Estimation of Quantitative Genetic Parameters Using Marker-Inferred Relatedness in Japanese Flounder: A Case Study of Upward Bias

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Abstract

Marker-based methods for estimating heritability have been proposed as an effective means to study quantitative traits in long-lived organisms and natural populations. However, practical examinations to evaluate the usefulness and robustness of a regression method are limited. Using several quantitative traits of Japanese flounder Paralichthys olivaceus, the present study examined the influence of relatedness estimator and population structure on the estimation of heritability and genetic correlation under a regression method with 7 microsatellite loci. Significant heritability and genetic correlation were detected for several quantitative traits in 2 laboratory populations but not in a natural population. In the laboratory populations, upward bias in heritability appeared depending on the relatedness estimators and the populations. Upward bias in heritability increased with decreasing the actual variance of relatedness, suggesting that the estimates of heritability under the regression method tend to be overestimated due to the underestimation of the actual variance of relatedness. Therefore, relationship structure and precise estimation of relatedness are critical for applying this method.

An understanding of phenotypic variation of quantitative traits is of fundamental significance in evolutionary biology, conservation biology, as well as applied genetics. Heritability and genetic correlation are principal genetic parameters for understanding variation of quantitative traits (Falconer and Mackay 1996). Genetic correlation between traits can arise by 2 mechanisms: One is pleiotropy and the other is gametic phase disequilibrium between genes affecting different traits (Lynch and Walsh 1998). Heritability and genetic correlation are classically estimated from the degree of phenotypic resemblance between relatives. This requires a controlled breeding program or information on pedigree, making it difficult to estimate heritability and genetic correlation in organisms which are long-lived, difficult to culture, or residing in natural populations (Ritland 2000).

DNA polymorphisms have been extensively employed as a means of assessing genetic diversity of organisms and have made it possible to study previously inaccessible questions. Efforts have been made to use molecular markers for estimating relatedness among individuals of unknown pedigree, which is subsequently useful to estimate heritability (Thompson 1975; Lynch 1988; Queller and Goodnight 1989; Ritland 1996b; Mousseau et al. 1998; Lynch and Ritland 1999; Thomas and Hill 2000, 2002; Smith et al. 2001; Wang 2002, 2004). Methods for estimating relationship among individuals from molecular markers fall into 3 major classes: pairwise likelihood estimators, group likelihood estimators, and methods-of-moments estimators. Pairwise likelihood estimators calculate the likelihood for a pair falling into alternate relationship classes (Thompson 1975; Mousseau et al. 1998). The use of pairwise likelihood estimators is limited because this approach requires previous knowledge of the size of relationship classes, such as a mixture of full sibs versus unrelated individuals. Group likelihood methods consider all individuals in the entire sample and partition them simultaneously into distinctive genetic groups of variable sizes, such as full sibs, half sibs, and unrelated individuals (Thomas and Hill 2000, 2002; Smith et al. 2001; Wang 2004). Smith et al. (2001) reported the data on Atlantic salmon Salmo salar showing that most individuals were correctly classified into full-sib groups using this approach with 4 microsatellite loci. Thomas and Hill (2000) proposed the use of a group likelihood method to partition populations into sib groups that was then used in restricted maximum likelihood (REML) technique under an animal model to estimate heritability. The usefulness of
this approach was demonstrated in Soay sheep *Ovis aries* (Thomas et al. 2002) and rainbow trout *Oncorhynchus mykiss* (Wilson et al. 2003).

On the other hand, the methods-of-moments estimators are designed for estimating relatedness, which is the probability that genes are identity-by-descent. Several investigators have developed pairwise relatedness estimators (Queller and Goodnight 1989; Ritland 1996b; Lynch and Ritland 1999; Wang 2002). Marker-based relatedness estimators have sampling variance due to variance in identity-by-descent among loci and variance in identity-by-state for alleles that are not identical by descent (Lynch and Ritland 1999; Van de Casteele et al. 2001). In natural populations of 2 bird and 1 mammal species, Van de Casteele et al. (2001) showed that microsatellite-based estimates explained between 25% and 79% of variation in true relatedness. On the basis of the methods-of-moments estimators, Ritland (1996a) has proposed a method for estimating heritability which involves regressing quantitative trait similarity on marker-estimated relatedness between individuals. Therefore, this approach does not require any information on explicit pedigree, but it relies on the estimation of relatedness between individuals. However, practical information on the estimation of heritability and genetic correlation using the regression method has been limited (Ritland and Ritland C 1996; Klapier et al. 2001; Thomas et al. 2002; Wilson et al. 2003; Ritland and Travis 2004; Andrew et al. 2005; Shikano 2005; van Kleunen and Ritland 2005). In addition, few studies reported reliable heritabilities and genetic correlations using this approach (Ritland K and Ritland C 1996). More practical examinations using various organisms and populations with several quantitative traits will be necessary to discuss the usefulness and robustness of the regression method. This is partly because genetic structure of a population differs remarkably among taxa due to several factors such as ecological and reproductive characteristics between organisms. Furthermore, performance of the regression method may be influenced by both pairwise relatedness estimators and the genetic structure of a population. The lack of such examinations has made it difficult to evaluate usefulness and robustness of the regression method.

