Estimating Trait Heritability in Highly Fecund Species

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ABSTRACT Increasingly, researchers are interested in estimating the heritability of traits for nonmodel organisms. However, estimating the heritability of these traits presents both experimental and statistical challenges, which typically arise from logistical difficulties associated with rearing large numbers of families independently in the field, a lack of known pedigree, the need to account for group or batch effects, etc. Here we develop both an empirical and computational methodology for estimating the narrow-sense heritability of traits for highly fecund species. Our experimental approach controls for undesirable culturing effects while minimizing culture numbers, increasing feasibility in the field. Our statistical approach accounts for known issues with model-selection by using a permutation test to calculate significance values and includes both fitting and power calculation methods. We further demonstrate that even with moderately high sample-sizes, the p-values derived from asymptotic properties of the likelihood ratio test are overly conservative, thus reducing statistical power. We illustrate our methodology by estimating the narrow-sense heritability for larval settlement, a key life-history trait, in the reef-building coral Orbicella faveolata. The experimental, statistical, and computational methods, along with all of the data from this study, are available in the R package multiDimBio.

KEYWORDS heritability nonmodel organisms common garden binary variable traits coral settlement

Organisms with high fecundity, small propagule size, and limited parental investment, also referred to as r-selected species, often exhibit higher levels of nucleotide diversity and/or standing genetic variation compared with k-selected species (Romiguier et al. 2014). Many marine species, including fish and invertebrates, exhibit these r-selected life history characteristics (Doherty and Fowler 1994) and indeed have been shown to exhibit high levels of genetic diversity (Bay et al. 2004; Davies et al. 2015). However, this high genetic diversity does little to predict how a population will respond to environmental perturbations, such as those caused by climate change. Instead, the key question is not how much variation is present, but what is the heritability of the traits under selection after the perturbation. Quantifying narrow-sense heritability, the proportion of phenotypic variance attributable to additive genetic effects (Lynch and Walsh 1998), for nonmodel organisms presents both experimental and statistical challenges. Most experiments aiming to quantify narrow-sense heritability involve multigenerational breeding programs and large numbers of crosses with many culture replicates to account for “jar effects,” both of which are rarely feasible in nonmodel species.

Here we present a quantitative genetic methodology for estimating the narrow-sense heritability of traits in highly fecund species. The method does not require the onerous sampling schemes usually required for these types of experiments. Instead, our approach leverages high fecundity by completing independent fertilizations with large quantities of eggs equally divided among sires to account for sperm competition (Figure 1). These cultures are then mixed into a single bulk culture (common garden) and divided into three replicate tanks per dam. Bulk larvae are then sorted according to the trait of interest, which in this study is a binary trait (whether or not the larvae underwent metamorphosis in response to settlement cue). Single larvae that “succeeded” and “failed” are then individually genotyped and their sire assignments are compared with the predicted distribution of sire assignments in the original design. This experimental design allows for all sires to be
cultured in “common garden” conditions, which greatly reduces the number of cultures compared with a standard approach, where each family would be cultured individually, resulting in a culture number of 3× the number of sires. The narrow-sense heritability of these data can be estimated using a generalized linear mixed model with a binomial error distribution. However, as we discuss herein, appropriately determining statistical significance is nontrivial. This method of quantifying heritability of binary traits is broadly applicable to many traits of interest including—but not limited to—stress tolerance, dispersal potential, and disease susceptibility. Furthermore, the framework we have developed—including the statistical methods—can be readily adapted to traits with different distributions, e.g., normally distributed phenotypes.

