Kaplan–Meier survival curve for all the full-sib families in the *V. harveyi* challenge test.

Table 2
Variance components and heritabilities in different statistical models for the *V. harveyi* challenge test.

Model	Trait	σ_a^2	σ_s^2	σ_d^2	σ_c^2	σ_e^2	h^2 ± SE
LAM	Trait 1	0.046	–	0.035	–	0.17	0.19 ± 0.16
	Trait 2	3.69	–	1.42	–	10.59	0.24 ± 0.19
LSM	Trait 1	–	0.025	0.0012	0.019	0.19	0.22 ± 0.09
	Trait 2	–	2.10	0.16	1.24	12.43	0.28 ± 0.09
TAM	Trait 1	0.92	–	–	1.43	3.29	0.16 ± 0.02
TSM	Trait 1	–	0.10	0.10	0.10	3.29	0.11 ± 0.02

0.33 ± 0.06 and 0.25 ± 0.05, respectively.

3.3. Genetic correlations

The genetic and phenotypic correlations (evaluated based on Trait 1 and Trait 2) between survival data and growth traits (BW and TL) are shown in Table 3. The estimated genetic correlations between survival and growth traits were moderately positive and significantly different from zero (ranging from 0.27 to 0.51) although with large standard errors. The phenotypic correlations between these traits were also in a moderate level and significantly different from zero (ranging from 0.29 to 0.33) but with very small standard errors.

3.4. Model comparison

The Pearson and Spearman correlation coefficients (evaluated based on Trait 1) of full-sib families EBVs between different models were presented in Table 4. The Pearson and Spearman correlation coefficients between different models were all very high (~1), indicating a near identical ranking of families (Table 4).

4. Discussion

The Chinese tongue sole is an important aquaculture species and is under threat from a range of bacterial pathogens including *Vibrio parahaemolyticus*, *Listonella anguillarum* (Wang et al., 2007; Chen et al., 2012; Hu et al., 2014a, 2014b), and *V. harveyi* which has proven to be highly pathogenic resulting in several outbreaks with resultant losses over recent years. Selective breeding is an effective approach that can be taken to improve survival. The genetic evaluations on disease resistance, however, are still very finite. Unlike other quantitative traits (e.g. weight and length), disease resistance trait cannot be measured directly. In practical selective breeding programs, disease resistance in fish is generally assessed based on challenge tests and survival following intraperitoneal injection of the pathogen (Ødegård et al., 2011a). There are, however, some caveats due to the limitations of the challenge test procedure used here. First, the injection of pathogen is not a natural mechanism of infection, though it is expected that the improvement from selection in the nucleus by challenge test will be transferred to the commercial populations. Second, there is no replicated tank for each family and thus the reproducibility of the heritability is uncertain and probably biased if tank effect represents a significant source of variation for vibriosis resistance. Further research, therefore, is needed to corroborate the estimates and to elucidate if genetic resistance mechanisms involved in intraperitoneal injection are similar to those implicated in natural infection.

Table 3
The genetic (above diagonal) and phenotypic (below diagonal) correlations between survival and growth traits (i.e. BW and TL).

Trait	Trait 1	Trait 2	BW	TL
Trait 1	–	–	0.51 ± 0.19	0.29 ± 0.22
Trait 2	–	–	0.27 ± 0.36	0.43 ± 0.13
BW	0.33 ± 0.03	0.30 ± 0.04	–	–
TL	0.29 ± 0.03	0.32 ± 0.02	–	–

Table 4
The Pearson rank correlation coefficients (above diagonal) and Spearman rank correlation coefficients (below diagonal) of EBVs of full-sib families between the different models.