One of the main potential applications of marker-based methods for estimating quantitative genetic parameters is for systems where individuals are difficult to track. Teleosts are one of the most important taxa in aquatic systems where pedigrees are hard to build. Japanese flounder is one such fish that is economically important and widely distributed around Japan. Enormous numbers of juveniles are produced and released to the ocean for stock enhancement. Genetic analysis on several quantitative traits will contribute to studying ecological and conservation questions of this species (Minami 1997; Kinoshita et al. 2000). As their generation time is approximately 3–4 years, a straightforward and precise method is desired for the genetic analyses of quantitative traits. The present study focused on the influence of population structure and relatedness estimator on the estimation of quantitative genetic parameters under a regression method reported by Ritland (1996a). Using 4 relatedness estimators with 7 microsatellite loci, heritability and genetic correlation were estimated in 1 natural and 2 laboratory populations of Japanese flounder. Because heritability varies largely among traits (Mousseau and Roff 1987; Roff and Mousseau 1987; Falconer and Mackay 1996; Roff 1997), this study examined several quantitative traits, including growth-related traits, morphometric traits, body color, and tolerance to environmental stress. In addition to the regression method, heritability and genetic correlation were estimated from REML using pedigree information reconstructed by a likelihood method. Potential causes of upward bias in heritability under the regression method were discussed.

### Materials and Methods

#### Animals

One natural population and 2 laboratory populations were used in this study. Samples of the natural population (FS) were caught at the coast of Fukushima prefecture, Japan, on 9 October 2002. An otolithic analysis showed that all the specimens were 1 year old. Two laboratory populations were produced by different groups of parents: 1 was wild-caught individuals (FSNR-N) and the other was domesticated individuals (FSNR-D). The FSNR-N population was produced by a parental stock of 19 individuals, which were caught at the coast of Fukushima prefecture and reared at Fukushima Prefectural Nursing Research Institute, Japan. The FSNR-D population was produced by a parental stock of 23 individuals, which originated from the wild fish caught at the coast of Iwate prefecture, Japan, and domesticated for one generation at Fukushima Prefectural Nursing Research Institute. The FSNR-N and FSNR-D populations were simultaneously reared under the same environmental conditions. In each population, fish were reared in a 100-l tank with flow-through seawater from Days 0 to 45 and a 1000 l tank from Days 45 to 130. In each population, 500 individuals were moved from the 100-l tank to the 1000-l tank at Day 45. The water temperature of the tanks ranged from 15.6 °C to 23.9 °C. Fish were fed *Brachionus plicatilis* from Days 4 to 21, *Artemia salina* from Days 11 to 39, and dry pellets after Day 18. Fish samples were collected from the each stock at 130 days after birth.

#### Quantitative Traits

Quantitative traits examined in laboratory populations were growth-related traits (standard body length, body weight, and condition factor), morphometric traits (vertebral count and dorsal and anal fin ray counts), body color (brightness and hypermelanosis area), and tolerance to environmental stress conditions (high temperature tolerance and salinity tolerance). In a natural population, growth-related traits and morphometric traits were examined.

Standard body length was measured as the distance from the anterior tip of snout to the posterior edge of hypural. Condition factor was calculated as 1000(BW/BL³), where BW and BL were body weight in milligrams and standard body length in millimeters, respectively.
length in millimeters, respectively. Vertebral count and dorsal and anal fin ray counts were measured under a Super Soft X-ray apparatus (CMDW-2, Softex, Tokyo). The morphometric traits were analyzed using individuals with normal vertebrae.

To measure body brightness and hypermelanosis area, the ocular and blind sides of the fish body were scanned by a scanner (GT-9300UF, Epson, Tokyo) with 150 dpi resolution in color mode. Body brightness on the ocular side was measured at 10 points of a 5 × 5 pixel area for each individual, using software (Adobe Photoshop 6.0, Adobe Systems, San Jose, CA; GIMP 1.2, written by S. Kimball and P. Mattis, available at http://www.gimp.org/). Hypermelanosis is typically observed on the blind side of the fish reared under artificial breeding conditions (Seikai 1997).

Hypermelanosis area was measured as 100(HA/BA), where HA and BA are hypermelanosis area and body area excluding fins, respectively, with image analysis software (NIH Image 1.0, developed at US National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/).