To demonstrate this methodology, we estimated the heritability of dispersal potential in reef-building coral larvae. The majority of corals—like many other marine invertebrates—release gametes into the water annually. These gametes develop into planktonic larvae that are dispersed by ocean currents, representing each coral’s only dispersal opportunity (Baird et al. 2009). The now pelagic larvae can travel great distances before settling on a reef, but once the larva settles, it will remain there for the duration of its life. Therefore, selection for dispersal potential is limited to optimizing larval traits, which can be investigated through classical quantitative genetics, e.g., Meyer et al. (2009). Specifically, we determined how much variation in the early larval responsiveness to settlement cue depends on the genetic background of larvae. The experiments were performed on larvae of the hermaphroditic mountainous star coral, Orbicella faveolata, which is an important but endangered Caribbean reef-building coral. To analyze these data, and estimate the narrow-sense heritability of this binary trait, we developed a Monte Carlo method for performing model selection and calculating statistical power with generalized linear mixed models. The code and data are available in the R package multiDimBio (Scarpino et al. 2014).

MATERIALS AND METHODS

Experimental framework

Our experimental framework, which is summarized in Figure 1, proceeds in four steps. First, we perform crosses between the desired number of parents. Second, all offspring from a single dam are reared in the same environment (“common garden”). Third, offspring are phenotyped for the trait of interest and genotyped to determine paternity. Fourth, these data are analyzed by the use of random-effects models and a permutation test to determine statistical significance. What follows is a detailed description of how to estimate the narrow-sense heritability of coral settlement using this framework.

Application of the experimental framework to coral settlement

Crossing design and larval rearing: One day before the annual coral spawn on August 7, 2012, 10 independent O. faveolata colony fragments (10 cm × 10 cm) were collected from the East Flower Garden Banks National Marine Sanctuary, Gulf of Mexico. Colonies were maintained in flow through conditions aboard the vessel and were shaded from direct sunlight. Colonies were at least 10 m apart to avoid sampling clones, as clones within reefs have been detected in this genus (Severance and Karl 2006; Baums et al., 2010). However, intracolony variation (chimerism) in scleractinian corals is very rare (Puill-Stephan et al., 2009), so each sire was assumed to only produce sperm of a single genotype. Before spawning, at 20:00 Central Daylight Time (CDT) on August 8, 2012, colonies were isolated in individual bins filled with 1 μL of filtered seawater and were shaded completely. Nine colonies spawned at approximately 23:30 CDT. From these spawning colonies, we collected gamete bundles and separated eggs and sperm with nylon mesh. Each colony was used as an independent sire, with no additional sperm/sires included in this study. Samples from each sire were preserved in ethanol for genotyping.

Divers collected gamete bundles directly from three colonies during spawning and eggs were separated to serve as maternal material (N = 3 dams). Eggs were divided equally among fertilization bins...
garden Banks National Marine Sanctuary permit #FGBNMS-2012-002.

of Texas at Austin on August 10, 2012. After spawning, colony frag-
culture containers (N = 3 per dam). Larvae were transferred to the University
into three replicate culture vessels at one larva per 2 mL for a total of nine
common culture. Densities were determined and larvae were stocked
utilization, at 08:00 CDT, excess sperm was removed by rinsing with ny-

presented larvae exhibiting
removed and recruits were individually preserved in 96% ethanol, rep-
et al.

On August 14, 2012, 6-d-old,
Common garden settlement assay: On August 14, 2012, 6-d-old,

agreement that self-fertilization was not detectable in our samples. After fer-
tilization, at 08:00 CDT, excess sperm was removed by rinsing with nylon
mesh, and embryos for each dam across all sires were pooled in one
common culture. Densities were determined and larvae were stocked
into three replicate culture vessels at one larva per 2 mL for a total of nine
culture containers (N = 3 per dam). Larvae were transferred to the University
of Texas at Austin on August 10, 2012. After spawning, colony frag-
ments were returned to the reef. All work was completed under the Flower
garden Banks National Marine Sanctuary permit #FGBNMS-2012-002.

Common garden settlement assay: On August 14, 2012, 6-d-old,
precompetent larvae from the three replicate bins for a single dam were
divided across three settlement assays. Four hundred healthy larvae per
culture replicate were added to a sterile 800-mL container with five
conditioned glass slides and finely ground, locally collected crustose
coraline algae, a known settlement inducer for this coral genus (Davies
et al. 2014). Cultures were maintained for 3 d, after which each slide was
removed and recruits were individually preserved in 96% ethanol, rep-
resenting larvae exhibiting “early” responsiveness to settlement cue. Culture
water was changed, new slides were added with additional

larval DNA extraction: Larval DNA extraction followed a standard
phenol-chloroform iso-amyl alcohol extraction protocol, see Davies
et al. (2013), with modifications to accommodate for the single larva
instead of bulk adult tissue.