Model	LAM	LSM	TAM	TSM
LAM	1	0.995	0.993	0.992
LSM	0.995	1	0.989	0.997
TAM	0.996	0.991	1	0.994
TSM	0.993	0.997	0.993	1

In this paper, for better understanding the genetic parameters of disease resistance to *V. harveyi*, four models (longitudinal and cross-sectional threshold models) were fitted by using different trait definitions. The evidence shows that longitudinal models could obtain a somewhat better precision of estimation results (e.g. EBV), compared with simpler cross-sectional models (Gitterle et al., 2006; Ødegård et al., 2006, 2007). Linear models (LAM and LSM) which take time to death into account are more precise than threshold models (using binary trait definition). Similar conclusions have been reported by other studies for disease resistance in Atlantic salmon (Ødegård et al., 2006, 2007; Yáñez et al., 2013). In this case, however, time until death is only known for non-survivors, while the censoring (survivors at end of test) does not contain any information besides the minimum survival time (right-censored data) (Ødegård et al., 2011a). That is, if the bacteria appear to be non-pathogenic or at least non-lethal to parts of the population (i.e., resistant or tolerant fish), the analyses may be biased (Ødegård et al., 2011a). In aquaculture species, therefore, new methods accounting for such factors in challenge test data are needed to be developed.

The aim of this study was to evaluate the genetic components of survival to *V. harveyi*. The estimated underlying heritabilities for disease resistance were moderate (0.19–0.28) based on the linear models regardless which kind of trait definition was used. The heritabilities, however, were slightly lower (0.11–0.16) when estimated based on threshold models by using binary survival definition. This was highly consistent with the results of other two bacteria challenge test (i.e. *Vibrio anguillarum* and *Edwardsiella tarda*) in Chinese tongue sole (Liu et al., 2016). The results from these studies however appear to contradict the findings in other aquaculture species that heritabilities estimated by threshold models were larger than linear models (Ødegård et al., 2006, 2007; Bangera et al., 2014; Xiong et al., 2017). In aquaculture fish species, the heiritabilies of bacteria disease resistance traits usually range from 0 to 0.62 (Ødegård et al., 2011a). However, due to the differences in infection mode, trait definition, mortality level, infectious agent and statistic model across different studies, a proper

comparative analysis of these studies were not available (Antonello et al., 2009). In this paper, despite the differences in heritability estimates, the ranking of families was highly identical in all the four models used, as indicated by the correlations (~1) between predicted full-sib family EBVs (assessed based on the entire dataset and Trait 1 definition). This result also meant that different model selections had little influence on the ranking of the families. Numerous previous studies have reported the similar results (Gitterle et al., 2006; Ødegård et al., 2006, 2007; Bangera et al., 2014).

The potential to improve disease resistance of aquaculture species depends on the magnitude of additive genetic variance present in the population and genetic interrelationship between the disease and other traits (e.g. growth) in the breeding goal. Favourable genetic correlations (low to moderately positive) have been estimated between growth traits and survival in both field and challenge tests (Gjedrem et al., 1991; Perry et al., 2004; Gjedrem and Olesen, 2005; Antonello et al., 2009; Nielsen et al., 2010). Conversely, low to moderately negative genetic correlations between growth traits and resistance to specific pathogens have also been reported (Henryon et al., 2002; Bangera et al., 2011; Yáñez et al., 2014; Evenhuis et al., 2015; Yáñez et al., 2016). Recently, Flores-Mara et al. (2017) revealed that resistance against infectious pancreatic necrosis is not genetically correlated with harvest weight in rainbow trout, *Oncorhynchus mykiss*. In general, it is reasonable that the relationship between growth-related traits and resistance to specific pathogens varies among species or within the same species in genetic terms. Here, the genetic correlations between growth and resistance to *V. harveyi* are moderate (0.27–0.51), indicating that genetic selection to increase genetic resistance to *V. harveyi*, will have an effect on growth (i.e. BW and TL) in tongue sole, and vice versa.

5. Conclusion

To the best of our knowledge, this is the first genetic analysis of *V. harveyi* based on challenge test data by using different trait definitions implemented in different models in Chinese tongue sole. The low to moderate heritabilities indicated that there is a large potential for genetic improvement in vibrio resistance by challenge test. The correlations of full-sib family EBVs between different models were all close to unity which might reveal the similar predictive ability of the four models. The levels of genetic variation and the magnitude of the genetic correlations between resistance to *V. harveyi* and growth traits presented in this study demonstrate the feasibility for the improvement of these traits simultaneously by means of selective breeding.

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