Survival times in extremely high temperature or high salinity were measured to examine high temperature tolerance or salinity tolerance, respectively. Such measurements were useful to examine high temperature adaptability (Kanda et al. 1992; Okabe et al. 2004) or seawater adaptability (Shikano et al. 1997; Shikano and Fujio 1998). In addition, tolerance to high temperature or salinity can be a useful indicator of vigor in fish (Fujio et al. 1995; Shikano and Taniguchi 2003). High temperature tolerance was measured as survival time after transfer from 22.5 °C to 33.0 °C. Salinity tolerance was measured as survival time after transfer from 34 ppt seawater to 80 ppt artificial seawater (Aquasalz, Nissei, Tokyo) at 22.5 °C. Before the tests of high temperature tolerance and salinity tolerance, fish were maintained at water temperatures of 22.2–22.8 °C for 5 days. Fish were put into 100-l aquariums with up to 30 individuals per aquarium for the test of high temperature tolerance or salinity tolerance. Dead fish were recorded at 15-min intervals until all fish died. High temperature tolerance or salinity tolerance of the FSNR-N and FSNR-D populations was simultaneously examined under the same experimental conditions. Statistical comparisons for quantitative traits between FSNR-N and FSNR-D were assessed using f test.

**Microsatellite Analysis**

DNA was extracted from fin tissue stored in ethanol using phenol–chloroform extraction (Taggart et al. 1992). Seven microsatellite loci (Po1, Po3, Po4, Po13, Po42, and Po91) reported by Takagi et al. (1999) and Sekino and Hara (2000) were used in this study. Polymerase chain reaction (PCR) was conducted in an 8-μl reaction volume containing 10–20 ng of template DNA, 20 pmol of each primer set (1 fluorescently labeled), 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, 0.5 units of Taq DNA polymerase (TAKARA BIO Inc., Otsu, Japan), and 0.8 μl of 10× PCR buffer. The reactions were proceeded as follows: an initial denaturation step at 94 °C for 2 min, followed by 40 s at 94 °C, 40 s at annealing temperature (50 °C for Po1, Po3, and Po4, 55 °C for Po13, Po42, and Po91, and 58°C for Po13), and 90 s at 72 °C for 30 cycles with a final extension at 72 °C for 5 min. PCR products were visualized using an automated sequencers (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA), and alleles were scored using GeneScan 2.0 (Applied Biosystems). Microsatellite data were analyzed using Arlequin 2.0 (Schneider et al. 2000). The amount of differentiation of allele frequencies was quantified using standardized FST of Weir and Cockerham (1984). Statistical significance of the F value was determined by bootstrapping of 1000 replicates. Statistical comparison for heterozygosity was assessed using Kruskal–Wallis test and Mann–Whitney U test.

**Quantitative Genetic Parameters from Regression Method**

Pairwise relatedness was measured using 4 estimators (Queller and Goodnight 1989; Ritland 1996b; Lynch and Ritland 1999; Wang 2002). The calculation was performed with the microsatellite data at 7 loci using the Mark program (written by K. Ritland, available at http://genetics.forestry.ubc.ca/ritland/programs.html).

Heritability and genetic correlation was estimated according to Ritland (1996a, 2000) with the microsatellite data at 7 loci using the Mark program. The estimation of heritability was on the basis of a simple regression of pairwise trait similarity on pairwise relatedness. The phenotypic similarity of a quantitative trait Y for 2 individuals i and j was calculated as

\[ Z_{ij} = \frac{(Y_i - U)(Y_j - U)}{V}, \]

where U and V were the sample mean and variance of Y, respectively, in the population. Among all pairs, the average Z̄ij equals the phenotypic correlation. Genetic components of similarity were estimated as

\[ Z_{ij} = 2r_{ij}b^2 + e_{ij}, \]

where rij was the relatedness coefficient, b2 was the heritability, and eij was the random error. In this study, 4 relatedness estimators were used (Queller and Goodnight 1989; Ritland 1996b; Lynch and Ritland 1999; Wang 2002). Heritability was estimated as

\[ \hat{h}^2 = \frac{Cov(Z_{ij}, r_{ij})}{2Var(r_{ij})}, \]

where Cov(Zij, rij) was the covariance between estimated relatedness and phenotypic similarity and Var(rij) was the actual variance of relatedness. The estimation of the actual variance of relatedness was described in detail in Ritland (1996a, 2000). For 2 characters (1 and 2), the phenotypic covariance between individuals (i and j) was determined as

\[ Y_{12ij} = (Y_{1i} - U_1)(Y_{2j} - U_2). \]

Additive genetic correlation was estimated as

\[ r_{A12} = \frac{r_{ij}Y_{12ij}}{\sqrt{r_{ij}Y_{11ij}r_{ij}Y_{22ij}}}. \]

Significance of genetic parameters estimated was determined by bootstrapping over individuals with a bootstrap.
number of 1000. Estimates were deemed significant if 95% of the bootstrap values were found to be positive for heritability and positive or negative for genetic correlation. Standard errors (SEs) of genetic parameters were computed with the bootstrap method, where individuals were the unit of resampling.