Parental genotyping: Sire genotyping was completed with the use of
nine loci from Davies et al. (2014) and four loci from Severance et al.
(2004) following published protocols. Amplicons were resolved on
agarose gel to verify amplification and molecular weights were analyzed
using the ABI 3130XL capillary sequencer. GeneMarker V2.4.0 (Soft
Genetics) assessed genotypes and loci representing the highest allelic
diversities among the sires were chosen as larval parentage markers. To
ensure that each sire was a unique multilocus genotype (MLG) and that
the relatedness between sires was negligible, we compared the allelic
composition of each sire across six microsatellite loci (MLG) and cal-
culated the Probability of Identity at each locus in GENALEX v6.5 from
Peakall and Smouse (2006).

Larval parentage: To compensate for the low larval DNA concentra-
tions, 3 µL of each single extracted larva (unknown concentration) was
amplified in a multiplex reaction with six loci from Davies et al. (2013)
with the following modifications: 1µM of each fluorescent primer pair
(N = 6) and 20-µL reaction volumes (Table 1). Alleles were called in
GeneMarker V2.4.0 and offspring parentage was assigned based on
presence/absence of sire alleles. Data were formatted into a dataframe
consisting of the number of early settlers and swimming larvae that
were assigned to each sire (A-J) from each of three replicate bins (1–3).

Statistical methods

Estimating narrow-sense heritability from binary data: In principle,
estimating narrow-sense heritability for a binomially distributed trait,
such as coral settlement, is straightforward, see Gilmour et al. (1985);
Foulley et al. (1987); Vazquez et al. (2009); Biscarini et al. (2014, 2015).
The desired quantity is the among-sire variance, denoted as \( \tau^2 \), which
can be estimated with a generalized linear mixed model with a binomial
error distribution. Although this a departure from the standard thresh-
old approach for estimating the heritability of binomial traits, it is now
fairly common in the quantitative genetics literature, see Foulley
et al. (1987) and Vazquez et al. (2009).

Suppose we have binary observations \( y_{ij} \in \{0,1\} \) where \( i \) index
units (sires) and \( j \) indexes observations within units. The model is
simple Bernoulli sampling, parameterized by log odds:

\[
P(y_{ij} = 1) = \frac{1}{1 + \exp(-\psi_{ij})}.
\]

We will assume that the log odds have a sire-level random effect:

\[
\psi_{ij} = \alpha + \beta_i, \quad \beta_i \sim N(0, \tau^2). \]

Thus we have a simple binary logit model with a single random effect.
A standard result on logit models is that we can represent the outcomes
\( y_{ij} \) as thresholded versions of a latent continuous quantity \( z_{ij} \) (Holmes
et al. 2006):

| Table 1 Summary of the six microsatellite loci from Davies et al. (2013) used in paternity assignment |
|-------------------------------------------------|
| Locus  | Observed length (bp) | \( N_s \) | Fluorescence |
|--------|----------------------|---------|-------------|
| M_fav4 | 375-391              | 5       | FAM         |
| maMS2-5 | 280-328             | 20      | FAM         |
| maMS8  | 197-203              | 3       | FAM-HEX-NED |
| M_fav6 | 387-429              | 11      | HEX         |
| M_fav7 | 453-498              | 9       | HEX         |
| maMS2-8 | 187-205             | 10      | NED         |

\( N_s \) is the number of alleles. FAM, HEX, and NED are fluorescent dyes used for
labeling the markers so that they can be multiplexed.

To compensate for the low larval DNA concentra-
tions, 3 µL of each single extracted larva (unknown concentration) was
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consisting of the number of early settlers and swimming larvae that
were assigned to each sire (A-J) from each of three replicate bins (1–3).

| Table 2 Summary of paternity assignment results |
|-----------------------------------------------|
| Sire   | Locus 1 MaMS8 | MaMS8.1 Sev5 | Locus 2 Sev5.1 | Locus 3 Mfav4 | Mfav4.1 | Locus 4 Mfav6 | Mfav6.1 | Locus 5 Mfav7 | Mfav7.1 | Locus 6 Sev8 | Sev8.1 |
|--------|---------------|---------------|----------------|----------------|---------|---------------|---------|---------------|---------|---------------|---------|
| A      | 200           | 200           | 280            | 322            | 379     | 391           | 391     | 453           | 465     | 190           | 196     |
| B      | 200           | 200           | 292            | 322            | 379     | 391           | 391     | 453           | 465     | 190           | 196     |
| C      | 200           | 200           | 283            | 313            | 375     | 375           | 419     | 429           | 453     | 471           | 486     |
| D      | 197           | 200           | 301            | 322            | 375     | 379           | 423     | 423           | 465     | 486           | 190     |
| E      | 200           | 200           | 283            | 316            | 375     | 379           | 389     | 389           | 453     | 474           | 190     |
| F      | 197           | 197           | 307            | 313            | 375     | 375           | 391     | 391           | 462     | 471           | 190     |
| G      | 197           | 200           | 301            | 328            | 379     | 379           | 391     | 391           | 474     | 193           | 205     |
| H      | 197           | 200           | 280            | 307            | 383     | 383           | 389     | 389           | 453     | 453           | 190     |
| J      | 197           | 200           | 280            | 313            | 379     | 379           | 389     | 389           | 477     | 498           | 193     |

Values are the microsatellite lengths for each of six loci from Davies et al. (2014)
exploiting the facts that the sire-level random effect.

where $e_{ij}$ follows a standard logistic distribution. Note this nonstandard form of latent-threshold model, wherein the errors $e_{ij}$ are logistic rather than normally distributed. Upon integrating out the $z_{ij}$ values (which are often referred to as latent or data-augmentation variables), we recover exactly the logistic regression model of Equation (1) with a sire-level random effect.

In light of this, we can interpret narrow-sense heritability in terms of the ratio of predictable to total variation in our logistic random-effects model. This is often referred to as the Bayesian $R^2$, by analogy with the classical coefficient of determination in a regression model:

$$R^2 = \frac{\text{var}(\beta_i)}{\text{var}(z_{ij})} = \frac{\text{var}(\beta_i)}{\text{var}(\beta_i) + \text{var}(e_{ij})} = \frac{\tau^2}{\tau^2 + \pi^2/3},$$

exploiting the facts that the $\beta_i$ and $e_{ij}$ are independent and that the variance of the standard logistic distribution is $\pi^2/3$. The aforementioned equation for the Bayesian $R^2$ is the narrow-sense heritability for the animal model. Therefore, the among-sire variance can be transformed into an approximation of narrow-sense heritability under the sire model by multiplying the Bayesian $R^2$ by four, see Foulley et al. (1987) and Vazquez et al. (2009) for a more detailed derivation and Lynch and Walsh (1998) for a discussion of the assumptions this approximation relies on.

However, under this model, determining whether statistical support exists for an among-sire variance greater than zero remains a challenge. Traditionally, an approach to the problem would be to fit two models, one where $\tau^2$, the among-sire variance, is a free parameter and one where it is constrained to zero. These models can then be compared, and model selection performed, with a likelihood ratio test, or in this case the difference in each model’s deviance, which is equivalent to a likelihood ratio test for nested models. Although, critically, this is a special kind of likelihood ratio test because the null hypothesis resides on the edge of the parameter space. The large sample reference distribution for this type of test is usually considered to be a 50% mixture of a point at mass at zero and a $\chi^2(1)$ (Self and Liang 1987). However there is still substantial debate in the literature about what mixture should be used—e.g., Crainiceanu et al. (2003)—and it is not clear whether any of these mixtures are valid null distributions for finite sample sizes.