Quantitative Genetic Parameters from Pedigree Reconstruction

Individuals were putatively partitioned into full sibs and half sibs with nested full sibs using a likelihood method reported by Wang (2004). This estimation was performed with the genotype data at 7 microsatellite loci using the COLONY 1.1 program (written by J. Wang, available at http://www.zoo.cam.ac.uk/ioz/software.htm).

Reconstructed pedigrees were subsequently used under an animal model to estimate the additive genetic and residual (co)variances for quantitative traits, using the Multiple Trait Derivative-Free Restricted Maximum Likelihood program (Boldman et al. 1995). The animal model for single- and multiple-trait analyses included random effects for animal and residual effects. Estimation of genetic parameters in this model involved partitioning phenotypic covariance between relatives into its components using the degree of relationship between individuals (Boldman et al. 1995).

Results

Relatedness

Total number of alleles detected in 1 natural and 2 laboratory populations was 222 with 43 at Pol1, 31 at Pol3, 35 at Pol4, 29 at Pol13, and 25 at Pol42. The total number of alleles was 195 in the FS natural population, whereas it was 95 and 82 in the FSNR-N and FSNR-D laboratory populations, respectively. The number of alleles per locus was significantly higher in the natural population than in the laboratory populations (P < 0.01), but there was no difference between the laboratory populations (P > 0.05). Mean observed heterozygosity significantly differed from 0.871 to 0.978 among the populations (P < 0.05). FSNR-N showed significantly higher mean heterozygosity than FSNR-D did (P < 0.05).

Table 1. Actual variance of relatedness in several estimators and sibship reconstructed by a likelihood method in one natural and 2 laboratory populations

| Population   | N   | QG (1989)       | R (1996b)       | LR (1999)       | W (2002)       | Half sib | Pure full sib |
|--------------|-----|----------------|----------------|----------------|----------------|----------|---------------|
| Natural      |     |                |                |                |                |          |               |
| FS           | 50  | 0.0001 ± 0.0003| 0.0000 ± 0.0000| 0.0000 ± 0.0001| 0.0000 ± 0.0001| 16 (38)  | 12            |
| Laboratory   |     |                |                |                |                |          |               |
| FSNR-N       | 134 | 0.0120 ± 0.0013**| 0.0028 ± 0.0004**| 0.0095 ± 0.0012**| 0.0125 ± 0.0010**| 8 (27)   | 4             |
| FSNR-D       | 105 | 0.0052 ± 0.0010**| 0.0020 ± 0.0002**| 0.0044 ± 0.0008**| 0.0053 ± 0.0010**| 13 (45)  | 4             |

QG, Queller and Goodnight (1989); R, Ritland (1996b); LR, Lynch and Ritland (1999); and W, Wang (2002). Number of nested full-sib families within half-sib families is included in parentheses. Significantly positive actual variance at ** P < 0.01.

Table 2. Mean and coefficient of variance of various quantitative traits in 1 natural and 2 laboratory populations

| Trait          | Natural | Laboratory | Laboratory | Laboratory |
|----------------|---------|------------|------------|------------|
|                | FS      | FSNR-N     | FSNR-D     |            |
| N              |         |            |            |            |
| Growth-related |         |            |            |            |
| Body length (mm)| 50 279.3 ± 13.7 4.9 | 134 83.2 ± 9.8 11.8 | 105 100.3 ± 8.9 8.9 |
| Body weight (g) | 50 364.2 ± 51.7 14.2 | 134 7.9 ± 2.8 35.4 | 105 14.7 ± 4.0 27.2 |
| Condition factor | 50 16.6 ± 1.2 7.2 | 134 13.2 ± 1.3 9.8 | 105 14.2 ± 1.4 9.9 |
| Morphometric trait |         |            |            |            |
| Vertebral count | 50 38.3 ± 0.5 1.3 | 129 36.8 ± 0.7 1.9 | 99 36.8 ± 0.5 1.4 |
| Dorsal fin ray count | 50 72.9 ± 2.5 3.4 | 129 76.8 ± 2.5 3.3 | 99 77.7 ± 2.2 2.8 |
| Anal fin ray count | 50 56.0 ± 2.0 3.6 | 129 58.4 ± 2.0 3.4 | 99 58.9 ± 1.9 3.2 |
| Body color |         |            |            |            |
| Brightness | — — — — | 134 29.9 ± 3.9 13.0 | 105 25.3 ± 3.8 15.0 |
| Hypermelanosis area (%) | — — — — | 134 67.6 ± 17.2 25.4 | 105 63.7 ± 21.6 33.9 |
| Tolerance |         |            |            |            |
| High temperature (m) | — — — — | 57 133.9 ± 22.1 16.5 | 52 143.1 ± 16.9 11.8 |
| Salinity (m) | — — — — | 70 76.3 ± 6.6 8.7 | 44 95.1 ± 10.7 11.3 |

CV, coefficient of variance; SD, standard deviation.
Pairwise $F_{ST}$ was 0.089 among the 3 populations ($P < 0.01$). Pairwise $F_{ST}$ was 0.054 between FS and FSNR-N, 0.069 between FS and FSNR-D, and 0.115 between FSNR-N and FSNR-D ($P < 0.01$).

Actual variance of relatedness was examined using 4 different relatedness estimators (Table 1). In all the relatedness estimators, actual variance of relatedness was significantly greater than zero in the 2 laboratory populations ($P < 0.01$) but not in the natural population ($P > 0.05$). The actual variance of relatedness ranged between 0.0028 and 0.0125 in the FSNR-N, whereas it ranged between 0.020 and 0.0053 in the FSNR-D. In both the laboratory populations, the actual variance of relatedness was lower in the estimator of Ritland (1996b) than in the other estimators.

Sibship was reconstructed by a likelihood method using the genotype data at the 7 microsatellite loci (Table 1). All individuals of FS were classified into different nested full sibs or pure full sibs, although some individuals were identified as half sibs. Individuals of FSNR-N were partitioned into 8 half sibs with 27 nested full sibs and 4 pure full sibs. Individuals of FSNR-D were partitioned into 13 half sibs with 45 nested full sibs and 4 pure full sibs.

**Heritability**

Table 2 shows mean and coefficient of variation in 10 quantitative traits. Coefficient of variation was lower in morphometric traits, especially in vertebral count, than in the other quantitative traits in all the 3 populations. In contrast,
coefficient of variation in body weight was large in all the populations. A significant difference in the mean was observed in all the quantitative traits except vertebral count and anal fin ray count between the FSNR-N and FSNR-D \( (P < 0.05\) for high temperature tolerance and \( P < 0.01\) for the others). Both growth-related traits and tolerance were greater in the FSNR-D than in the FSNR-N.

Table 3 shows estimates of heritability under the regression method with 4 relatedness estimators and the reconstructed pedigree method. Under the regression method, large sampling variance of heritability was observed in the natural population in all the quantitative traits regardless of the relatedness estimators. No significantly positive heritability was detected in the natural population \( (P > 0.05)\). Variance of heritability in the laboratory populations was smaller than that in the natural population in all the quantitative traits and the relatedness estimators. In the 2 laboratory populations, estimates of heritability ranged between \(-0.11\) and \(1.63\) with the estimator of Queller and Goodnight (1989), between \(-0.09\) and \(3.08\) with the estimator of Ritland (1996b), between \(-0.06\) and \(1.61\) with the estimator of Lynch and Ritland (1999), and between \(-0.07\) and \(1.50\) with the estimator of Wang (2002). In all the relatedness estimators, heritability was significantly positive for vertebral count, dorsal fin ray count, brightness, and hypermelanosis area in both the populations \( (P < 0.01\) or \( P < 0.05)\). Significantly positive heritability was also found for anal fin ray count in the FSNR-N and for high temperature tolerance in the FSNR-D \( (P < 0.01\) or \( P < 0.05)\). Estimates of heritability were greater than one for 1 out of 10 quantitative traits in all the relatedness estimators except Ritland (1996b) in the FSNR-N. On the other hand, heritability was greater than one for several quantitative traits in the FSNR-D regardless of the relatedness estimators. In the traits with significant heritability, SEs tended to be larger in the estimates with the estimator of Ritland (1996b) than those with the other estimators.

Relationship of the actual variance of relatedness with covariance between estimated relatedness and phenotypic similarity in laboratory populations. The dashed line indicates where heritability is equal to 1.0.

![Figure 1. Relationship of the actual variance of relatedness with covariance between estimated relatedness and phenotypic similarity in laboratory populations. The dashed line indicates where heritability is equal to 1.0.](image)

Heritability and genetic correlation were not estimated in the natural population under the reconstructed pedigree method because all individuals of the natural population were divided into different full sibs. Estimates of heritability ranged between \(0.00\) and \(0.97\) in the FSNR-N and between \(0.00\) and \(0.55\) in the FSNR-D. Significantly positive heritability was observed for vertebral count, dorsal fin ray count, and hypermelanosis area in both the populations and for anal fin ray count in the FSNR-N. The reconstructed pedigree method indicated significant heritability for traits mostly in common with the regression method. Estimates of heritability for some quantitative traits were significant under the regression method but were not significant under the reconstructed pedigree method. Correlations between heritabilities estimated under the reconstructed pedigree method and those from the regression method with different relatedness estimators were examined in the 2 laboratory populations (Figure 2). Regardless of the relatedness estimators, there were significantly positive correlations between them in both the populations \( (P < 0.01\) or \( P < 0.05)\). Among the relatedness estimators, the regression slope varied between 1.087 and 2.698 in the FSNR-N and between 2.098 and 2.899 in the FSNR-D. Significant departure of the slope from one was observed for the estimator of Ritland (1996b) in the FSNR-N and for the estimators of Ritland (1996b) and Lynch and Ritland (1999) in the FSNR-D \( (P < 0.05)\). The regression slopes in the estimator of Ritland (1996b) tended to be greater than those in the other estimators in both the populations. Furthermore, the slope tended to be greater in the FSNR-D than in the FSNR-N in all the relatedness estimators. The regression slope negatively correlated with the actual variance of relatedness \( (P < 0.01)\).