Instead, our approach is to construct a permutation-based method for calculating a p-value for the likelihood ratio test and performing model selection. This test is simple to implement, because it only involves randomly shuffling the identity of each offspring’s sire a large number of times (say, 500) and refitting the random-effects model to each shuffled data set. This avoids making assumptions about the asymptotic distribution of the test statistic that may fail to hold for finite sample sizes.

Monte Carlo simulation for the likelihood ratio test: Our simulations assume a fixed probability of settlement, $p_{settle}$, to be equal across all sires, in this case $p_{settle} = 0.285$ (the global mean), and simulate 1000 data sets where the number of offspring for each sire in each of three bins is drawn from a negative binomial distribution with $\mu = 4.63$ and size $= \mu^2 / (\sqrt{12.63} - \mu)$, again these are the empirically observed values across sires. The resulting 1000 data sets have the same structure as the observed data, but the only among sire variability comes from sampling, the true $\tau^2 = 0$. For each simulated data set, we calculated the likelihood-ratio test statistic. This provides a Monte Carlo approximation to the true sampling distribution of the test statistic under the null.

Power analysis: With any novel experimental design, it is desirable to construct a method for estimating its statistical power. Using the Monte Carlo approach designed to calculate p-values for likelihood ratio tests, we can simulate data sets with an arbitrary number of sires, number and variance in offspring, among-sires variance, and number of bins. By repeatedly simulating data sets with fixed combinations of these parameters, the statistical power is simply the fraction of times we correctly reject the null hypothesis. Similarly, the false-positive rate is the fraction of times we falsely reject the null hypothesis.

Data availability

All code and data developed for this study are available in the R package multiDimBio (Scarpino et al. 2014). The statistical models were fit using the R packages stats in R version 3.2.1 (R Core Team 2015) and lme4 version 1.1-8 (Bates et al. 2015).

RESULTS

Sire independence

Each sire was determined to be a unique MLG across the six microsatellite loci indicating that no clones were collected (Table 2). To ensure
that each sire could be considered independent, we calculated the Probability of Identity at each locus and found that these probabilities ranged from 3.2E-01 for a single locus down to 2.0E-06 when all six loci are considered and therefore each sire was considered independent.

Parentage
Larvae that amplified at >2 loci were considered successful amplifications. A total number of 55 recruits (binary successes) were collected and of these 47 were amplified and 37 were assigned parentage. A total number of 129 swimming larvae (binary failures) were extracted and of these 112 amplified successfully and 81 were assigned parentage (Figure 2).

Monte Carlo simulation for the likelihood ratio test
To test whether the procedure proposed in this study provided any benefits over the traditional approach to performing a likelihood ratio test, we first simulated the true sampling distribution of the likelihood ratio statistic under the null hypothesis. This was accomplished by repeatedly simulating data from a model where the true among-sire variance ($\sigma^2$) was zero. The cumulative distribution function of this random variable is shown as a black curve (actual null) in Figure 3. We then calculated two approximations to this sampling distribution; these cumulative distribution functions are also plotted in Figure 3. First, the red curve (theoretical null) shows a mixture distribution of a point mass at 0 (with probability 0.5) and $\chi^2(1)$ random variable (with probability 0.5). This is the asymptotic approximation to the true null used in the traditional likelihood-ratio test of a variance component in a mixed-effects model. Second, the dotted gray curve (permutation null) shows the estimated null distribution obtained by running the permutation test on a single simulated data set. The permutation null is clearly a better approximation to the actual null than is the theoretical null, whose distribution is shifted to the right. This fact suggests that—at least for data sets similar to ours—the asymptotic approximation is too conservative, and will therefore lead to reduced power at a specified false-positive rate.

Statistics
Using the described experimental design and statistical methods, we were unable to detect a significant random effect of sire, although there was a trend in overall variation in early settlement among sires (Figure 2). However, by bootstrapping the data, we were able to obtain an estimated $\sigma^2$ of approximately 0.176 (0.42 standard deviation), corresponding to a narrow-sense heritability of around 0.2 (95% confidence interval 0.0–1.0). Considering the number of sires used and offspring sampled in our study, the true narrow-sense heritability would have to be well above 0.6 to achieve 80% power (Figure 4A). Nevertheless, this experimental set up should be sufficiently powered to correctly fail to reject the null hypothesis if in fact the true among sire variance was zero (Figure 4B).

Power analysis
Power analysis results suggest that increasing the number of sires is the most effective mechanism to increase statistical power. Unfortunately, for heritabilities less than 0.4, very large numbers of sires will be required. The intuition is that substantial amounts of variability between sires is expected just due to sampling alone, and therefore statistical support for a nonzero heritability requires large sample sizes. Despite the lack of statistical power, this approach does have the desirable property of low false-positive rates. For example, even with nine sires, we expect to have a nearly 90% chance of failing to reject the null hypothesis on data sets simulated with an among-sire variance equal to zero (Figure 4B). Lastly, if sequencing additional offspring is an option, statistical power can be improved (Figure 5).

DISCUSSION
In this paper, we present an experimental and statistical methodology for estimating the heritability of traits in nonmodel, highly fecund organisms. We applied this approach to determine whether settlement is a heritable trait in the reef-building coral O. faveolata. Although we did not find statistical support for a nonzero heritability in this trait, a power analysis suggests we lacked a sufficient number of individuals.
Our computational method includes code for fitting model parameters, performing model selection using a permutation test, and calculating the expected statistical power for proposed or completed studies. The power calculation method is especially important for studies requiring animal care and use approval and/or those with complex or expensive collection demands.

Previous work suggests that heritable variation exists for a variety of traits across many marine organisms (Foo et al. 2012; Johnson et al. 2010; Kelly et al. 2013; Lobon et al. 2011; McKenzie et al. 2011; Parsons 1997), including corals (Kenkel et al. 2011; Meyer et al. 2009). These studies have found significant heritability for nearly every trait measured in corals (Carlson et al. 2011; Kenkel et al. 2011; Meyer et al. 2009, 2011), but see Casazar et al. (2010). In fact, one study specifically quantified the additive genetic variance in settlement rates of the Pacific reef-building coral Acropora millepora and found $h^2 = 0.49$; however no variance around this mean was estimated (Meyer et al. 2009). It would not be surprising from an evolutionary standpoint if an ecologically important life-history trait such as larval settlement was heritable in other coral species, such as O. faveolata. However, in this study we were unable to detect heritable variation, likely due to insufficient numbers of individuals.

There is a rich quantitative genetics literature on estimating the heritability of binomial traits dating back to Wright (1917) and Fisher (1918); however, the first use of generalized linear models fit to observed presence/absence data are from Gilmour et al. (1985), with key future contributions from Foulley et al. (1987) and Vazquez et al. (2009). These methods originally were developed for agricultural breeders, where fewer constraints exist on the number of families used to estimate the heritability, for example, the viability of poultry (Robertson and Lerner 1949), common genetic disorders of Holstein cows (Urine et al. 1995), and root vigor in sugar beets (Biscarini et al. 2014, 2015). Urbe et al. (1995) estimated sire and residual variance components by using restricted maximum likelihood, or REML, modeling of 7416 paternal half-sib cows and found that heritability of common diseases in cows ranged from 0 to 0.28. These sorts of numbers are unreasonable to sample in natural populations of corals because parentage is rarely known unless controlled crosses are completed and then the costs associated with genotyping thousands of individuals are prohibitive.

A priori expectations from models of heritability lead to a powerful negative result. If the null hypothesis is true, then a permutation test should detect a significant deviation from the observed data with high probability. If the test statistic is too large, we conclude that the hypothesis is not true. We used a modified distribution function. Future work should focus on adapting existing methods and developing new methods to allow for smaller sample sizes. This effort is meant to be a project that will grow and develop organically; therefore, we welcome suggestions and contributions and plan regular updates to the statistical methods.

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