Genetic Correlation

Genetic correlation was estimated between quantitative traits in each category (Table 4). Under the regression method, significant genetic correlation was not detected in the natural population \( (P > 0.05)\) but observed among morphometric traits in the FSNR-N and also between vertebral count and dorsal fin ray count and between brightness and hypermelanosis area in the FSNR-D.
Figure 2. Correlations between heritabilities estimated under the reconstructed pedigree method and the regression method with relatedness estimators of QG (Queller and Goodnight 1989, A and E), R (Ritland 1996a, Band F), LR (Lynch and Ritland 1999, C and G), and W (Wang 2002, D and H) in FSNR-N population (A–D) and FSNR-D population (E–H). The dashed lines indicate 1:1.
There is little possibility that the individuals flounder contains free-swimming egg and larval phases. Because the life cycle of Japanese population were classified into different full sibs using a likelihood method. Actual variance of relatedness in the natural population did not significantly differ from zero in all the estimators. This may be the main reason why the regression method did not give useful estimates of heritability in the natural population.

The laboratory populations used in this study were produced by limited numbers of parents by natural spawning. Therefore, relationship between the offspring may be classified into 3 categories: full sibs, half sibs, and unrelated individuals. Individuals of the laboratory populations were partitioned into pure full sibs and half sibs with nested full sibs using a likelihood method. Amount variance of relatedness was found to be significantly greater than zero in both the laboratory populations regardless of the relatedness estimators, as expected for populations in which there is some mixture of relatives. In the laboratory populations, the regression method indicated significantly positive heritability for several quantitative traits mostly in common with the reconstructed pedigree method. Using a sib analysis in a traditional quantitative genetic design, Furutsuka-Uozumi and Tabata (1999) reported that estimates of heritability were 0.0 for body size and 0.5 for body color abnormality in juvenile Japanese flounder. In addition, heritability was estimated as 0.0 for body size and 0.7 for dorsal and anal fin ray counts using a diallel cross of 4 dams and 4 sires (Shimada Y, Shikano T, unpublished data). In accordance with these conventional experiments, heritability estimated under the regression method was significantly positive for morphometric traits and color-related traits in the laboratory populations.

Estimates of heritability for some quantitative traits were greater than one under the regression method regardless of the relatedness estimators. Upward bias in heritability under the regression method was also found in other studies (Ritland K and Ritland C 1996; Wilson et al. 2003). In the present study, the actual variance of relatedness was particularly lower in the estimator of Ritland (1996b) than in the other estimators used in this study contained some relatives. Therefore, some individuals may have been incorrectly identified as half sibs due to errors in pedigree reconstruction, which are typically observed in likelihood methods (Smith et al. 2001; Thomas and Hill 2002; Wilson et al. 2003). As expected for large populations such as natural populations of an organism living in the ocean, the actual variance of relatedness in the natural population did not significantly differ from zero in all the estimators. This may be the main reason why the regression method did not give useful estimates of heritability in the natural population.

Figure 3. Correlation of the actual variance of relatedness with the regression slope between heritabilities under the reconstructed pedigree method and the regression method (see also Figure 2). The dashed line indicates a regression slope of 1.0. 

\[ y = 3.077 - 169.285x \]
\[ r^2 = 0.961 \]
\[ N = 8 \]
\[ P < 0.01 \]

\( P < 0.01 \) or \( P < 0.05 \). The reconstructed pedigree method indicated significant genetic correlation for the same combinations as the regression method. In some relatedness estimator, significantly positive genetic correlation was also observed in body color in the FSNR-N and in morphometric traits in the FSNR-D \( P < 0.05 \). In the combinations with significant genetic correlation, estimates of genetic correlation were similar among the relatedness estimators. However, SEs of the estimates tended to be higher in the estimator of Ritland (1996b) than in the other estimators.

### Discussion

The present results showed that all estimates of heritability in a natural population of Japanese flounder lay outside of the true parameter space (0–1) with large sampling variance in all quantitative traits, regardless of the pairwise relatedness estimators. Because this method is established on the regression between pairwise relatedness measured by molecular markers and pairwise phenotypic similarity of a quantitative trait, large variance of the 2 measures is essential for correct estimation of heritability (Ritland 1996a, 2000). Actual variance of relatedness occurs when there is some mixture of relatives, such as full sibs versus unrelated individuals (Ritland 2000). Although some individuals were identified as half sibs, all individuals of the natural population were classified into different full sibs using a likelihood method. Because the life cycle of Japanese flounder contains free-swimming egg and larval phases (Minami 1997), there is little possibility that the individuals

\( y = 3.077 - 169.285x \)
\[ r^2 = 0.961 \]
\[ N = 8 \]
\[ P < 0.01 \]
a low precision of the relatedness estimator of Ritland (1996b) in these laboratory populations.

The degree of upward bias in heritability differed between the laboratory populations as well as the relatedness estimators. Larger upward bias in heritability was observed in the FSNR-D population than in the FSNR-N population. In this context, the actual variance of relatedness in the FSNR-D population was considerably lower than that in the FSNR-N population. An important feature of these populations is that the FSNR-N population was developed by wild-caught fish, whereas the FSNR-D population was produced by domesticated fish. Genetic differences between these populations were indicated by the data of microsatellites and quantitative traits. Especially, mean heterozygosity of the FSNR-D population was significantly lower than that of the FSNR-N population. As the performance of relatedness estimators is affected by several factors including the number of loci, allele frequency distribution and the degree of actual relationships (Lynch and Ritland 1999; Van de Casteele et al. 2001), lower heterozygosity might result in poorer estimates of relatedness.

Based on relationship of the actual variance of relatedness with covariance between relatedness and phenotypic similarity, the present study indicated that upward bias in heritability increased with decreasing the actual variance of relatedness. In addition, the actual variance of relatedness was negatively correlated with regression slopes between heritabilities using the regression method and a reconstructed pedigree method. A critical feature observed through this study was that the upward bias was accompanied by low actual variance of relatedness, suggesting that the upward bias was mainly caused by the underestimation of the actual variance of relatedness. Ritland (1996a) indicated that the actual variance of relatedness affects the estimation of heritability but has no effect on the estimation of genetic correlation. In the present study, no correlation was observed between the degree of the actual variance of relatedness and the estimates of genetic correlation. This result strongly supported the presumption.

Until now, reliable estimates of heritability using the regression method was reported in a plant (Ritland K and

Table 4. Estimates of genetic correlation for various quantitative traits using a regression method with different relatedness estimators and are constructed pedigree method in one natural and two laboratory population

| Population | Trait                  | Regression method | Reconstructed pedigree method |
|------------|------------------------|-------------------|-------------------------------|
|            |                        | QG (1989) | R (1996a) | LR (1999) | W (2002) |       |
| Natural    |                        |           |           |           |           |       |
| FS         | Growth-related trait   |           |           |           |           |       |
|            | BL and BW              | 0.79 ± 15.39 | 0.40 ± 10.91 | 7.63 ± 152.94 | 0.79 ± 4.75 | —     |
|            | BL and CF              | -0.67 ± 42.28 | -0.33 ± 35.58 | -0.06 ± 30.39 | 5.29 ± 94.97 | —     |
|            | BW and CF              | -0.29 ± 3.21 | -0.75 ± 37.51 | 0.48 ± 23.36 | 7.43 ± 252.32 | —     |
|            | Morphometric trait     |           |           |           |           |       |
|            | VC and DFC             | 3.09 ± 86.31 | 3.91 ± 70.07 | 1.74 ± 64.21 | 10.77 ± 172.50 | —     |
|            | VC and AFC             | 0.39 ± 48.86 | 5.15 ± 175.50 | 1.50 ± 58.68 | -0.60 ± 50.34 | —     |
|            | DFC and AFC            | -0.26 ± 29.34 | 0.46 ± 35.95 | 0.35 ± 42.39 | 3.36 ± 84.01 | —     |
| Laboratory |                        |           |           |           |           |       |
| FSNR-N     | Growth-related trait   |           |           |           |           |       |
|            | BL and BW              | 1.06 ± 1.52 | 1.02 ± 2.76 | -0.12 ± 20.93 | 1.19 ± 5.53 | 0.01 ± 0.23 |
|            | BL and CF              | 1.34 ± 51.29 | 4.74 ± 82.64 | 9.23 ± 272.34 | 2.44 ± 83.46 | 0.00 ± 0.23 |
|            | BW and CF              | 0.10 ± 53.36 | 1.94 ± 78.13 | 2.12 ± 68.74 | 1.81 ± 56.55 | 0.01 ± 0.19 |
|            | Morphometric trait     |           |           |           |           |       |
|            | VC and DFC             | 1.01 ± 0.24** | 1.12 ± 2.66** | 1.09 ± 0.38** | 1.03 ± 0.28** | 1.00 ± 0.37* |
|            | VC and AFC             | 0.98 ± 0.24** | 1.15 ± 1.24** | 1.00 ± 0.25** | 1.03 ± 0.22** | 1.00 ± 0.40* |
|            | DFC and AFC            | 1.00 ± 0.12** | 1.03 ± 0.09** | 0.98 ± 0.11** | 1.00 ± 0.12** | 1.00 ± 0.16* |
|            | Body color             |           |           |           |           |       |
|            | BR and HA              | 2.09 ± 19.08 | 1.10 ± 1.36* | 1.04 ± 1.33* | 2.25 ± 28.36 | 0.03 ± 0.15 |
| FSNR-D     | Growth-related trait   |           |           |           |           |       |
|            | BL and BW              | 1.40 ± 10.66 | 0.59 ± 27.92 | 1.21 ± 20.42 | 1.38 ± 13.27 | 0.09 ± 0.21 |
|            | BL and CF              | 2.59 ± 45.60 | 1.65 ± 76.91 | 2.60 ± 95.40 | 2.99 ± 91.98 | 0.08 ± 0.21 |
|            | BW and CF              | 1.71 ± 34.29 | 1.81 ± 38.00 | 1.67 ± 47.11 | -0.82 ± 19.43 | 0.11 ± 0.24 |
|            | Morphometric trait     |           |           |           |           |       |
|            | VC and DFC             | 0.94 ± 0.28** | 0.91 ± 1.10** | 0.90 ± 0.72** | 1.05 ± 0.27** | 0.67 ± 0.43* |
|            | VC and AFC             | 0.62 ± 0.80* | 1.34 ± 13.72 | 0.74 ± 8.38 | 0.86 ± 0.92* | 1.00 ± 2.68 |
|            | DFC and AFC            | 0.92 ± 2.58* | 1.03 ± 5.98 | 0.78 ± 6.09 | 0.81 ± 0.62* | 1.00 ± 2.87 |
|            | Body color             |           |           |           |           |       |
|            | BR and HA              | 0.50 ± 3.91* | 0.54 ± 3.44* | 0.48 ± 1.98* | 0.34 ± 4.76* | 0.75 ± 0.50* |

QG, Queller and Goodnight (1989); R, Ritland (1996b); LR, Lynch and Ritland (1999); W, Wang (2002). BL, Body length; BW, body weight; CF, condition factor; VC, vertebral count; DFC, dorsal fin ray count; AFC, anal fin ray count; BR, brightness; HA, hypermelanosis area. Values indicate genetic correlation and SE. Significantly different from zero at *P < 0.05**P < 0.01 in regression methods. *Significant departure from zero in a likelihood method.
Ritland C 1996), but studies of other plants (Klaper et al. 2001; Ritland and Travis 2004) and animals (Thomas et al. 2002; Wilson et al. 2003) resulted in the unreliable estimation. The different consequences may be caused by several factors including population genetic structures, relatedness estimators, genetic markers, sample sizes, and quantitative traits. However, it was difficult to evaluate these factors because there were few studies that showed reliable estimates of heritability using this method. As demonstrated in the present study, the actual variance of relatedness should be one of the most important factors for reliable estimation of heritability. Ritland K and Ritland C (1996) found positive heritability for some quantitative traits in a monkeyflower population, where the actual variance of relatedness was 0.044. Significantly positive heritability was also reported in the study of Japanese flounder, where the actual variance of relatedness was 0.030 (Shikano 2005). In the studies which failed estimating reliable heritability, on the other hand, the actual variance of relatedness was 0.0005 or lower (Klaper et al. 2001; Thomas et al. 2002) and 0.002 to 0.005 (Wilson et al. 2003). These facts emphasize the importance of the actual variance of relatedness for the estimation of heritability under the regression method.

Few studies have reported significant genetic correlation using the regression method (Ritland K and Ritland C 1996). In the laboratory populations, significantly positive genetic correlation was detected for some combinations of quantitative traits in common with the reconstructed pedigree method. Although these estimates of genetic correlation were similar among the relatedness estimators, their SEs were considerably larger in the relatedness estimator of Ritland (1996b) than in the other estimators. This may be caused by a low precision of the estimator of Ritland (1996b) in these laboratory populations as suggested in the estimation of heritability. The high genetic correlation observed for some quantitative traits in common with the reconstructed pedigree shows the reliability of the relatedness estimator. However, it was difficult to evaluate these factors because there were few studies that showed reliable estimates of heritability using this method. As demonstrated in the present study, the actual variance of relatedness should be one of the most important factors for reliable estimation of heritability. Ritland K and Ritland C (1996) found positive heritability for some quantitative traits in a monkeyflower population, where the actual variance of relatedness was 0.044. Significantly positive heritability was also reported in the study of Japanese flounder, where the actual variance of relatedness was 0.030 (Shikano 2005). In the studies which failed estimating reliable heritability, on the other hand, the actual variance of relatedness was 0.0005 or lower (Klaper et al. 2001; Thomas et al. 2002) and 0.002 to 0.005 (Wilson et al. 2003). These facts emphasize the importance of the actual variance of relatedness for the estimation of heritability under the regression method.

The present study demonstrated that the performance of heritability estimation using a regression method largely depends on the actual variance of relatedness that is influenced by both pairwise relatedness estimators and the genetic structure of a population. The results of this study suggested that the estimates of heritability tend to be overestimated due to the underestimation of the actual variance of relatedness. Therefore, relationship structure and precise estimation of relatedness are critical for applying this method.

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